THE CARDIOVASCULAR PHYSIOLOGY OF WINTER FLOUNDER (*Pseudopleuronectes americanus*): CONTROL MECHANISMS AND ENVIRONMENTAL INFLUENCES

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The Cardiovascular Physiology of Winter Flounder (*Pseudopleuronectes americanus*): Control Mechanisms and Environmental Influences

by

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A thesis submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Ocean Sciences Centre

Memorial University of Newfoundland

November 20, 2009

St. John's, Newfoundland

Abstract

In this thesis, I used a multi-level approach (whole animal, organ/tissue and cellular) to determine the functional relationships between maximum cardiac performance, heart morphology, and adrenergic capacity of the winter flounder (*Pseudopleuronectes americanus*) heart. Further, I studied the capacity of the flounder cardiovascular system to respond to two ecologically relevant environmental stressors: hypoxia and an acute temperature increase. In the first series of experiments, an *in situ* heart preparation was developed for this species, and *in situ* cardiac function and *in vitro* pressure-volume curves were determined. This research showed that maximum stroke volume per gram of ventricle is the highest reported for teleosts, and that this elevated stroke volume is related to a pronounced and extended Starling curve, more compliant heart chambers, and a high bulbus:ventricle mass ratio.

In the second part of my thesis I determined how flounder *in vivo* cardiovascular function is regulated by nervous and humoral mechanisms. Although resting cholinergic tonus (26%) was comparable to other teleosts, bretylium (an adrenergic nerve blocker) injection resulted in an increase in heart rate, and there was little evidence that catecholamines support cardiac function at rest or following an exhaustive chase. Further, the high myocardial β-adrenoreceptor density (252.8 fmol mg^{-1} protein), yet low affinity (1.02 nM), measured for cardiac β-adrenoreceptors raises the possibility that the flounder heart is populated primarily by β_3-adrenoreceptors, not β_1- or β_2-adrenoreceptors as has been reported for most other teleosts.

Finally, although flounder acclimated to 8°C underwent a typical reflex bradycardia when exposed to hypoxia, and this species' cardiac response to an acute elevation in water temperature to critical thermal maximum was in general typical of that shown for teleosts, a number of observations do not fit with the pattern established for other species. Specifically, the onset of bradycardia at 8°C occurred earlier than expected for an inactive and hypoxia-tolerant species (60% water O_2 saturation), resting cardiac output was similar in flounder acclimated to 8 and 15°C, and hypoxic bradycardia was absent at 15°C. These observations raise the possibility that behaviour is an important component of this species' response to environmental changes.

Acknowledgements

I would like to start by thanking my supervisor, Dr. Kurt Gamperl, for his guidance and support during my Ph.D. and without whom this thesis would not have been possible. I would also like to recognize my supervisory committee, Dr. William Driedzic, Dr. Reza Tabrizchi and the late Dr. Joe Brown for their role in the development of this thesis.

A very special thanks to my laboratory members: Gaylene Genge for her invaluable help during my excruciating flounder heart preparations; Eric Deitch (and AquaBounty) for supplying Atlantic salmon hearts for my experiments; and Isabel Costa for spending many hours teaching me the nooks and crannies of the HPLC analysis and for being my little piece of home in Newfoundland. I am also extremely grateful to other laboratory members - Dr. Juan Pérez Casanova, Dr. Matt Gollock and Dr. Shaun Killen – for their friendship through "thick and thin" and for making our lab feel like a well knitted family. Outside of the Gamperl lab., I would like to acknowledge Dr. Tyson MacCormack and Dr. Jay Treberg for their insightful scientific observations and clarifications, and the other members of the dearly departed "annex grad room" for welcoming me with open arms and making the Ocean Sciences Centre such a great work place. The amazing staff at the Ocean Sciences Centre (a.k.a. Superheroes), in particularly, the Field Services, the Laboratory Services, the Computer Services and the Main Office, have greatly contributed to the success of my Ph.D. by always helping me with the small and bigger tasks/dramas that I put in their hands (even when they came in centimetres instead of inches).

To my "non-scientist" friends, particularly to Diana Cardoso, I am extremely grateful for patiently listening day after day about the progresses (and retreats) of my work, and for showing me that there is life outside of the laboratory. To Chris Harnett – words can not convey the appreciation I feel for the unconditional support you show in every endeavour I take. Thank you for showing me that everything is indeed possible if we put our mind to it.

Finally, I would like to express the extreme gratitude, pride and love that I have towards the best family a person could have (Mine!): "A vossa força e apoio foi extremamente importante durante estes anos. Obrigada por todos os esforços que fizeram para eu ter chegado até aqui. Este momento não é só meu, é nosso!".

Co-authorship Statement

In this doctoral thesis, the author was responsible for the intellectual design, execution of the experiments, data analysis and the preparation of the manuscripts. The only exception is the study presented in Chapter 2. In this study, Gaylene Genge and Eric Deitch performed the *in situ* heart preparations for the Atlantic cod and Atlantic salmon, respectively. Both Ms. Genge and Mr. Deitch are co-authors on the manuscript. The analysis of the data obtained from all *in situ* preparations was done by the author.

Publication Statement

The work presented in Chapter 2 is published in its entirety as follows;

Mendonça PC, Genge AG, Deitch EJ and Gamperl AK. 2007. Mechanisms responsible for the enhanced pumping capacity of the in situ winter flounder heart (*Pseudopleuronectes americanus*). *Am. J. Physiol.* 293(5):R2112-2119.

The work presented in Chapter 3 is published in its entirety as follows;

Mendonça PC and Gamperl AK. 2009. Nervous and humoral control of cardiac performance in the winter flounder (*Pleuronectes americanus*). *J. Exp. Biol.* 212:934-944.

The work presented in Chapter 4 is published in its entirety as follows;

Mendonça PC and Gamperl AK. *In press.* The effects of acute changes in temperature and oxygen availability on cardiac performance in winter flounder (*Pseudopleuronectes americanus*). *Comp. Biochem. Physiol.* A.

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List of Abbreviations

A adrenaline

ANOVA analysis of variance

ANCOVA analysis of covariance

A:V atrium:ventricular mass ratio

B_{max} cell surface beta-adrenoreceptor density

B:V bulbus:ventricular mass ratio

CA total catecholamines

CTM critical thermal maximum

DHBA 3,4-Dihydroxybenzylamine

ECG – electrocardiogram

f_H heart rate

Hct haematocrit

HPLC high performance liquid chromatography

IC_{50} concentration of ligand that reduced [^{3}H]CGP binding by 50%

ID inner diameter

K_d dissociation constant for [^{3}H]CGP 12177

MS-222 Tricaine methanesulfonate

NA noradrenaline

NANC non-adrenergic non-cholinergic

OD outside diameter

ODS octadecyl silane

P_H cardiac power output

pH negative logarithm of the hydrogen-ion concentration

P_{IN} input pressure

P_{DA} dorsal aortic pressure

PO_2 oxygen partial pressure

P_{VA} ventral aortic pressure

P_WO_2 water oxygen partial pressure

P_{OUT} output pressure (afterload)

Q cardiac output

Q_{10} temperature coefficient

RAM relative atrial mass

RBM relative bulbar mass

R_{sys} systemic vascular resistance

RVM relative ventricular mass

s.e.m. standard error of the mean

U_{crit} critical swimming speed

V_S stroke volume

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1. Background and Literature Review

Fish are the most successful vertebrate group both in terms of biomass and number of species, and they occupy a wide range of environments. For example, the benthic Antarctic teleost *Pagothenia bernacchii* lives at a relative constant temperature (from -1.9 to 1°C) all year round (di Prisco et al., 1991; Axelsson et al., 1992). *Bathygobius soporator* is a small marine teleost which lives in tide pools along the east coast of South America, and can be exposed to acute temperature shifts from 25°C to greater than 40°C (Rantin et al., 1998). The winter flounder (*Pseudopleuronectes americanus*) can usually be found throughout the year in coastal regions of the Atlantic provinces (Fletcher, 1975; Pereira et al., 1999), and is physically adapted for living on mud bottoms (Fletcher, 1975; Moyle and Cech, 1996). Finally, tunas are pelagic fishes that are anatomically, physiologically and biochemically adapted to achieve exceptionally high maximum aerobic metabolic rates (Brill and Bushnell, 1991). In all adult vertebrates, the cardiovascular system is essential for survival since it ensures adequate oxygen and nutrient delivery to the tissues, and removes carbon dioxide and other metabolic wastes. As a result of the above variation in lifestyles, and consequently demands placed on the heart, the cardiovascular function and design of fishes shows a multiplicity of modifications with regards to structure, and responses to temperature, oxygen availability and other environmental factors (Farrell and Jones, 1992; Sánchez-Quintana et al., 1995). To provide some information on the range of inter-specific differences that have been

reported for this group, this section reviews the basic features of teleost heart design and performance, and explains several aspects that control cardiac function.

1.1 Teleost heart

Fishes have a closed, single-circuit, circulation where the heart ejects blood at a relatively high pressure towards the gills (Agnisola and Tota, 1994). The teleost heart is composed of four chambers in series (the sinus venosus, atrium, ventricle and bulbus arteriosus), that are contained within a semi-rigid pericardial sac (Randall, 1968; Farrell and Jones, 1992; Farrell, 1993; Agnisola and Tota, 1994). The venous blood that returns to the heart is collected by the sinus venosus and is pumped sequentially by the atrium into the ventricle, and by the ventricle into the bulbus arteriosus (Farrell, 1993). In teleosts, three distinct ventricular shapes are found: tubular, saccular and pyramidal (Sánchez-Quintana et al., 1996). The tubular ventricle has an almost cylindrical form. The saccular ventricle is rounded and flattened dorsoventrally, and is typical of fish with a benthic ecophysiological lifestyle. Teleost species with saccular and tubular ventricles (e.g., flounder and cod; Santer et al., 1983) are also characterized by having an exclusively spongy (or trabeculated) myocardium in which nutrients and oxygen delivery are obtained exclusively from the venous blood that moves through the lumen and inter-trabecular spaces (Sánchez-Quintana et al., 1996). In contrast, active pelagic species such as the tuna tend to have a more pyramidal shaped ventricle (Sánchez-Quintana et al., 1995, 1996) and a myocardium with one or more layers of compact tissue (which is perfused by capillaries derived from the coronary artery), in addition to the spongy

myocardial tissue (Farrell and Jones, 1992; Farrell, 1993; Agnisola and Tota, 1994). Hence, it seems evident that the presence of an outer compact layer of myocardium is a morphological adaptation which allows for the enhanced cardiac output that is required by the elevated metabolic rates of active fish species (Brill and Bushnell, 2001; Santer and Greer Walker, 1980).

The performance of the heart is also related to the relative size of the ventricle (relative ventricular mass, RVM; g ventricle body mass^{-1}). Several studies have demonstrated that higher RVMs are associated with an enhanced cardiac performance, and that more active species have a higher RVM (Farrell and Jones, 1992). For instance, the tuna heart is unusually large compared with most fish species having a RVM of ~0.30% (Brill and Bushnell, 1991). In contrast, benthic species such as the winter flounder have a RVM of 0.05% (Joaquim et al., 2004), one of the smallest among teleost species. Further, Farrell and Jones (1992) suggested that large ventricular masses are important, among other things for: (i) developing the higher blood pressures measured in active fishes, (ii) compensating for the negative inotropic effect of low temperatures, and (iii) accommodating large cardiac stroke volumes.

1.2 Cardiovascular function: cardiac output, stroke volume and heart rate

Cardiac function in fishes has been mainly studied through the assessment of heart rate (f_H), cardiac output ($\dot{Q}$), and stroke volume (V_S). These parameters vary greatly between species (e.g., see Table 3 in Joaquim et al., 2004), and fish with higher levels of activity generally have significantly higher resting and maximum values than benthic

species (Farrell, 1991, 1993). Heart rate (f_H) is determined by the intrinsic rhythm of the pacemaker, by the activity of cholinergic and adrenergic nerves, and by circulating hormones (see reviews by Farrell, 1991, 1993). Further, non-adrenergic non-cholinergic (NANC) factor(s) also appear to modulate f_H in several species such as the eel (*Anguilla anguilla*) and Atlantic cod (*Gadus morhua*) (Peyraud-Waitzenegger and Soulier, 1989; McKenzie et al., 2009).

Cardiac output ($\dot{Q}$) is the volume of blood pumped by the heart per unit time and is presently measured directly using ultrasonic (Transonic®) or Doppler-flow probes that are placed around the ventral aorta. However, it was common for many years to use the Fick equation to indirectly calculate $\dot{Q}$. The Fick equation is based on the Fick principle which relates $\dot{Q}$ and oxygen (O_2) uptake (Metcalfe and Butler, 1982): $\dot{Q}$ = total rate of O_2 consumption / (O_2 content of arterial blood leaving the gills – O_2 content of venous blood entering the gills). However, $\dot{Q}$ estimates based on the Fick principle assume that all the oxygen consumed by the fish is taken up across the gills, and that all $\dot{Q}$ flows across the gills and enters the systemic circulation via the dorsal aorta. This is problematic, however, and may lead to inaccurate $\dot{Q}$ values because: 1) a large proportion (up to ~35%) of the total oxygen consumed by some fishes is taken up across the skin (Nonnotte and Kirsch, 1978; Steffensen et al., 1981), and as such calculated $\dot{Q}$ values will be overestimated (Metcalfe and Butler, 1982; Joaquim et al., 2004); 2) in many other fish species (and under various conditions), some of the venous blood is shunt away from the gills and returns directly to venous circulation, in which case the Fick principle will underestimate $\dot{Q}$ (Hughes et al., 1982; Metcalfe and Butler, 1982).

Large increases in Q are seen under a variety of conditions, such as exercise and acute increases in temperature. For example, prolonged swimming induces large increases in Q (up to 3-fold higher than resting Q), the magnitude of this increase being species dependent and for temperate species associated with increases in both V_S (approx. 25-200%) and f_H (approx. 7-50%; Farrell, 1991; Farrell and Jones, 1992).

Stroke volume (V_S), the volume of blood ejected with each heart beat, is a major contributor to changes in Q during exercise (Farrell and Jones, 1992; Olson and Farrell, 2006). V_S is determined by end-diastolic volume (preload), the force of contraction and arterial pressure (afterload) (Olson and Farrell, 2006). When end-diastolic volume is increased, the myocardial muscle stretches, which consequently increases the force of contraction and V_S (Frank-Starling mechanism; Allen and Kentish, 1985; Shiels and White, 2008). End-diastolic volume is determined by the volume of blood delivered from the atrium which is achieved through *vis-à-fronte* (force from in front) and *vis-à-tergo* (force from behind) mechanisms (Farrell and Jones, 1992; Farrell, 1993). *Vis-à-fronte* atrial filling uses some of the energy of ventricular contraction to directly distend, and thus fill the atrium. *Vis-à-tergo* atrial filling utilizes the potential and kinetic energy that remains in the venous circulation or that is generated by the sinus and atrial contractions (Farrell and Jones, 1992; Farrell, 1993). Earlier it was suggested that during resting conditions the v*is-à-fronte* mechanism was predominant, while the importance of v*is-à-tergo* mechanisms would rise at higher V_S (Farrell et al., 1988*b*; Farrell and Jones, 1992). However, it is presently thought that, at least in trout, v*is-à-tergo* mechanisms are the primary determinant of cardiac filling (Minerick et al., 2003; Altimiras and Axelsson,

2004) and *vis-à-fronte* is only important when elevated Q is required (Minerick et al., 2003).

1.3 Neurohormonal control of fish cardiac function

1.3.1 Cholinergic and adrenergic innervation

Cardiac innervation in fish also shows great variation; from myxinoid cyclostomes with aneural hearts (Laurent et al., 1983; Axelsson et al., 1990; Taylor et al., 1999), to elasmobranchs in which the heart is mainly innervated by cholinergic fibres (Taylor et al., 1999; Agnisola et al., 2003), and finally, to the complex and well developed system in most teleosts where both cholinergic and adrenergic fibres control cardiac function (Gannon and Brunstock, 1969; Cameron, 1979; Donald and Campbell, 1982; Laurent et al., 1983; Axelsson et al., 1987). In teleosts, the inhibitory cholinergic fibres run in the vagus nerve (Gannon and Brunstock, 1969; Chan and Chow, 1976; Holmgren, 1977), whereas cardiac adrenergic innervation can be supplied both *via* the vagus nerve and directly via spinal nerves (Holmgren, 1977; Laurent et al., 1983).

Heart rate in teleost fish is primarily mediated by variations in vagal tone. In most fishes, such the Atlantic cod (*Gadus morhua*), ballan wrasse (*Labrus berggylta*) and the sea bream (*Sparus aurata*) cholinergic tone is higher than adrenergic tone during resting conditions (10-12°C; Axelsson et al., 1987; Axelsson, 1988; Altimiras et al., 1997). Still, exceptions do exist, and in species such the pollack (*Pollachius pollachius*), the eel pout (*Zoarces viviparus*) and the shorthorn scorpion (*Myoxocephalus scorpius*), resting

adrenergic tone is higher than cholinergic tone (Axelsson et al., 1987). During exercise, cholinergic tone tends to decrease while adrenergic tone increases (Axelsson et al., 1987; Axelsson, 1988). With regards to environmental challenges, changes in nervous tone vary greatly. For example, while hypoxia-induced bradycardia is primarily mediated by increases in cholinergic tone (Farrell and Jones, 1992; Farrell, 1993), increases in acclimation temperature can be associated with increases in the adrenergic (rainbow trout, *Oncorhynchus mykiss*; Wood et al., 1979*b*) or cholinergic (sole, *Solea vulgaris*; Sureau et al., 1989) contribution to f_H. However, one needs to take into account that the nature of the adrenergic tone on the heart can also be humoral (i.e. through circulating catecholamines) when looking at f_H control under varied environmental conditions. A brief review on the role of circulating catecholamines in modulating fish cardiac function follows.

1.3.2 Circulating catecholamines

Humoral adrenergic stimulation of the heart is mediated by the catecholamines adrenaline (A) and noradrenaline (NA). These hormones/neurotransmitters are synthesized and stored in the chromaffin tissue, located in the head kidney within the walls of the posterior cardinal vein (Reid et al., 1998). The release of adrenaline and noradrenaline into the circulation is, in teleosts, mainly mediated by pre-ganglionic sympathetic fibers that release acetylcholine, which in turn binds to cholinoreceptors on the surface of the chromaffin cells (Randall and Perry, 1992; Reid et al., 1998; Perry and Bernier, 1999). Although, resting levels of plasma catecholamines are normally less than

10 nM, in response to severe stress, their concentration can increase to levels in excess of 300 nM (Randall and Perry, 1992; Gamperl et al., 1994*a*). In fact, A levels of 2601 nM and NA levels of 841 nM have been reported in Antarctic fish (Nototheniids) after capture by trawling (Whiteley and Egginton, 1999). The release of catecholamines into the circulation promotes metabolic and circulatory adjustments that allow the fish to cope with the heightened energetic demands that occur during stressful events (Randall and Perry, 1992; Gamperl et al., 1994*a*; Perry and Gilmour, 1999). However, the levels of circulating catecholamines vary with the severity and type of stressor (e.g., mild vs. severe hypoxia, moderate exercise vs. critical swimming speed) and, in general, species with a more active lifestyle exhibit a greater increase in circulating catecholamines when exposed to stressors than benthic/sluggish species.

The cardiac response to catecholamines also depends on the density, sensitivity (affinity) and subtype of β-adrenoreceptors present on the surface of myocardial cells (Randall and Perry, 1992). An increase in the number of β-adrenoreceptors is associated with enhanced cardiac responsiveness to catecholamines (Keen et al., 1993). However, binding affinity also varies between species and environmental conditions (Keen et al., 1993; Gamperl et al., 1994*b*; Olsson et al., 2000; Hanson et al., 2005), and thus, the capacity of the heart to respond to these hormones is determined by both β-adrenoreceptor characteristics. At present, three subclasses of β–adrenoreceptors have been identified in the fish heart, β_1–, β_2- and β_3–adrenoreceptors. The β_1–adrenoreceptors predominantly mediate the inotropic and chronotropic effects of NA, while β_2–adrenoreceptors mediate A-induced effects (Ask et al., 1980). Recently, β_3–

adrenoreceptors were identified in the trout and eel (*Anguilla anguilla*) (Nickerson et al., 2003; Imbrogno et al., 2006) and, at least in the eel, they mediate negative inotropic effects (Imbrogno et al., 2006). In trout, atrial and ventricular β–adrenoreceptors seem to be predominantly of the β_2 type (Ask et al., 1980; Ask, 1983; Gamperl et al., 1994*b*), which is consistent with the fact that plasma A is the predominant circulating catecholamine (Perry et al., 1996). Cobb and Santer (1973), also found that the isolated heart of the flounder *Pleuronectes platessa* was more sensitive to A than to NA, however, atrial preparations from the flounder *Platichthys flesus* have equal affinity for A and NA (Ask, 1983). This latter finding led Ask (1983) to suggest that both β_1– and β_2–adrenoreceptors mediate adrenergically-mediated responses in the flatfish heart.

1.4 Environmental effects on fish cardiac performance

1.4.1 Hypoxia

Fish can encounter waters in which oxygen content has been substantially reduced by biological oxygen demand, increased temperature, or anthropogenic activity (Fernandes, 1996; Pereira et al., 1999). Typically, fish try to avoid hypoxic aquatic environments by swimming away (Claireaux et al., 1995). However, if escape is not possible they will undergo physiological adjustments to maintain function.

In fish, the most common cardiac response to aquatic hypoxia is reflex bradycardia due to an increase in cholinergic inhibitory tone on the cardiac pacemaker (Farrell and Jones, 1992; Farrell, 1993). Earlier studies suggested that a decrease in f_H during hypoxia would improve oxygen transfer across the gills by decreasing Q and,

consequently, by increasing blood residence time in the gills (Randall and Shelton, 1963). Further, in species in which Q remained unchanged with bradycardia, due to a concomitant increase in V_S, it was proposed that bradycardia would increase branchial vascular resistance and, consequently, ventral and dorsal pulse pressures, which would increase lamellar perfusion (i.e. increased blood volume in gills) and/or recruit additional lamellae (Randall, 1982; Taylor, 1985; Farrell, 2007). These mechanisms decrease the diffusion distance between the fish's blood and the water, and increase gill surface area, thus enhancing O_2 uptake across the gills (Randall, 1982; Taylor, 1985; Farrell, 2007). However, in several species hypoxic bradycardia does not enhance O_2 uptake across the gills (Holeton and Randall, 1967; Taylor et al., 1977; Perry and Desforges, 2006). Further, while it is true that most fish respond to severe hypoxia with an increase in branchial vascular resistance, not every species increases ventral aortic pressure (P_{VA}) and P_{DA}. For instance, the tuna [*Thunnus albacares*, *Katsuwonus pelamis*; (Bushnell and Brill, 1992)] maintains P_{VA} and P_{DA} at water oxygen tensions (PO_2) as low as 33% air saturation, and in the Japanese eel (*Anguilla japonica*, 22°C; Chan, 1986; and in the lingcod, 9-10°C; Farrell, 1982) both parameters decrease with hypoxia. Finally, in the winter flounder, P_{DA} decreases while P_{VA} does not change at 46% air saturation (22°C; Cech et al., 1977). Collectively, these data argue against bradycardia improving branchial gas exchange.

Farrell (2007) proposed that hypoxic bradycardia has direct benefits to the fish heart. Drop in f_H increases the residence time of the blood in the lumen of the heart, thus enhancing O_2 diffusion time and improving O_2 supply to the myocardium. Further, this increase in blood residence time raises end-diastolic volume (i.e. increase V_S) and thus,

increases myocardial stretch/contractility and reduces oxygen diffusion distance (Farrell, 2007). By directly extending the time available for O_2 diffusion and by indirectly decreasing diffusion distance, hypoxic bradycardia improves O_2 extraction in the fish heart. This is particularly important for fish whose ventricle is composed entirely of trabecular myocardium (Santer et al., 1983; Tota, 1983) and in which deoxygenated blood is its only source of oxygen (Farrell and Jones, 1992; Farrell, 2007).

In addition to bradycardia and an increase in branchial vascular resistance, venous tone appears to be involved in regulating cardiac function in teleosts during hypoxia (Altimiras and Axelsson, 2004; Sandblom and Axelsson, 2005). For example, when exposed to hypoxia that did not elicit bradycardia, both the rainbow trout and the swamp eel (*Synbranchus marmoratus*) show elevations in central venous pressure that allow for the maintenance or increase in V_S due to an increase in cardiac filling pressure (Sandblom and Axelsson, 2005; Skals et al., 2006). This increase in central venous pressure, which is likely the result of increased sympathetic tone, causes the reallocation of blood to central vascular compartments, and consequently, increases cardiac preload and V_S (Sandblom and Axelsson, 2005; Skals et al., 2006)

1.4.2 Temperature

Cardiac function is also intimately linked to water temperature. For example, the decline in dissolved O_2 with elevations in water temperature can limit O_2 supply to the heart through a mismatch between O_2 supply and O_2 demand, and thus limit fish thermal tolerance (Portner, 2002). Temperature effects on the fish heart are usually assessed by

calculating Q_{10} values: the factorial change in a rate variable over a 10°C change in temperature. Reported Q_{10} values range from 1.5 to 3.0 for f_H and Q (Farrell et al., 1996). However, caution needs to be taken when predicting cardiac alterations promoted by fluctuations in temperature since Q_{10} values vary depending upon the section of the fish's thermal range under consideration, and that temperatures above a fish's preferred temperature can have a limited predictive value (Farrell et al., 1996).

Acclimatization/acclimation to warmer temperatures normally induces a positive chronotropic effect, and depending on the fish species, a positive or negative inotropic effect. For example, crucian carp (*Carassius carassius*) hearts *in vitro* and the common carp (*Cyprinus carpio*) *in vivo* show increases in both V_S and f_H with an increase in acclimation temperature from 5 to 15°C (Matikainen and Vornanen, 1992; Stecyk and Farrell, 2006). In contrast, although *in situ* hearts from rainbow trout acclimated to 22°C have a higher f_H than trout acclimated to 15°C (a 20% increase), V_S falls by 22% between 15 and 22°C (Farrell et al., 1996). When acclimated to cold temperatures, higher values for V_S are sometimes associated with cardiac hypertrophy (Driedzic and Gesser, 1994; Driedzic et al., 1996; Farrell et al., 1988*a*; Keen et al., 1993). For example, in Antarctic fishes relative ventricular mass can be as high as 0.39% of body mass (Tota et al., 1991). A larger ventricular mass would compensate for the decrease in contractility normally seen in fish muscle at lower temperatures (Axelsson et al., 1992). However, cardiac growth with acclimation to cold temperatures is not universal amongst fishes. For example, the RVM of 0 and 10°C cod is similar (Lurman et al., unpublished) and carp hearts are 10% smaller during the winter compared to the summer months (Matikainen

and Vornanen, 1992). β-adrenoreceptors density is also temperature-dependent, with the number of cardiac β-adrenoreceptors inversely related to acclimation temperature (Keen et al., 1993). Thus, an increase in myocardial β-adrenoreceptor density and/or cardiac β-adrenoreceptors binding affinity (Hanson et al., 2005) might also explain the maintenance or increase of V_S at cooler temperatures in some fish species.

Acute temperature changes can have different effects on fish hearts than acclimatization/acclimation. Acute increases in temperatures *in vitro* or *in situ* are associated with increases in f_H and decreases in V_S (Axelsson et al., 1992; Farrell et al., 1996; Shiels et al., 2002). The decrease in V_S with elevations in f_H, may be related to a limitation on cardiac filling or a decrease in contractility (negative staircase effect) (see review by Shiels et al., 2002). However, it appears that this effect may largely be an artefact of isolated preparations. In the intact cod (Gollock et al., 2006), winter flounder (Cech et al., 1976), and the chinook (*Oncorhynchus tshawytscha*; Clark et al., 2008) and sockeye salmons (*Oncorhynchus nerka*; Steinhausen et al. 2008), V_S is maintained constant during acute increases in temperature to near the fish's critical thermal maximum (CTM). *In vivo* data thus show that high contraction rates do not jeopardize the heart ability to develop force. A preservation of V_S would assist with meeting the oxygen demands, since increases in water temperature are associated with significant increases in metabolic rate (Q_{10} ~1.8 to 3) (Watters and Smith, 1973; Kramer, 1987; Brodeur et al., 2001; Gollock et al., 2006; Killen et al., 2008). Moreover, water oxygen solubility and haemoglobin-oxygen binding affinity decrease with increasing temperature (Watters and Smith, 1973; Perry and Reid, 1994; Gollock et al., 2006).

1.5 The winter flounder / Study rationale

The winter flounder (*Pseudopleuronectes americanus)* is member of the pleuronectiformes (flatfishes), a diverse and abundant group with over 570 species in 11 families (Moyle and Cech, 1996). Adult flatfish are laterally compressed, negatively buoyant (swimbladder is absent), and physically adapted for living on the bottom (Fletcher, 1975; Duthie, 1982; Moyle and Cech, 1996; Pereira et al., 1999). The winter flounder, in particular, can usually be found throughout the year in most Atlantic coastal regions of North America (Fletcher, 1975; Pereira et al., 1999). Despite their abundance and diversity, flatfish cardiovascular biology/physiology has not been extensively studied, and much of the data on cardiac function now appears to be inaccurate due to the use of indirect measurement techniques for $\dot{Q}$ (e.g., the Fick principle).

The relatively inactive lifestyle of flatfish (Duthie, 1982) is generally reflected by their cardiorespiratory physiology and anatomy. For example, the flatfish heart is characterized by a saccular ventricle with type Ib myocardium (Santer et al., 1983; Tota, 1983), it has one of the smallest RVM among teleost species (0.05%; Joaquim et al., 2004), and pumps against relatively low arterial blood pressures (2.5-3 kPa; Wood et al., 1979*a*). Standard metabolic rate is much lower in comparison to more active species such as the trout (Wood et al., 1979*a*; Priede and Holliday, 1980; Duthie, 1982). Finally, flatfish have a low haematocrit (20.3%; Wood et al., 1979*a*) and blood haemoglobin levels (3.5 g %; Cech et al., 1976), and a limited gill surface area (1 cm^2 g^{-1}; Nonnotte and Kirsch, 1978). However, despite their extremely small RVM and simple cardiac anatomy, cardiac performance in the winter flounder is equal to or exceeds that in other more active

teleosts. For instance, although resting Q and f_H are generally similar to those measured for other temperate species (Joaquim et al., 2004), these authors reported that resting V_S in the winter flounder (1.0 ml g ventricle^{-1}) is 3-fold greater than in rainbow trout (0.3 ml g ventricle^{-1}; Farrell and Jones, 1992), and that with f_H as high as 52.4 beats min^{-1} (10°C) the flounder is capable of maintaining V_S relatively constant (Joaquim et al., 2004). Finally, maximum V_S (1.68 ml g^{-1} ventricle, 4°C; Joaquim et al., 2004) is even higher than recorded for the Antarctic fish *Pagothenia bernacchii* (1.35 ml g^{-1} ventricle at 0°C; Axelsson et al., 1992)

The high cardiac performance observed in pleuronectiformes is also surprising given that adrenergic cardiac innervation appears to be absent (Cobb and Santer, 1973; Donald and Campbell, 1982; Ask, 1983), and that while in most fishes β_2–adrenoreceptors seem to mediate adrenergically-mediated cardiac responses (Ask et al., 1980; Gamperl et al., 1994*b*), Ask (1983) suggested that the flounder atrium had a mixed population of β_1– and β_2–adrenoreceptors. Collectively, these data suggest that adrenergic control of the flatfish heart is primarily *via* circulating catecholamines, and may resemble that of *Chondrichthyes* instead of that of teleosts. However, neurohormonal control of flatfish cardiac function has never been directly studied.

A fish's environmental history and/or activity level is often reflected by their tolerance to environmental hypoxia and/or to changes in water temperature. The winter flounder is physically adapted for living on a mud bottom, and can encounter extremes in ambient oxygen concentration and temperature (-1.0 to 20°C) (Fletcher, 1975; Duthie, 1982; Moyle and Cech, 1996; Pereira et al., 1999). Cech et al (1977) reported that when

winter flounder were exposed to hypoxic stress (~46% air-saturated seawater at 10°C) they increased $\dot{Q}$, increasing cardiac work. Further, when this species was subjected to an acute 5°C increase in temperature, Q_{10} values for resting $\dot{Q}$ were 1.56, 2.12 and 2.35 for 5, 10 and 15°C-acclimated individuals, respectively (Cech et al., 1976). These studies suggest that the winter flounder (flatfish) has considerable capacity for adjusting cardiac function in response to environmental perturbation. However, the reported magnitude of these adjustments is questionable because all $\dot{Q}$ values were determined using the Fick equation.

1.6 Objectives of thesis

Given the very limited, and potentially inaccurate, data that is available on flatfish cardiac function, the goals of this thesis were to use a multi-level approach (whole animal, organ/tissue and cellular) to: 1) determine the functional relationships between maximum cardiac performance, heart morphology and adrenergic capacity; and 2) examine how changes in environmental parameters (hypoxia and temperature) affect cardiac function in winter flounder (*P. americanus*).

This is significant work since it deals with an ecologically important species whose cardiovascular biology/physiology remains elusive. Further, this research will help to understand how the cardiac physiology of the flounder relates to its life history, and provide valuable information on which cardiovascular variables can be used as indices of environmental stress/quality in flatfishes. This information is critical to the development of telemetric devices for the physiological monitoring of free-living fishes (as in cod;

Webber et al., 1998). Flatfish are a prime candidate for the testing these types of devices, and for the measurement of metabolism and "stress" in free-living fishes for several reasons. First, the negative buoyancy and benthic habit of these fish means that they can carry relatively large devices (>2% body wt.), without affecting their biology. Second, cardiovascular parameters are sensitive biomarkers of environmental health, and flatfish may be particularly sensitive to marine pollution because they show strong site fidelity, reside in bottom sediments where toxicants accumulate, and often utilize near-shore habitats (Pereira et al., 1999). Finally, flatfish aquaculture is being developed in many regions of the world (e.g. Atlantic Canada, Asia, Mediterranean countries) so information about how holding conditions (temperature and oxygen levels, stocking density, etc.) affect their physiology is needed before the aquaculture industry will see significant growth.

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2. Mechanisms Responsible for the Enhanced Pumping Capacity of the *In Situ* Winter Flounder Heart (*Pseudopleuronectes americanus*)

2.1 Abstract

In situ Starling and power output curves and *in vitro* pressure-volume curves were determined for winter flounder hearts, as well as the hearts of the Atlantic salmon and Atlantic cod. *In situ* maximum cardiac output was not different between the three species (approx. 62 ml min^{-1} kg^{-1}). However, because of the small size of the flounder heart, maximum stroke volume in millilitre per gram of ventricle was significantly greater (2.3) as compared with cod (1.7) and salmon (1.4), and is the highest reported for teleosts. The maximum power output of the flounder heart (7.6 mW g^{-1}) was significantly lower than that measured in the salmon (9.7) and similar to the cod (7.8), but was achieved at a much lower output pressure (4.9 vs. 8.0 and 6.2 kPa, respectively). Although the flounder heart could not perform resting levels of cardiac function at subambient pressures, it was much more sensitive to filling pressure, a finding supported by pressure-volume curves, which showed that the flounder's heart chambers were more compliant. Finally, I report that the flounder's bulbus:ventricle mass ratio (0.59) was significantly higher than in the cod (0.37) and salmon (0.22). These data, which support previous studies suggesting that the flatfish cardiovascular system is a high-volume, low-pressure design, show that: *vis-à-fronte* filling is not important in flatfish, and that some fish can achieve high levels of cardiac output by *vis-à-tergo* filling alone; and suggest that a large compliant bulbus

assists the flounder heart in delivering extremely large stroke volumes at pressures that do not become limiting.

2.2 Introduction

Flatfish are a diverse and abundant group of fishes with over 570 species in 11 families (Moyle and Cech, 1996). However, despite their abundance and diversity, the cardiovascular physiology of flatfish has not been extensively studied, and at present, data on cardiac function in this taxa are quite variable. For example, Joaquim et al. (2004) made the first direct measurements of resting and maximum *in vivo* cardiac function in flatfish using a custom-designed swimming flume and Transonic flow probes placed around the ventral aorta, and they reported values for maximum cardiac output ($\dot{Q}$) and stroke volume (V_S) of 39 ml min^{-1} kg^{-1} and 0.74 ml kg^{-1} (at 10°C), respectively. In contrast, previously reported values for maximum $\dot{Q}$ were 93.5 and 79.4 ml min^{-1} kg^{-1} (Watters and Smith, 1973; Wood et al., 1979). This discrepancy may be due to the use of indirect methodologies (Fick Principle) by Watters and Smith (1973) and Wood et al. (1979) and that flatfish can consume approx. 30% of their oxygen requirements directly though their skin (Nonnotte and Kirsch, 1978). However, there are other possible explanations for the difference in reported $\dot{Q}$ values between studies. First, Watters and Smith (1973) and Wood et al. (1979) made measurements on "resting fish," and induced increases in $\dot{Q}$ by increasing temperature (11–20°C) and by making the fish anaemic (haematocrit 14.5 to 0.8%), whereas Joaquim et al. (2004) swam their fish to exhaustion (U_{crit} test). Second, arterial pressures (afterload) in maximally swimming fishes increase considerably above those at rest (Jones and Randall, 1978) and may have limited

maximum Q in the study by Joaquim et al. (2004). Even if the values reported for maximum cardiac function by Joaquim et al. (2004) are underestimates, it is apparent that V_S (per gram of ventricle) is high in the winter flounder (resting V_S 0.94 ml g^{-1} ventricle; maximum V_S 1.5 ml min^{-1} g^{-1} ventricle at 10°C), compared with other teleosts (see Table 3; Joaquim et al., 2004). These authors suggest a number of possible mechanisms through which this elevated V_S could be achieved: (*1*) the lack of compact myocardium and thus the presence of a highly compliant ventricle; (*2*) a more pronounced Starling response associated with enhanced *vis-à-tergo* and/or *vis-à-fronte* cardiac filling; and (*3*) a reduced cardiac afterload, which could enhance ventricular emptying under conditions of maximal exercise. However, there are insufficient data on cardiovascular function in flatfishes and other teleosts to allow for a determination of which of these mechanisms is likely to be the most important. To accurately determine the maximum pumping capacity of the winter flounder (*Pseudopleuronectes americanus*) heart and to identify the factors contributing to the high V_S in this species, a comparative study was performed using the winter flounder and two other marine species, the Atlantic cod (*Gadus morhua*) and the Atlantic salmon (*Salmo salar*). These latter species were chosen because the cardiovascular physiology of cod (e.g., Axelsson and Nilsson, 1986; Axelsson, 1988; Claireaux et al., 1995*a*, 1995*b*) and salmonids (e.g., Farrell, 1979; Farrell et al., 1986, 1988; Gamperl et al.,1994*a*; Overgaard et al., 2004) has been extensively studied, and they have a morphology, physiology, and lifestyle very different from flatfishes. *In situ* perfused heart preparations (Farrell et al., 1982) were used to determine sensitivity to filling pressure (i.e., Starling curves), and maximum V_S, Q, and power output (P_H), for all three species. Pressure-volume curves were generated for the atrium, ventricle, and

bulbus arteriosus (Forster and Farrell, 1994) to examine whether differences in chamber compliance exist between species. Finally, the relative sizes of the heart chambers were measured to evaluate the contribution of heart morphology to pumping capacity and heart function.

2.3 Material and methods

2.3.1 Experimental animals

Ethical approval was obtained from the Animal Care Committee at Memorial University of Newfoundland (protocol no. 05-02-KG). Wild winter flounder were collected by divers (in Conception Bay, Newfoundland), while hatchery-reared cod and salmon were obtained from a cage-site operation (Bay D'Espoir, Newfoundland) and the Ocean Sciences Centre (OSC, Memorial University of Newfoundland), respectively. All fish were acclimated at 8 to 10±1°C for at least 4 weeks before experimentation in 1,200-liter tanks supplied with aerated seawater and natural photoperiod. Fish were fed three times a week with commercial pellets, except the winter flounder used for the *in situ* preparations, which were fed twice a week to apparent satiation with chopped frozen Atlantic herring (*Clupea harengus*).

2.3.2 *In situ* heart preparations

Fish were anesthetized in seawater containing methane sulfonic acid of m-aminobenzoate (MS- 222, 0.25 g l^{-1}), and then transferred to a surgery table, where their gills were irrigated with chilled (~4°C) and oxygenated seawater containing 0.1 g l^{-1} MS-222. *In situ* heart preparations were obtained for the salmon as described by Farrell et al.

(1986), with only minor modifications of this protocol required for cod. For example, the output cannula could not be secured in the ventral aorta of cod at a position between the 3rd and 4th gill arches due to the robustness of the cartilage in this area. Instead, the cannula was secured in place between the 1st and 2nd gill arches by tying directly to the ventral aorta, and the 3rd and 4th gill arches were subsequently occluded using cable ties. However, this is the first time that an *in situ* heart preparation has been used in the flounder, and thus, I briefly describe the procedure below.

The winter flounder's abdominal cavity was exposed by cutting the body wall along the lateral line. The gonadal veins were ligated with a 1-0 silk thread (American Cyanamid, Pearl River, NY, USA), and the gall bladder was drained. Umbilical tape (Baxter Healthcare, Deerfield, IL, USA) was tied around the gastrointestinal tract, inferior to the liver. The gonads and this isolated portion of the digestive tract were removed to allow for access to the hepatic veins. The hepatic vein on the "blind side" was occluded using a 3-0 silk tie (American Cyanamid). Then an input cannula (2.2 mm OD; 1.7 mm ID; steel chromatography tubing) was introduced into the sinus venosus via the hepatic vein on the "eyed side," and secured in place with 3-0 silk thread. At this point, the perfusate bottle was opened, and several gill arches were cut to prevent excessive pressure development by the ventricle. The height of the perfusate bottle relative to the heart determines atrial filling pressure, and this height was adjusted during surgery to ensure adequate cardiac output (~5 ml min^{-1}).

To place the output cannula (1.8 mm OD X 1.5 mm ID; steel chromatography tubing) in the ventral aorta, the fish's head was removed by cutting at a position just posterior to the eyes, and all gill arches except the 4th were removed. The isthmus was

then transacted between the 3rd and 4th gill arches, exposing the ventral aorta in cross section. An output cannula was inserted into the ventral aorta at a point confluent with the bulbus arteriosus and extended to within a few millimeters of the ventricular valve. The output cannula was then secured in place with a loop of 1-0 silk thread. Following this procedure the caudal, dorsal, and ventral fins of the fish were trimmed away.

Once *in situ* preparations for all species were obtained, the fish were placed into a saline bath maintained at 8–10°C, the input cannula was immediately connected to a tube delivering perfusate at constant pressure (0 to 0.1 kPa), and the output cannula was connected to tubing whose height could be adjusted to control the end-diastolic pressure developed by the ventricle. Initially, the output pressure head was set to produce an afterload (P_{OUT}) of 1 kPa. This prevented excessive cardiac work while input pressure (P_{IN}) was being adjusted to produce a basal level of cardiac output ($\dot{Q}$). Values for basal $\dot{Q}$ were selected to simulate the resting *in vivo* cardiac output of the three species: 14 ml min^{-1} kg^{-1} for the flounder (Joaquim et al., 2004), and 16 ml min^{-1} kg^{-1} for the cod and salmon (Axelsson and Nilsson, 1986; Gamperl et al., 1994*b*). The heart was allowed to equilibrate for 10 min at a P_{OUT} of 1 kPa. Thereafter, P_{OUT} was raised to 3 kPa for the flounder and 5 kPa for the cod and salmon; levels comparable to *in vivo* arterial pressures (Axelsson and Nilsson, 1986; Cech et al., 1976; Gamperl et al., 1994*b*). After 15 min, the Starling curve of the hearts was determined by raising P_{IN} in 0.05 kPa intervals until a stable maximum $\dot{Q}$ was achieved. Following this procedure, with the heart still at maximum $\dot{Q}$, P_{OUT} was decreased to 1 kPa then increased in 1 kPa intervals until $\dot{Q}$ reached 0 ml min^{-1}. Each increase in P_{IN} and/or P_{OUT} was maintained for ~30 s so that stable cardiac parameters could be obtained. After the heart's maximum output pressure

was measured, P_{OUT} was restored to *in vivo* arterial pressure levels (3.0 and 5.0 kPa), and the heart was allowed to recover for 5–10 min.

To ensure that the input cannula was securely tied within the sinus venosus and that the *in situ* heart was isolated from the saline in the experimental bath, two final tests were performed. First, it was confirmed that Q rapidly fell to 0 ml min^{-1} when the input cannula was clamped off using a pair of hemostats. Second, with the hemostats still clamping the input cannula, the tubing that connected the output cannula to the heart was raised to 10 kPa to confirm that Q was maintained at 0 ml min^{-1} (i.e., there is no backflow of perfusate).

2.3.3 Perfusate composition

The saline used to perfuse the hearts contained (in mM): 181.3 NaCl, 5.0 KCl, 2.30 $CaCl_2$ x $2H_2O$, 1.99 $MgSO_4$ x $6H_2O$, 2.58 TES acid, 7.33 sodium TES base, and 5.6 glucose (pH 7.75 at 8°C). All chemicals were purchased from Fisher Scientific (Fairlawn, NJ, USA), with the exception of TES salt, which was purchased from Sigma (St. Louis, MO, USA). The perfusate was continuously gassed with O_2 during surgery and experimentation, and adrenaline bitartrate (10 nM; Sigma) was added to the perfusate throughout the experiment to ensure long-term viability of the *in situ* heart (Gamperl et al., 1994*a*). Further, because adrenaline is light sensitive and deteriorates over time, it was added to a fresh perfusate bottle every 20 min.

2.3.4 Pressure-volume curves

Fish were over-anaesthetized in seawater containing MS-222 (0.35 g l^{-1}) and transferred to a surgery table where the pericardial sac was exposed through a ventral incision. Then, the pericardium was cut and the heart was dissected free from the animal, being careful to include part of the sinus venosus and ventral aorta. The heart was placed in a chamber with approx. 3 cm of ice on the bottom to help maintain the temperature at ~8°C. With the help of a microscope (Leica MZ 9.5, Leica Microsystems, Richmond Hill, ON, Canada) an input cannula (2.2 mm OD; 1.7 mm ID; steel chromatography tubing) was introduced into the atrium via the sinus venosus and secured in place with 0 silk thread. Steel chromatography tubing (1.0 mm OD) was then inserted via the ventral aorta into the bulbus arteriosus and was secured using 2–0 silk thread. Then a slightly curved micro-aneurysm clip (85 g pressure, 1 mm wide x 6 mm long; Harvard Apparatus, Holliston, MA) was carefully placed at the junction of the atrium and ventricle.

To generate pressure-volume curves for the atrium, the atrium was filled with saline at 3.05 ml h^{-1} using a calibrated syringe pump (A-99 model, Razel Scientific Instruments, Stamford, CT, USA), and pressure within the atrium was measured via a side arm in the input cannula, using a Gould Statham pressure transducer (Model P23 ID; Gould Statham, Oxnard, CA, USA). Once the pressure-volume curve for the atrium was generated, a small incision was made in the atrium to drain the chamber, the cannula was advanced into the ventricle, and the atraumatic clamp moved to the ventricle:bulbar junction. In a similar fashion, pressure-volume curves were generated for the ventricle, and then for the bulbus arteriosus. For all chambers, maximum pressure and volume were

taken at the point at which each chamber began leaking (i.e., pressure did not increase or began to fall with further increases in volume).

The determination of pressure-volume curves took about 90 min for each fish, and spontaneous cardiac contractions were often induced by the infusion of saline. Thus, chamber pressure was measured as diastolic pressure. During the measurements, the heart's surface was kept moist by frequent applications of saline at 8–10°C. At the conclusion of each experiment, the heart chambers were separated, individually weighed, and atrial:ventricular (A:V) and bulbus:ventricular (B:V) mass ratios were calculated to examine whether the size of the heart chambers or their relative size might influence the shape of the pressure-volume curves.

2.3.5 Data acquisition and calculations

P_{IN} and P_{OUT} were measured using Gould Statham pressure transducers (Model P23 ID, Gould Statham). For the *in situ* experiments, these pressure transducers were calibrated daily against a static water column, where zero pressure (0 kPa) was set equal to the saline level in the experimental bath. Further, the recorded P_{IN} and P_{OUT} were corrected using predetermined calibrations (Faust et al., 2004) to account for the resistance in the tubing between the points of pressure measurement and the heart. For the pressure-volume curves, 0 pressure was set to the level of the heart in the humidified chamber.

$\dot{Q}$ was measured using a Model T206 small animal blood flowmeter in conjunction with a precalibrated in-line flow probe (2N, Transonic Systems, Ithaca, NY, USA). Pressure and flow signals were amplified and filtered using a Model MP100A-CE

data acquisition system (BIOPAC Systems, Santa Barbara, CA, USA). The data acquired during the *in situ* protocol was stored and analyzed using Acq*knowledge* 3.7.2 Software (BIOPAC Systems, Santa Barbara, CA, USA) installed on a 300-MHz Toshiba laptop computer.

Cardiovascular function during the *in situ* experiments was continuously monitored by measuring $\dot{Q}$, P_{IN}, and P_{OUT}. Although data were continuously collected, cardiac function was only analyzed at specific intervals during each experiment. The P_{IN} required to maintain resting *in vivo* $\dot{Q}$ was measured prior to obtaining the Starling curve. In addition, all cardiac parameters ($\dot{Q}$, V_S and f_H) were measured at each level of P_{IN} during the Starling curve and at each level of P_{OUT} during the Max. Power test. Heart rate (f_H) was measured by counting the number of systolic peaks on the $\dot{Q}$ recording during a 30-s interval, and V_S (ml kg^{-1}) was calculated from $\dot{Q}$ (ml $kg^{-1}min^{-1}$)/f_H. V_S per gram of ventricular mass (V_S, ml g^{-1} ventricle) was calculated by dividing V_S (ml) by ventricular mass (g), and power output of the heart (P_H, mW g^{-1}) was calculated as [$\dot{Q}$ (ml min^{-1})/60] x [(P_{OUT} - P_{IN}) x 0.098]/ventricle wet mass (g).

Pressure values obtained during the generation of the pressure-volume curves were also stored and analyzed using Acq*Knowledge* 3.7.2 software installed on a Seanix computer. Chamber volumes were calculated based on the delivery rate of the syringe pump (3.05 ml h^{-1}) used to fill the heart chambers. Plots of ventricle, atrium, and bulbus arteriosus volume vs. pressure were used to describe the hemodynamic characteristics of each of the heart chambers, and maximum compliance values for each of the chambers were determined by calculating the slope of sections (>0.2 ml) of the mean volume-pressure relationships (see Fig. 2.2) at which the compliance was greatest (i.e., the slope

of each relationship was at a minimum). Finally, maximum distensibility was determined, by dividing maximum compliance by the initial volume for each chamber (i.e., the volume at the lower end of the range used to calculate maximum compliance).

2.3.4 Statistical analyses

After ln transforming the data for the Starling and pressure-volume curves, ANCOVA was used to test for homogeneity of slopes between species ($P<0.05$; SPSS Software). Maximum power output values were obtained by fitting 3rd-order regressions (SigmaPlot Software) to the pressure-flow data of each fish. Differences in maximum P_H, Q, f_H, V_S, maximum pressure and volumes, chamber masses, and A:V and B:V mass ratios between species were assessed by ANOVAs followed by pairwise Tukey post hoc tests (SPSS Software, $P<0.05$).

2.4 Results

2.4.1 *In situ* heart preparation

In situ heart rate was significantly lower in the cod, compared with the salmon and flounder, when the hearts performed at resting levels of cardiac performance. Although there was no significant difference in resting V_S between species (Table 2.1), the cod and salmon hearts could generate *in vivo* resting levels of Q at negative P_{IN} values, whereas the flounder heart required a positive P_{IN} of 0.04±0.02 kPa (Fig. 2.1*A*).

In situ maximum Q was not significantly different between the three species, averaging 62 ml min^{-1} kg^{-1}. However, because the relative ventricular mass (RVM) of the flounder was 40% less than for the other two species, the maximum V_S (per gram of

Table 2.1 Resting and maximum *in situ* cardiac performance of Atlantic salmon, Atlantic cod, and winter flounder hearts at 8–10°C. Values represent means±s.e.m. ($n \geq 7$). [a,b]Dissimilar letters indicate a significant difference ($P<0.05$) between species for a given parameter. Relative ventricular mass (RVM), heart rate (f_H), cardiac output (Q), stroke volume (V_S), and power output (P_H).

	RVM	Resting				Maximum				
	%	f_H bpm	Q ml min^{-1}kg^{-1}	V_S ml kg^{-1}	V_S ml g^{-1} vent.	f_H bpm	Q ml min^{-1}kg^{-1}	V_S ml kg^{-1}	V_S ml g^{-1} vent.	P_H mW g^{-1}
S. salar	0.07±0.002 ***a***	73.1±1.9 ***a***	16.3±0.36	0.22±0.01	0.33±0.01	66.8±1.4 ***a***	63.8±1.9	0.96±0.05 ***a***	1.4±0.08 ***a***	9.7±0.51 ***a***
G. morhua	0.07±0.04 ***a***	58.4±1.2 ***b***	16.8±0.23	0.29±0.01	0.40±0.02	51.3±0.77 ***b***	62.3±2.8	1.2±0.07 ***b***	1.7±0.16 ***a***	7.8±0.63 ***b***
P. americanus	0.05±0.001 ***b***	68.4±2.9 ***a***	14.3±2.8	0.21±0.04	0.44±0.09	54.0±1.5 ***b***	60.3±4.1	1.1±0.07 ***ab***	2.3±0.14 ***b***	7.6±0.33 ***b***

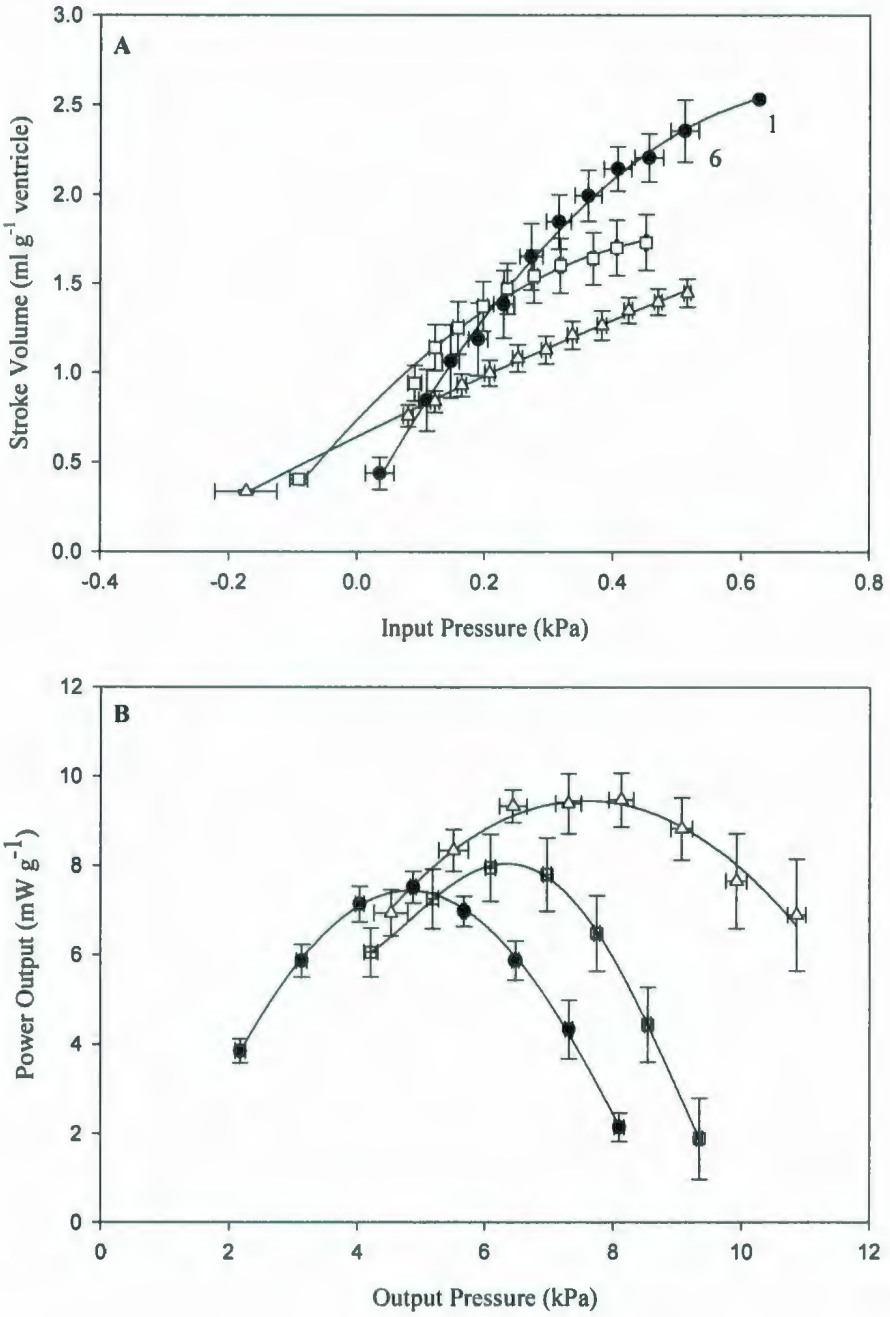

Figure 2.1 Starling (*A*) and power curves (*B*) for *in situ* winter flounder (●), Atlantic cod (□), and Atlantic salmon (Δ) hearts at 8–10°C. Values are expressed as means±s.e.m. n=7 or 8, except when numbers appear next to the data point.

ventricle) achieved by the winter flounder was significantly higher (2.3±0.1) compared with the Atlantic cod (1.7±0.2) and Atlantic salmon (1.4±0.1) (Fig. 2.1*A*; Table 2.1). In addition to having a higher maximum V_S, the flounder heart was much more sensitive to increases in filling pressure, and fewer increments in P_{IN} were required by the flounder heart to achieve elevated levels of V_S (Fig. 2.1*A*). For instance, to achieve a V_S of 1.4 ml g^{-1} ventricle (the maximum V_S for the salmon heart), the flounder heart only needed a P_{IN} increase of 0.19 kPa, whereas cod and salmon hearts required P_{IN} increases of 0.29 and 0.69 kPa, respectively.

The Atlantic salmon hearts achieved a much higher maximum P_H (9.7±0.5 mW g^{-1}) than the other two species, and could maintain significant flow at pressures in excess of 10 kPa (Fig. 2.1*B*). When comparing the power output curves of the flounder and cod, two things became apparent. First, maximum P_H was surprisingly similar in the two species (cod, 7.8±0.6 mW g^{-1}; flounder, 7.6±0.3 mW g^{-1}). Second, the power output curve for the flounder was shifted considerably to the left. This resulted in maximum P_H being achieved at a P_{OUT} of 4.89±0.17 kPa in the flounder, compared with 6.24 ±0.18 kPa in the cod (Fig. 2.1*B*; Table 2.1).

2.3.4 Pressure-volume curves

In several preparations one of the chambers leaked due to damage during surgery or advancement of the cannula, and thus, the data were not used. This resulted in different numbers of pressure-volume curves for each chamber (Table 2.2, Fig. 2.2). Only two ventricles were used to obtain the salmon pressure-volume curves, due to the difficulty in positioning the cannula in the centre of the small lumen of the salmon's heart chambers.

However, the data for these two ventricles were very similar and were incorporated for comparative purposes.

In all species the pressure-volume relationships for the atrium and ventricle generally had a characteristic J-shape, whereas those of the bulbus arteriosus were more sigmoidal (Fig. 2.2). Furthermore, with the exception of the salmon ventricle (which could be filled to a pressure approaching 10 kPa), the heart chambers of the three species attained similar maximum pressures; averaging 0.36, 5.3 (cod and flounder), and 10.6 kPa for the atrium, ventricle, and bulbus, respectively (Table 2.2; Fig. 2.2). Although the shape of the curves and the maximum pressure values obtained were similar between species, the pressure-volume curves show that all of the flounder's chambers are significantly more compliant and distensible (see Table 2.2), and in general, fill to significantly higher volumes (atrium: 0.65±0.03 ml; ventricle: 0.80±0.06 ml; bulbus: 0.50±0.05 ml) compared with the cod (atrium: 0.34±0.03 ml; ventricle: 0.46±0.04 ml; bulbus: 0.33±0.03 ml) and the salmon (atrium: 0.28±0.03 ml; ventricle: 0.75±0.01 ml; bulbus: 0.30±0.03 ml).

There were small or no differences in the atrial or ventricular masses (g), the relative atrial or bulbar masses (in % body mass), or the A:V mass ratio between species. However, the flounder's bulbar mass and B:V mass ratio (0.21±0.03 g, 0.59±0.06 g) were significantly greater than measured in the cod (0.14±0.01 g, 0.37±0.03 g) and salmon (0.11±0.01 g, 0.22±0.01 g) (Table 2.3). This enlarged bulbus may work in concert with the bulbus' higher compliance to keep systolic blood pressure low despite the large stroke volume delivered to the circulation.

Table 2.2 *In vitro* maximum pressures and volumes recorded when generating pressure-volume curves for the heart chambers of the three species (Atlantic salmon, Atlantic cod, and winter flounder). All chambers were filled at a rate of 3.05 ml/hr, and pressure was recorded through a side-arm in the input cannula. Values represent mean±s.e.m. ($n \geq 7$), except for the salmon ventricle, where $n=2$). [a,b,c]Dissimilar letters indicate a significant difference ($P<0.05$) between species for each chamber. Values for maximum compliance and distensibility were calculated from the mean values presented in Figure 2.2 (see text).

	Atrium			Ventricle			Bulbus		
	S. salar	*G. morhua*	*P. americanus*	*S. salar*	*G. morhua*	*P. americanus*	*S. salar*	*G. morhua*	*P. americanus*
Maximum Pressure kPa	0.39±0.07	0.39±0.07	0.29±0.06	11.3 9.3	5.1±0.13 ***b***	5.5±0.66 ***b***	11.0±0.35	10.3±0.20	10.5±0.18
Maximum Volume ml	0.28±0.03 ***a***	0.34±0.03 ***a***	0.65±0.03 ***b***	0.76 0.74	0.46±0.04 ***b***	0.80±0.06 ***a***	0.30±0.03 ***a***	0.33±0.03 ***a***	0.50±0.05 ***b***
Maximum Volume ml kg^{-1}	0.52±0.05 ***a***	0.76±0.04 ***b***	1.1±0.07 ***c***	1.2 1.3	1.0±0.12	1.1±0.05	0.52±0.04 ***a***	0.64±0.04 ***ab***	0.80±0.08 ***b***
Maximum Volume ml g^{-1} chamber	3.6±0.29 ***a***	4.7±0.34 ***a***	9.4±0.47 ***b***	1.6 1.7	1.3±0.12 ***a***	2.1±0.12 ***b***	2.7±0.15	2.3±0.20	2.5±0.21
Maximum Compliance ml kPa^{-1}	1.2	1.1	3.9	0.40	0.11	0.71	0.06	0.05	0.10
Maximum Distensibility Fold Change kPa^{-1}	25	43	164	15	4.0	26	0.56	0.40	5.4

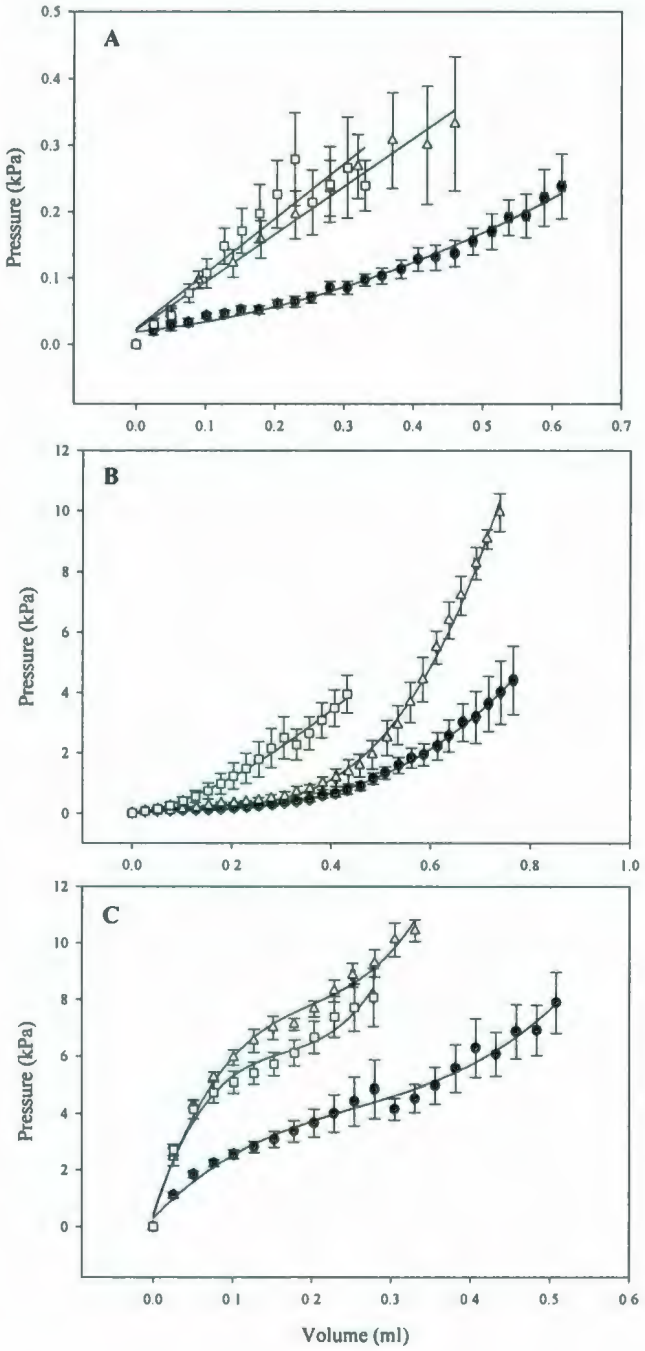

Figure 2.2 *In vitro* pressure-volume curves for the atrium (*A*), ventricle (*B*), and bulbus arteriosus (*C*) of the winter flounder (●), Atlantic cod (□), and Atlantic salmon (Δ) at 8–10°C. Values are expressed as means±s.e.m.; $n \geq 6$, except salmon ventricle where $n=2$. Data points for all but the salmon ventricle were not plotted when $n \leq 4$.

Table 2.3 Body and heart morphometrics, RVM, RAM, RBM, A:V, and B:V ratios recorded for 8–10°C acclimated Atlantic salmon, Atlantic cod, and winter flounder. Values are expressed as means±s.e.m. ($n \geq 7$). [a,b]Dissimilar letters indicate a significant difference ($P<0.05$) between species for a given parameter. RVM, relative ventricular mass; RAM, relative atrial mass; RBM, relative bulbar mass; A:V, atrium:ventricular mass ratio; B:V, bulbus:ventricular mass ratio.

	Body Weight	Ventricular Mass	Atrium Mass	Bulbus Mass	RVM	RAM	RBM	A:V	B:V
	g	g	g	g	%	%	%		
S. salar	568.3±14.1 *ab*	0.44±0.02	0.08±0.004	0.11±0.01 *a*	0.08±0.003 *a*	0.01±0.001 *ab*	0.019±0.001 *a*	0.18±0.01	0.22±0.01 *a*
G. morhua	492.6±25.6 *a*	0.37±0.02	0.07±0.01	0.14±0.01 *a*	0.08±0.01 *a*	0.02±0.001 *a*	0.026±0.001 *a*	0.21±0.01	0.37±0.03 *b*
P. americanus	650.3±30.8 *b*	0.39±0.03	0.07±0.004	0.21±0.03 *b*	0.05±0.003 *b*	0.01±0.001 *b*	0.034±0.004 *b*	0.22±0.01	0.59±0.06 *c*

2.5 Discussion

In situ heart preparations have been used for the past two-and-a-half decades to investigate aspects of cardiac function without any physical disturbance to the heart (e.g. Farrell et al., 1982, 1986, 1983; Davie and Farrell, 1991; Forster, et al.; 1991; Blank et al., 2002). However, this was the first time that an *in situ* heart preparation had been used to investigate flatfish or cod cardiac function. Although the *in situ* cod heart preparation was relatively easy to obtain, the laterally compressed body morphology of adult flounder made the perfused heart surgery exceptionally difficult and resulted in a low surgical success rate (~35%). Despite this, however, the flounder *in situ* heart proved to be a tractable preparation for examining cardiac function independent of hormonal and/or nervous control mechanisms.

2.5.1 Interspecific differences in cardiac performance

The mass-specific (per kilogram body mass) values for Q and V_S in the flounder are comparable to those measured for the cod and salmon, two considerably more active species. Moreover, the maximum *in situ* V_S (2.3±0.14 ml g^{-1} ventricle) that we recorded for the winter flounder is the highest ever reported for fish, even considerably higher than that measured for the Antarctic fish *P. bernacchii* (1.4 ml g^{-1} ventricle at 0°C; Axelsson et al., 1992). This *in situ* evidence for an enhanced pumping capacity of the winter flounder heart is supported by the pressure-volume curves (Fig. 2.2), which show that the flounder ventricle is able to fill to a maximum volume of ~2.1 ml g^{-1} ventricle (~0.8 ml). The high maximum Q (60.3±4.1 ml kg^{-1} min^{-1}) and V_S (1.1±0.07 ml kg^{-1}) reported for the flounder heart may seem surprising, considering the winter flounder's benthic and relatively

inactive lifestyle (Fletcher, 1975; Moyle and Cech, 1996) and the low values reported for aerobic capacity in flatfishes (Duthie, 1982; Lefrançois and Claireaux, 2003). However, flatfish are also reported to have lower haematocrit levels (≤20%; Wood et al., 1977, 1979; Duthie, 1982; Turner and Wood, 1983) compared with other teleosts (e.g. 24–30% for cod and salmonids; Milligan and Wood, 1987; Plante et al., 1998; Gallaugher et al., 2001; Deitch et al., 2006). Thus, it is likely that this enhanced cardiac function offsets the effects of reduced hematocrit on blood oxygen transport and thus allows these fish to achieve moderate levels of activity (critical swimming speed of 0.73 bl s^{-1} at 10°C; Joaquim et al., 2004). Further, the large V_S in flounder may be advantageous during severe hypoxia (e.g. <20% O_2 saturation), a situation where f_H is reduced by 41% of normoxic conditions (see Chapter 4). This latter point may be particularly important for coastal flatfishes like the flounder, which can periodically face hypoxia due to high nutrient loading (Pereira et al., 1999; Tallqvist et al.; 1999) and can be found buried several centimeters (12–15 cm) into the substrate (Fletcher, 1975).

Previous authors have reported that maximal values for Q in rainbow trout, determined using the *in situ* perfused heart preparation, are within ~20% of the highest *in vivo* Q values measured during prolonged swimming (e.g. Farrell et al., 1986; Thorarensen et al., 1996; Claireaux et al., 2005). Our *in situ* Q max values for the Atlantic salmon and cod (~63 ml min^{-1} kg^{-1}) are within 2 and 29% of those obtained for these two species when swum to exhaustion (Atlantic salmon, 63.8 ml min^{-1} kg^{-1}; Deitch et al., 2006; cod, 44.5 ml min^{-1} kg^{-1}; L. H. Petersen and A. K. Gamperl, unpublished data), and thus our data are consistent with those for the rainbow trout. In contrast, the maximum Q (60.3 ml min^{-1} kg^{-1}) and V_S (2.3 ml g^{-1} ventricle) I measured are more than 50% higher

than the values reported for the winter flounder by Joaquim et al. (2004) during a critical swimming speed (U_{crit}) test at 10°C (39.2 ml min^{-1} kg^{-1} and 1.51 ml g^{-1} ventricle, respectively). The reason(s) for the discrepancy is (are) unknown. However, the difference may be related to the inability of the flounder heart to deliver blood to the circulation at high pressures. First, while maximum P_H values for the cod and the salmon hearts were measured at ~6 and 8 kPa, respectively, the flounder ventricle was unable to completely empty at arterial pressures above 4.8 kPa. Second, arterial pressures (afterload) increase considerably (by 25 to 65%) when fish are forced to swim at or near maximal speeds (Axelsson and Nilsson, 1986; Jones and Randall, 1978), and Joaquim et al. (2004) showed that cardiac parameters in the flounder were at maximal levels at slow swimming velocities and remained constant until U_{crit}. Thus, these data suggest that the flounder heart cannot deal with the high-pressure demands of continuous exercise and that flounder are unable to fully exploit the flow potential of their hearts while swimming.

Atrial filling in fishes is achieved by *vis-à-fronte* and *vis-à-tergo* mechanisms. In *vis-à-fronte* filling, the energy of ventricular contraction creates a subambient intrapericardial pressure, and consequently, a negative atrial transmural pressure gradient that is used to distend the atrium and thus assist in its filling (Farrell, 1991, 1993; Farrell and Jones, 1992). In contrast, *vis-à-tergo* filling of the atrium is dependent on central venous pressure, as well as potentially contraction of the sinus venosus and atrium. It was suggested, for many fish species with a rigid pericardium that *vis-à-fronte* filling was the primary determinant of Q under resting conditions and that at higher V_S there was a transition from *vis-à-fronte* to *vis-à-tergo* (venous pressure) filling (Farrell et al., 1988; Farrell and Jones, 1992). In contrast, recent work by Minerick et al. (2003) suggests that,

at least for the rainbow trout, *vis-à-tergo* filling is the primary determinant of cardiac filling and that *vis-à-fronte* filling is only important in situations, such as high-intensity exercise in which elevated cardiac output is required. My results do not add to the debate about which of these two mechanisms is dominant at rest or during situations demanding elevated cardiac performance in salmonids. However, they support the present dogma that *vis-à-fronte* filling is only present in active (non-benthic) species (Farrell and Jones, 1992) and strongly suggests that *vis-à-fronte* filling is not a requirement for achieving high values of V_S. During my study, resting V_S could be achieved at subambient filling pressures in the cod and Atlantic salmon (both active pelagic species), whereas a positive P_{IN} of 0.04 kPa was needed for resting Q values in the winter flounder (Fig. 2.1*A*). This requirement for a positive input pressure to achieve resting Q in flounder is consistent with studies which reported that *in situ* eel (*Anguilla dieffenbachii*; Davie et al., 1992; Franklin and Davie, 1991), sea raven (*Hemitripterus americanus*; Farrell et al., 1982), and ocean pout (*Macrozoarces americanus*; Farrell et al., 1983) hearts were unable to maintain resting levels of Q at subambient filling pressures. In these previous studies on benthic (inactive) teleosts, maximum values for V_S and Q were $\leq$0.6 ml kg^{-1} and 20-30 ml min^{-1} kg^{-1}, and thus it appeared that *vis-à-fronte* filling was required for the high cardiac performance exhibited by more active teleosts such as the trout (maximum values ~1.0 ml kg^{-1} and $\geq$50 ml min^{-1} kg^{-1} at similar temperatures; Thorarensen, 1996; Farrell et al., 1988; Claireaux et al., 2005). Clearly, my results for the in situ flounder heart suggest that this is not the case and that high cardiac outputs can be achieved by *vis-à-tergo* filling mechanisms alone.

At present, I do not have an explanation for why the flounder heart is not capable of filling through *vis-à-fronte* mechanisms. First, the flounder pericardium is not saclike, is relatively rigid, and is closely associated with the body wall and musculature. Thus, it is morphologically similar to the pericardium found in salmonids and not benthic species such as the eel (Farrell and Jones, 1992; Franklin and Davie, 1991). Second, Farrell and Jones (1992) indicate that for the atrium and sinus venosus to act as variable-volume reservoirs within a rigid (semirigid) pericardium, thus facilitating *vis-à-fronte* filling, the maximum end-diastolic volume of these chambers must be equal to the maximum V_S plus the difference between resting and maximum V_S (Forster and Farrell, 1994). Although I did not construct pressure-volume curves for the flounder's sinus venosus, the maximum diastolic volume of the flounder atrium is equal to maximum V_S (1.1 ml kg^{-1}), and in the trout, maximum diastolic volume of the sinus venosus is 75% of that for the atrium (Forster and Farrell, 1994). Thus, it is likely that the combined maximum diastolic volumes of the flounder's sinus and atrium are sufficient to meet maximum pumping demands.

As with most active fish, the salmon ventricle is composed of a spongy layer and an outer compact layer of myocardium (30–45% of myocardial mass; Deitch et al., 2006; Santer and Greer Walker, 1980); the latter generally considered to be important for the enhanced cardiac performance required by active fish species. Thus, it was not surprising that the salmon had a significantly higher maximum P_H (9.7 mW g^{-1}) when compared with the cod (7.8 mW g^{-1}) and the flounder (7.6 mW g^{-1}). It was somewhat unexpected that the cod (a benthopelagic species) and the flounder (a benthic and relatively sedentary species) would have similar values for maximum P_H. However, the hearts of both of these

species are composed entirely of spongy myocardium, and Fig. 2.1*B* shows that the flounder heart reaches maximum P_H at a significantly lower output pressure (4.9 kPa) than the cod (6.2 kPa). Thus, the similarity in P_H values is due to the enhanced flow capacity of the flounder heart.

2.5.2 Mechanisms allowing for enhanced cardiac function in flounder

Through this study, I have begun to elucidate how a species with a RVM 30–50% smaller than most salmonids and other pelagic species (Axelsson and Nilsson, 1986; Thorarensen et al., 1996; Gallaugher et al., 2001) can achieve comparable levels of body mass-specific V_S and Q. First, I show that the flounder heart has a more pronounced Starling curve, meaning that it needs smaller increases in preload to achieve similar, or even higher, values of V_S. This greater sensitivity of the flounder heart to filling pressure undoubtedly reflects the high distensibility/compliance of its chambers (Fig. 2.2, Table 2.2) and the fact that *in vivo* end-systolic volume is normally zero at physiological output pressures (Franklin and Davie, 1992). For example, the flounder atrium only requires 18–23% of the *in vitro* input pressure required by cod and salmon to reach equivalent diastolic volumes, and flounder ventricular pressures at the cod's maximum diastolic volume (0.46 ml) are only ~30% of that recorded in the other two species. This increased distensibility/compliance may, in fact, compensate for the lack of *vis-à-fronte* filling by still allowing the flounder to rapidly attain large end-diastolic volumes, and increase V_S in situations demanding elevated cardiac performance.

Second, I report that there are several features of the flounder's bulbus arteriosus that would allow the heart to effectively deliver its enhanced end-diastolic volume into

the circulation. The primary function of the bulbus arteriosus is to depulsate the blood ejected from the ventricle, permitting an almost continuous blood flow in the ventral aorta and gills, and to minimize increases in ventral aortic pressure that are associated with ventricular ejection (Farrell, 1979; Jobling, 1996). The bulbar pressure-volume relationship is shifted downward for the winter flounder compared with the other two species at all chamber volumes, and the flounder's bulbus is most compliant over a range of pressures from ~2.5 to 5 kPa, compared with ~5 to 8 kPa for the cod and salmon (Fig. 2.2*C*). Further, the winter flounder has a B:V ratio (0.59) ~3 times larger than the salmon (0.22) and ~2 fold larger than that of cod (0.37) (Table 2.3). Thus, it appears that the flounder's bulbus has adapted, both in terms of compliance/distensibility and size, to permit large V_S at pressures that do not become limiting to ventricular function. This conclusion is consistent with Clark and Rodnick (1999), who proposed that alterations in ventricular function should be matched by morphofunctional changes in the bulbus and suggested that a disjoint between ventricular and bulbar function in mature male rainbow trout led to hypertension and promoted ventricular hypertrophy. In addition, this would at least partially explain why flounder have arterial pressures of ~3 kPa (Cech 1976, 1977; Wood et al., 1979), considerably less than measured in most other teleosts (approx. 3.9 to 5.3 kPa; Farrell and Jones, 1992).

Interestingly, f_H decreased by 14 beats min^{-1} (21%) in the flounder compared with 12% and 8.6% in the cod and salmon, respectively, between resting and maximum levels of cardiac performance (Table 2.1). A decrease in f_H with increased preload, was not unexpected, as the fish's pacemaker is located at the sinoatrial junction and stretch sensitive (Lillywhite et al., 1999), and an increase in end-diastolic volume would increase

the degree of myocardial stretch and consequently promote a drop in f_H. Further, one might have expected a larger decrease in f_H in the flounder, as its heart is considerably smaller compared with the other two species, and thus equivalent increases in V_S (ml kg^{-1}) would result in greater myocardial stretch compared with the cod and salmon. However, this difference in f_H responsiveness does not explain why maximum V_S was greater in the flounder or why this species had a steeper Starling curve. For example, while the flounder's f_H was ~19% lower than measured in the salmon at maximum Q, the flounder's maximum V_S (ml g^{-1} ventricle) was 65% higher. In addition, f_H at maximum Q was not significantly different between the cod and flounder (Table 2.1, Fig. 2.1).

Although my study provides some information on how the flounder heart achieves such high stroke volumes, further investigation is required to: (*1*) ascertain the cellular basis behind the steepness and extension of the flounder Starling curve and (*2*) determine whether alterations in myocardial contractility/myofilament Ca^{2+} sensitivity, in addition to bulbus morphophysiology and low arterial blood pressures, permit the flounder ventricle to deliver such large stroke volumes into the circulation. On the basis of the research presented here, one could hypothesize that for a specific length, flounder myocytes have a decreased passive tension and an increased active tension compared with the trout and cod. Indeed, the enhanced distensibility of the flounder's heart chambers, at least when compared with the cod heart, which also lacks a compact myocardium, may well be related to changes in myocardial connective tissue (e.g., titin and collagen) content or isoform (Granzier and Irving, 1995; Wu et al., 2000). Further, research suggests that low resting tensions and length/stretch-dependent increases in myofilament Ca^{2+} sensitivity are important cardiomyocyte features in fish species such as the trout,

which are capable of large increases in V_S as compared with mammals (Shiels et al., 2006). However, such an adaptation in myocyte physiology may not be required in the flounder, compared with the trout. The trout/salmon heart has a distinct lumen, and an outer layer of compact myocardium that is largely responsible for its enhanced pressure generating capacity (Franklin and Davie, 1992). Thus, the Law of Laplace would apply to the whole trout heart and make it difficult for this species to develop enough wall tension at very large stroke volumes, so that zero or minimal end-systolic volumes are maintained. In contrast, the flounder heart has essentially no lumen and is composed entirely of spongy myocardium. Because the radius of the lacunae comprising this spongy myocardium is small, Laplacian relationships dictate that pressure may be generated at "considerable mechanical advantage compared with hearts consisting solely or partially of compact myocardium" (Farrell and Jones, 1992).

Overall, this work shows that the V_S measured in the winter flounder (per gram of ventricle) is extremely high and that this high V_S is related to: (*1*) a pronounced and extended Starling curve; (*2*) more compliant heart chambers; and (*3*) a high bulbus:ventricle mass ratio. My data support the *in vivo* data of Joaquim et al. (2004), which showed that the cardiovascular system of flatfish is a high volume, low-pressure design.

However, this also raises several questions whose answers may lead to significant advances in our understanding of fish cardiac physiology. Thus, I plan to perform single cardiac myocyte length-tension curves to examine the passive (between contractions) and active (during contraction) properties of flounder cardiac muscle and thus determine

whether these cells possess unique physiological adaptations that allow for easy ventricular expansion (filling), yet the development of substantial contractile force.

2.6 Acknowledgments and grants

The authors are grateful to Dr. Trevor Avery for statistical advice and to AquaBounty (Canada) for supplying the Atlantic salmon used in this research. Three anonymous reviewers are also thanked for their constructive comments.

This work was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery grant to A. K. Gamperl and an NSERC Industrial Postgraduate Scholarship scholarship to E. J. Deitch. Paula C. Mendonça was supported by a doctoral fellowship SFRH/BD/8082/2002 from Portuguese Foundation for Science and Technology (FCT) co-financed through POCI 2010 (Programa Operacional Ciência e Inovação) and FSE (Fundo Social Europeu).

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3. Nervous and Humoral Control of Cardiac Performance in the Winter Flounder (*Pseudopleuronectes americanus*)

3.1 Abstract

Previous studies have suggested that flatfish lack adrenergic cardiac innervation and have a limited humoral adrenergic stress response. However, data on neurohormonal control of flatfish cardiac function is scarce, and has never been directly studied *in vivo*. Hence, I (1) injected neural and humoral antagonists into flounder (*Pseudopleuronectes americanus*) *in vivo* to determine the contribution of autonomic innervation and circulating catecholamines to the control of resting cardiac function; (2) measured pre- and post-stress (90 s chase) catecholamine levels in this species; and (3) constructed *in vivo* catecholamine dose–response curves for cardiovascular function based on the results of the second experiment. In addition, I quantified the density (B_{max}) and ligand-binding affinity (K_d) of flounder ventricular cell-surface β-adrenoreceptors, and established whether they were of β_1 or β_2 subtype using pharmacological antagonists. The cholinergic contribution to resting flounder heart rate was comparable to other teleosts (cholinergic tonus 26%). Interestingly, however, bretylium increased heart rate, resulting in a negative resting adrenergic tonus (-11.9%), and I was unable to demonstrate that catecholamines supported cardiac function at rest or at circulating concentrations approximating those following an exhaustive chase (adrenaline, 21 nM; noradrenaline, 14 nM). Myocardial B_{max} was very high in the flounder (252.8 fmol mg^{-1} protein), and it appears that flounder ventricular β-adrenoreceptors are predominantly of the β_2 subtype [based on the inability

of atenolol to displace [^{3}H]CGP from the β-adrenoreceptors, and the IC_{50} value for ICI 118551 ($1.91X10^{-6}$ mol l^{-1})]. However, the extremely low affinity (K_d 1.02 nM) for these receptors raises the possibility that the flounder heart is also populated by a significant number of β_3-adrenoreceptors.

3.2 Introduction

Numerous studies have examined the nervous and humoral control of the fish heart. For instance, myxinoid cyclostomes have aneural hearts (Laurent et al., 1983; Axelsson et al., 1990; Taylor et al., 1999) and, in elasmobranchs, nervous control of the heart is mainly attributed to the degree of cholinergic vagal tonus (Taylor et al., 1999; Agnisola et al., 2003). In most teleosts, the heart is innervated by both inhibitory parasympathetic fibres, running in the vagus nerve, and postganglionic sympathetic fibres, reaching the heart directly or *via* the vagus (Donald and Campbell, 1982; Laurent et al., 1983; Farrell and Jones, 1992). Humoral adrenergic stimulation of the heart is also generally important and is mediated by catecholamines released from the chromaffin tissue into the circulatory system (Gamperl et al., 1994*c*; Reid et al., 1998). Resting levels of plasma catecholamines [adrenaline (A) and noradrenaline (NA)] are usually less than 10 nM. However, in response to severe stress, the concentration of circulating catecholamines can increase dramatically (to levels in excess of 300 nM), promoting metabolic and circulatory adjustments to cope with increasing energetic demands (Randall and Perry, 1992; Gamperl et al., 1994*c*; Perry and Gilmour, 1999).

Interestingly, the potential for catecholamines to modulate cardiac performance varies with the severity and type of stressor, and among species. For instance, in Atlantic

cod (*Gadus morhua*), A and NA concentrations increase by 11.5-fold and 5.6-fold, respectively, at this species' critical swimming speed (U_{crit}) (Butler et al., 1989), whereas no change in plasma catecholamine concentrations was found when cod were swum at only two-thirds of U_{crit} (Axelsson and Nilsson, 1986). Increases in A and NA, of 46-fold and 11.5-fold, respectively, were reported in rainbow trout (*Oncorhynchus mykiss*) after 10 min of chasing (Tang and Boutilier, 1988), while chasing till exhaustion can raise A and NA by as much as 92-fold and 20-fold, respectively (Perry et al., 1996). Circulating catecholamine concentrations also differ greatly between fish species and, in general, species with a more active lifestyle exhibit greater increases in plasma catecholamine levels when exposed to stressors as compared with benthic/sluggish species. Thus, it is not surprising that maximum A and NA levels in the sea raven (*Hemitripterus americanus*; a North Atlantic benthic species) after 1 min of air exposure followed by 1 min of chasing are only ~8 nM (Vijayan and Moon, 1994), and that A and NA levels are only ~30 and 37 nM, respectively, in the starry flounder (*Platichthys stellatus;* Milligan and Wood, 1987) after 10 min of chasing. By contrast, the A concentration in rainbow trout can be as high as 275 nM after being chased to exhaustion (Perry et al., 1996), and circulating A concentrations of 565 nM have been found in Atlantic cod after exposure to severe acute hypoxia (L. H. Petersen and A.K.G., unpublished data).

Flatfishes (order Pleuronectiformes) are unique because, in contrast to most teleosts, electrophysiological and histochemical studies suggest that adrenergic cardiac innervation is absent (Cobb and Santer, 1973; Donald and Campbell, 1982; Ask, 1983). Furthermore, flounder appear to have a limited humoral adrenergic stress response (Milligan and Wood, 1987), a characteristic probably related to their benthic and inactive

lifestyle (Pereira et al., 1999). Given the lack of cardiac sympathetic innervation and the low post-stress circulating catecholamine levels reported, and that neurohormonal control of flatfish cardiac function has never been directly studied, it is not clear how this taxa regulates cardiac function. Thus, the purpose of this study was to determine how *in vivo* cardiovascular function is regulated in the winter flounder (*Pseudopleuronectes americanus* Walbaum 1792) by nervous and humoral mechanisms. To accomplish this a number of experiments were undertaken: (1) a series of neural and humoral antagonists were used to determine the contribution of autonomic innervation and circulating catecholamines to the control of *in vivo* heart function; (2) maximal post-stress circulating catecholamines were measured in the flounder following two different stressors (a 60 s net stress and a 90 s chase); (3) *in vivo* dose–response curves for catecholamines (A and NA) were produced to examine the ability of circulating catecholamines to stimulate the winter flounder heart; and (4) flounder ventricular β-adrenoreceptors were typed and quantified, to better understand how β-adrenoreceptors relate to, and mediate, the effects of catecholamines on the heart of this species.

3.3 Material and methods

3.3.1 Animals

Ethical approval for the following studies was obtained from the Animal Care Committee at Memorial University (protocol # 05-02-KG). Winter flounder were caught by divers using a hand net in Conception Bay (Newfoundland, Canada) at a depth of 4-6 m and transported to the Ocean Sciences Centre (OSC, Memorial University of Newfoundland). Flounder were acclimated to 8±1°C for at least 4 weeks prior to

experimentation in 1200 l rectangular fibreglass tanks supplied with aerated seawater and natural photoperiod. Fish were fed three times a week with commercial pellets, but were fasted for 24h prior to surgery.

3.3.2 *In vivo* experiments

3.3.2.1 Surgery

Fish were anaesthetized (average mass 0.46±0.13 kg) in seawater containing methane sulfonic acid of *m*-aminobenzoate (MS-222; 0.25 g l^{-1}), and then transferred to a surgical table, where their gills were irrigated with chilled (~4°C) and oxygenated seawater containing 0.1g l^{-1} MS-222.

3.3.2.1.1 Implantation of flow probe

Implantation of the blood flow probe was performed as previously described by Crocker et al. (2000) for white sturgeon, with some modifications. In this procedure, the gills and operculum were retracted using umbilical tape (Baxter Healthcare Corporation, Deerfield, IL, USA) which was passed from a hole behind the fourth gill arch into the opercular cavity, the ventral aorta was exposed through a small incision (~0.5 cm) in the isthmus without disrupting the pericardium, and a flow probe (1.5RB, Transonic Systems; Ithaca, NY, USA) was placed loosely around the vessel. Finally, after verifying the quality of the cardiac output signal, the probe lead was sutured to the eyed side of the fish using 3–0 silk (American Cyanamid Company, Pearl River, NY, USA) at three locations.

3.3.2.1.2 Cannulation of caudal artery

Cannulation of the caudal artery for the measurement of dorsal aortic pressure was performed as previously described by Cech and Rowell (1976) with modifications. Briefly, a 2 cm long incision was made, just below the lateral line at about one third of the animal's length from the tail. The skin and underlying muscle tissue were then retracted to expose the caudal artery which lies between the haemal arches, and a heparinized cannula (PE 50, Clay Adams, Parsippany, NJ, USA; 80 cm long, volume 0.2 ml) with indwelling 14 gauge piano wire was inserted into the vessel. Finally, after removing the indwelling wire, and pushing the cannula approximately 8 cm anteriorly into the artery, the incision was closed with a continuous suture, and the cannula was filled with heparinised saline (181.3 mM NaCl, 5.0 mM KCl, 2.30 mM $CaCl_2$ $2H_2O$, 1.99 mM $MgSO_4$ $6H_2O$, 2.58 TES acid {*N*-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid} and 7.33 mM sodium TES base {*N*-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid sodium salt}) with 100 i.u. ml^{-1} heparin, and sutured to the fish's dorsal surface at two locations. There was minimal bleeding during cannula implantation, and the cannula was flushed regularly with heparinized saline to prevent clot formation.

Recovery from anaesthesia was initiated after surgery by artificially ventilating the fish with aerated, anaesthetic-free water. Once ventilatory activity had returned, the fish were placed into an opaque 45l cooler supplied with aerated 8°C seawater and filled with ~5 cm of sand. The flounder were then allowed to recover for at least 24 h prior to experimentation.

3.3.2.2 Experimental procedures

3.3.2.2.1 Neural control of cardiac function

Following the recovery period, cardiac output ($\dot{Q}$), dorsal aortic pressure (P_{DA}) and heart rate (f_H) were recorded for 1 h. After this initial recording period, a series of drugs were injected every 1 h 30 min in the following order: 1.2 mg kg^{-1} atropine sulphate (muscarinic receptor antagonist); 10 mg kg^{-1} bretylium tosylate (adrenergic nerve blocker), 213 μg kg^{-1} (RS)-atenolol (β_1- adrenoreceptor antagonist) and 250 μg kg^{-1} ICI 118551 hydrochloride (β_2-adrenoreceptor antagonist). These drug concentrations were selected based on previous fish studies (Smith et al., 1985; Altimiras et al., 1997; van Heeswijk et al., 2005), and all drugs were injected slowly (over approx. 15 s) through the caudal artery in a concentrated form using a 1 ml kg^{-1} carrier volume of saline. This initial injection was followed by an injection of enough saline (~0.3 ml) to ensure complete delivery of the drug into the animal.

3.3.2.2.1 Chasing and net stress

In order to determine maximal post-stress circulating catecholamines and haematocrit levels, two groups of flounder were used in which no surgical procedure was performed. One group of flounder (n=8) was held in a net for 60 s, while another group (n=8) was chased to exhaustion prior to sampling. In the chasing procedure, each fish was caught individually from their holding tanks using a net and chased immediately in a rectangular tank (1 m x 1 m x 0.5 m) for 90 s using a small wooden prod. Immediately after these procedures were finished, blood samples were taken from the flounder (0.6-0.9

ml) for the measurement of post-stress haematocrit and plasma catecholamine (A and NA) levels by caudal puncture. For the measurement of catecholamines, the blood was immediately placed into a chilled 1.5 ml Eppendorf centrifuge tube, and centrifuged at 10,000*g* for 30 s to obtain plasma. The plasma was then pipetted into a 1.0 ml cryovial containing glutathione and EDTA (5 µl of 0.2 M glutathione and 5 µl of 0.2 M EDTA per 100 µl of plasma), and immediately frozen in liquid N_2. All plasma samples were then stored at –80°C until analysis.

3.3.2.2.3 Catecholamine dose-response curves

To assess the sensitivity of the flounder cardiovascular system to circulating catecholamines, dose-response curves for A and NA were generated from *in vivo* measurements. As described above, fish (n=7) were implanted with a ventral aortic flow probe and a caudal artery cannula, and then allowed to recover for approx. 24 h. After recovery, Q, P_{DA} and f_H were recorded for 1 h, and then each fish was given a series of five 1 ml kg^{-1} saline injections. Each saline injection was separated by 1 h to assess the potential effects of haemodilution on the flounder's cardiovascular function, and thus discern sham injection effects from drug effects (see below). Subsequent to the saline injections, the flounder were allowed to recover for 18 h, after which they were injected with 0.1 and 0.05 (dose 1), 0.15 and 0.075 (dose 2), 0.2 and 0.1 (dose 3), 0.3 and 0.15 (dose 4), and finally 0.4 and 0.2 µg kg^{-1} (dose 5) of A and NA, respectively. These injections were given at 1 h intervals (an inter-injection period that allowed cardiovascular variables to return to resting values), and all doses of A and NA were injected slowly (over approx. 15 s) through the caudal artery in a concentrated form using

a 1 ml kg^{-1} carrier volume of saline. These concentrations were selected based on the maximum concentrations of A (21 nM) and NA (14 nM) found in winter flounder after chasing, and the relationship between injected dose and plasma A levels established by Gamperl et al. (1994*b*) for rainbow trout at similar temperatures. Cardiovascular variables and P_{DA} were measured for 10 min before and 45 min after each injection. Blood samples were also taken prior to the first injection (0.5 ml) and 2 min after the last injection (0.8 ml) for the determination of plasma A and NA concentrations induced by the injection of 0.4 and 0.2 μg kg^{-1} (dose 5) of A and NA, respectively. Immediately upon collection, blood samples were processed using the same protocol as in the chasing and net stress experiments.

3.3.2.3 Data analysis

3.3.2.3 Instrumentation for cardiovascular measurements

Dorsal aortic pressure (P_{DA}, in kPa) was measured using a Gould Statham pressure transducer (Model P23 ID, Oxnard, CA, USA) that was calibrated daily against a static water column, where zero pressure (0 kPa) was set equal to the water level in the experimental chamber. Cardiac output (Q; ml min^{-1} kg^{-1}) was monitored by connecting the flow probe lead to a small animal blood flow meter (Model T206, Transonic® Systems, Ithaca, NY, USA). Both pressure and flow signals were amplified and filtered using a Model MP100A-CE data acquisition system (BIOPAC Systems, Santa Barbara, CA, USA), and analyzed and stored using Acq*Knowledge* Software (BIOPAC Systems, Santa Barbara, CA, USA) installed on a 300 MHz Toshiba laptop computer.

3.3.2.3.2 Calculation of cardiovascular parameters

Cardiovascular function was continuously monitored throughout the 'neural control of cardiac function' and 'dose-response curve' experiments by measuring Q and P_{DA}. Heart rate (f_H; beats min^{-1}) was calculated by measuring the number of systolic peaks during 20–30s intervals. Mass specific stroke volume (V_S; ml kg^{-1}), was calculated as: V_S=cardiac output (ml min^{-1} kg^{-1})/heart rate (beats min^{-1}). Systemic vascular resistance (R_{sys}; kPa ml^{-1} min kg) was calculated as: dorsal aortic pressure (kPa)/cardiac output (ml min^{-1} kg^{-1}).

The 'intrinsic' (after the administration of all drugs) f_H (f_{Hint}; beats min^{-1}), cholinergic tonus (% f_{Hch}; %) and adrenergic tonus (% f_{Had}; %) were calculated for the winter flounder heart as described by Axelsson (1988):

$$\% f_{Hch} = [(f_H \text{ after atropine} - f_H \text{ before atropine}) / f_{Hint}] \times 100, \quad (1)$$

$$\% f_{Had} = [(f_H \text{ after atropine} - f_{Hint}) / f_{Hint}] \times 100. \quad (2)$$

3.3.2.3.3 Catecholamine analysis

The plasma catecholamines, adrenaline (A) and noradrenaline (NA), were measured using high performance liquid chromatography (HPLC; Bioanalytical Systems, West Lafayette, IN, USA) with electrochemical detection (LC Epsilon® detector, model E5, Bioanalytical Systems) after extraction with alumina (Woodward, 1982). For amine separation, a ODS reverse phase column (3.0 mm ID X 10 cm long, 3 μm pore size, model MF 8954) was used in conjunction with an aqueous mobile phase (containing per litre: 7.088 g monochloroacetic acid, 186.1 mg Na_2EDTA $2H_2O$, 15 ml acetonitrile and 232.3 mg sodium octyl sulphate, pH 3.00–3.05), pumped (PM 80, BAS) at a flow rate of

1 ml min^{-1}. DHBA (3,4-dihydroxybenzylamine) was used as an internal standard for all plasma samples and catecholamine standards. The recovery of DHBA (3,4-dihydroxybenzylamine) from the alumina was in the order of 50–80%, and was used to determine individual plasma catecholamine concentrations. The output from the detector was collected and analysed using a computer running ChromGraph Control and ChromGraph Report version 2.30 software (Bioanalytical Systems).

3.3.2.3.4 Chemicals

Components of the saline were purchased from Fisher Scientific (Fairlawn, NJ, USA), with the exception of TES salt, which was purchased from Sigma Chemical Co. (St. Louis, MO, USA). MS-222 was purchased from Syndel Laboratories (Vancouver, BC, Canada). Atropine sulphate salt, bretylium tosylate, (RS)-atenolol, ICI 118551 hydrochloride, (±) timolol, adrenaline bitartrate, noradrenaline bitartrate and all chemicals used in catecholamine extraction and analysis were also purchased from Sigma Chemical Company.

3.3.3 *In vitro* experiments

3.3.3.1 Cardiac β-adrenoreceptors

The punch-technique for the measurement of ventricular β-adrenoreceptors was performed as previously described by Gamperl et al. (1994*a*) for rainbow trout. Flounder (average mass 0.55±0.03 kg) were killed by a blow to the head, and the heart was quickly removed and allowed to beat for approximately 1 min in cold (0–2°C) saline to remove

erythrocytes from the ventricular lumen. The ventricle was then quickly removed, cut in half and frozen (in ~2 min) onto the tissue chopper (McIlwain tissue chopper, Brinkmann, Mississauga, ON, Canada) stage before being sliced into 400 μm thick cross sections. The tissue slices were then placed in a Sylgard-coated tissue culture dish filled with ice-chilled saline, and ventricular tissue punches (2 mm diameter, 0.9–1.25 mg) were taken from the slices using a sample corer (Fine Science Tools, Vancouver, BC, Canada). Finally, individual punches were placed in separate wells of a tissue culture plate (Becton Dickinson and Company, Franklin Lakes, NJ, USA), with each well containing 500 μl of saline.

β-Adrenergic receptor density and affinity were measured by incubating the punches in varying concentrations (0.05–2 nM) of the hydrophilic β-antagonist [^{3}H]CGP-12177 (specific activity 37 Ci mM; Amersham Biosciences, Amersham, Bucks, UK) for 2 h. Non-specific binding (NSB) was measured in the presence of 10^{-5} M timolol (β-adrenoreceptor antagonist), and was subtracted from total counts to determine specific binding. To express B_{max} (i.e. the density of β-adrenoreceptors on the cell surface) as fmol μg^{-1} protein, the protein content of representative punches was determined using a Bradford protein assay (Coomassie Plus, Pierce Biotechnology, Rokford, IL, USA) with bovine serum albumin (ICN Biomedical, Aurora, OH, USA) as a standard. During the incubation period all tissue culture plates were kept on ice (i.e. at 0°C) and covered with aluminum foil to prevent the photodegradation of [^{3}H]CGP and its competitors. Following incubation, aliquots of buffer were removed to determine the free concentration of [^{3}H]CGP-12177, and the tissue punches were washed twice in ice-chilled saline and placed into 7 ml scintillation vials containing 5 ml of Ecolume (ICN Canada,

Montreal, QC, Canada). All scintillation vials were shaken and allowed to sit for at least 18h prior to counting. Radioactivity was quantified using a liquid scintillation counter (Packard Tri-Carb 2100TR; Meriden, CT, USA). For the determination of [^{3}H]CGP-binding specificity (i.e. the construction of competition curves), punches were incubated with 1.5 nM [^{3}H]CGP and with various concentrations (10^{-4}-10^{-9} M) of atenolol (β_1-adrenoreceptor antagonist) or ICI 118551 (β_2-adrenoreceptor antagonist).

The small size of the winter flounder ventricle required the pooling of tissue punches from two individuals to construct each binding curve. To assess the specific binding of [^{3}H]CGP-12177 to ventricular β-adrenoreceptors, a total of eight binding curves were obtained and each ligand concentration had three (only at 0.05 nM) to six replicates. For both atenolol and ICI 118551 competition curves, a total of six curves were constructed and each competitor concentration had five to six replicates.

3.3.4 Statistical analyses

The reported variables are expressed as means±standard error of the mean (s.e.m.). Univariate general linear models were used to test for significant differences between the saline and drug injection groups and between the saline and catecholamine injection groups. When a significant difference between groups was detected, one-way ANOVAs were performed to test for differences between values obtained with saline and corresponding drug/catecholamine injections, and contrasts were performed to test for significant differences within groups. Differences between circulating catecholamine concentrations measured at rest, and after net stress, chasing and catecholamine dose 5 were assessed using one-way ANOVAs. Saturation-binding curves for CGP were

analysed, and values for K_d ([^{3}H]CGP dissociation constant; in nM) and B_{max} (fmol μg^{-1} protein) were determined using Scatchard plots as described by Zivin and Waud (1982). Competition curves were fitted, and IC_{50} values (i.e., the concentration of ligand that reduced [^{3}H]CGP binding by 50%) were determined using SigmaPlot Software for Windows version 10.0 (Systat Software, Chicago, IL, USA). All statistical analyses were performed using SPSS software for Windows version 15.0 (SPSS, Chicago, IL, USA) and $P<0.05$ was used as the level of statistical significance.

3.4 Results

3.4.1 *In vivo* experiments

3.4.1.1. Neural control of cardiac function

Cardiovascular parameters reached stable values at approx. 20 min after the injection of drugs, and the repeated injection of saline had only minor effects on cardiovascular parameters measured at 1h post-injection, indicating that the observed changes in cardiac function, P_{DA} and R_{sys} were directly related to the effects of the various drugs (Figs. 3.1 and 3.2). A significant positive chronotropic effect was induced by the administration of atropine, with f_H increasing from 25.3 to 36 beats min^{-1}. This increase in f_H was coincident with a significant decrease in V_S (from 0.41 to 0.3 ml kg^{-1}), and thus Q remained constant at approx. 10 ml min^{-1} kg^{-1} following atropine injection (Fig. 3.2). Interestingly, bretylium administration did not result in a decrease in f_H, but a further increase to 40 beats min^{-1}, and had no significant effects on either V_S or Q. Finally, none

of the measured cardiac parameters were affected by the injection of the β_1- and β_2-adrenoreceptor blockers (atenolol and ICI 118551, respectively). Based on the changes in f_H following administration of the various drugs, intrinsic f_H, and cholinergic and adrenergic nervous tones were calculated to be 40.9 beats min^{-1}, 26.1% and –11.9%, respectively.

P_{DA} increased from 3.03 to 3.37 kPa (by 11%) following atropine injection and, because Q was unchanged by administration of this pharmacological blocker, it was clear that this change in P_{DA} was directly related to an atropine-induced increase in systemic vascular resistance (R_{sys} increasing from 0.32 to 0.35 kPa ml^{-1} min kg). Neither P_{DA} nor *R*sys were affected by bretylium, atenolol or ICI 118551 (Fig. 3.2).

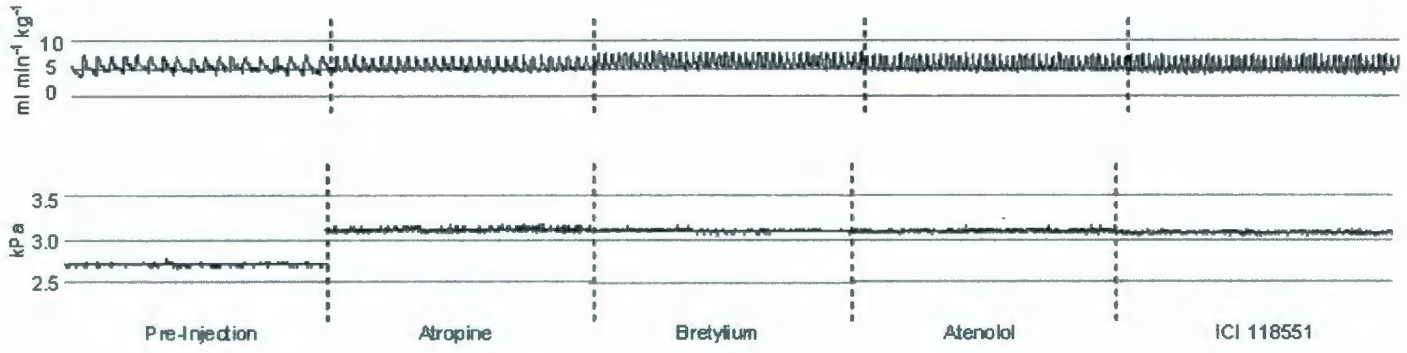

Figure 3.1 Cardiac output (ml min^{-1} kg^{-1}) and dorsal aortic pressure (kPa) traces for one winter flounder. These traces are approximately 30 s in duration and were obtained 1 h after each drug injection. The drugs were sequentially injected every 1 h 30 min in the following order: atropine (1.2 mg kg^{-1}), bretylium (10 mg kg^{-1}), atenolol (213 μg kg^{-1}) and ICI 118 551 (250 μg kg^{-1}).

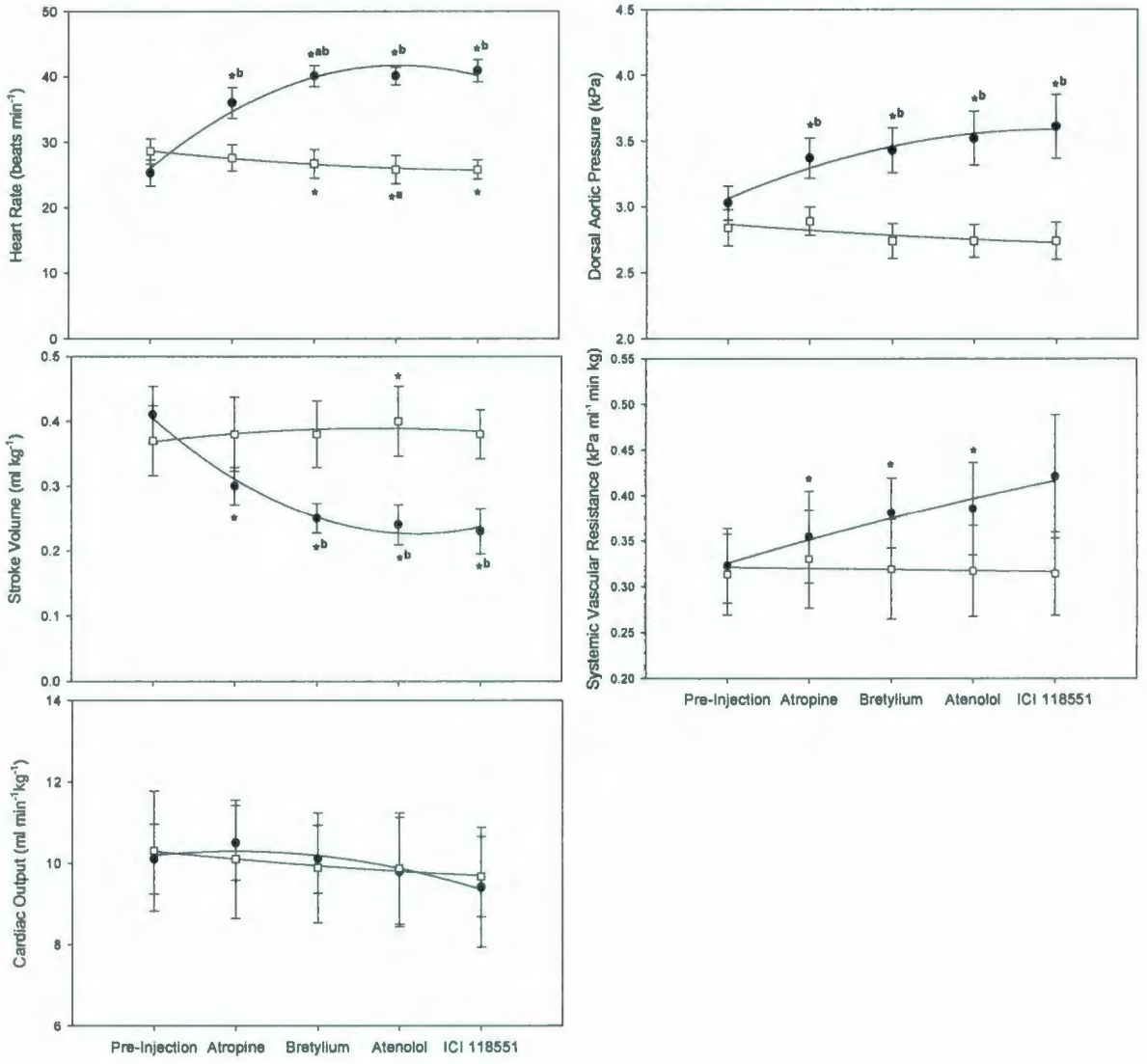

Figure 3.2 Cardiovascular responses to sequential drug (●) and saline (□) administration in the winter flounder. Saline and drug injections were made every 1 h 30 min. Drugs were administrated in the following order: atropine (1.2 mg kg^{-1}), bretylium (10 mg kg^{-1}), atenolol (213 μg kg^{-1}) and ICI 118 551 (250 μg kg^{-1}); measurements were taken 1 h after each injection. Values are means±s.e.m. Saline group, n=7 for all parameters; experimental group, n=8, except dorsal aortic pressure and total vascular resistance (n=6). *Values significantly different (P<0.05) from preinjection values. [a]Different from previous treatment. [b]Difference between saline and corresponding drug injections.

3.4.1.2 Net stress and chasing

Resting plasma levels of A and NA were both approx. 5 nM, resulting in a A:NA ratio of approx. 1.1. The 90 s chase resulted in slightly higher post-stress levels of catecholamines than the 60 s net stress (A, 20.7±5.1 *vs* 15.0±7.0; NA, 14.0±5.0 *vs* 11.6±3.4), however, these differences were not significant (P=0.52 for A and P=0.70 for NA). This lack of a statistical difference was also true for A:NA ratios, which were 2.0 and 1.3 following the 90 s chase and 60 s net stressors, respectively (Table 3.1).

3.4.1.3 Catecholamine dose–response curves

In these experiments, I was aiming for maximum circulating catecholamine concentrations of approx. 25 nM adrenaline and 14 nM noradrenaline based on the injection of 0.4 μg kg^{-1} A and 0.2 μg kg^{-1} NA (dose 5). The plasma levels achieved with this dose at 2 min post-injection were 13.7 nM for NA but only 11.5 nM for A. Although this was an increase over resting levels of only 8.5 and 7 nM, respectively, these values were significantly different from resting levels, and not significantly different from catecholamine concentrations measured after chasing and net stress (Table 3.1).

The injection of A and NA resulted in dose-dependent changes in cardiovascular parameters, with f_H decreasing, and Q and V_S increasing, by a maximum of 15, 30 and 27%, respectively (Fig. 3.3). However, because significant increases in Q (by 9%) and V_S (by 24%), and decreases in f_H (by 13%), were also associated with the injection of saline (Fig. 3.3), values were expressed as a percentage change from pre-injection, after subtracting the increase/decrease associated with saline injection. When this was done (Fig. 3.4), it was clear that the maximum post-injection increases in cardiovascular

Table 3.1 Plasma adrenaline and noradrenaline concentrations, adrenaline:noradrenaline ratio and total catecholamine concentrations, in the winter flounder at rest, after 60 s of net stress, after 90 s of chasing, and after an injection of catecholamines. A, adrenaline; NA, noradrenaline; A:NA, adrenaline:noradrenaline ratio; CA, total catecholamines (dose 5: 0.4 μg kg^{-1} of A and 0.2 μg kg^{-1} of NA). Values are presented as means±s.e.m. Different superscript letters indicate a significant difference ($P<0.05$) between treatments.

	A (nM)	NA (nM)	A:NA	Total CA (nM)
Rest	4.6±0.7 [a]	5.2±1.0 [a]	1.1±0.3 [a]	9.8±1.1 [a]
Net Stress	15.0±7.0 [b]	11.6±3.4 [b]	1.3±0.4 [ab]	26.6±9.5 [b]
Chasing	20.7 ± 5.1 [b]	14.0±5.0 [b]	2.0±0.4 [b]	34.6±8.4 [b]
Dose 5	11.5 ± 2.3 [b]	13.7±3.6 [b]	1.0±0.1 [a]	25.2±5.8 [b]

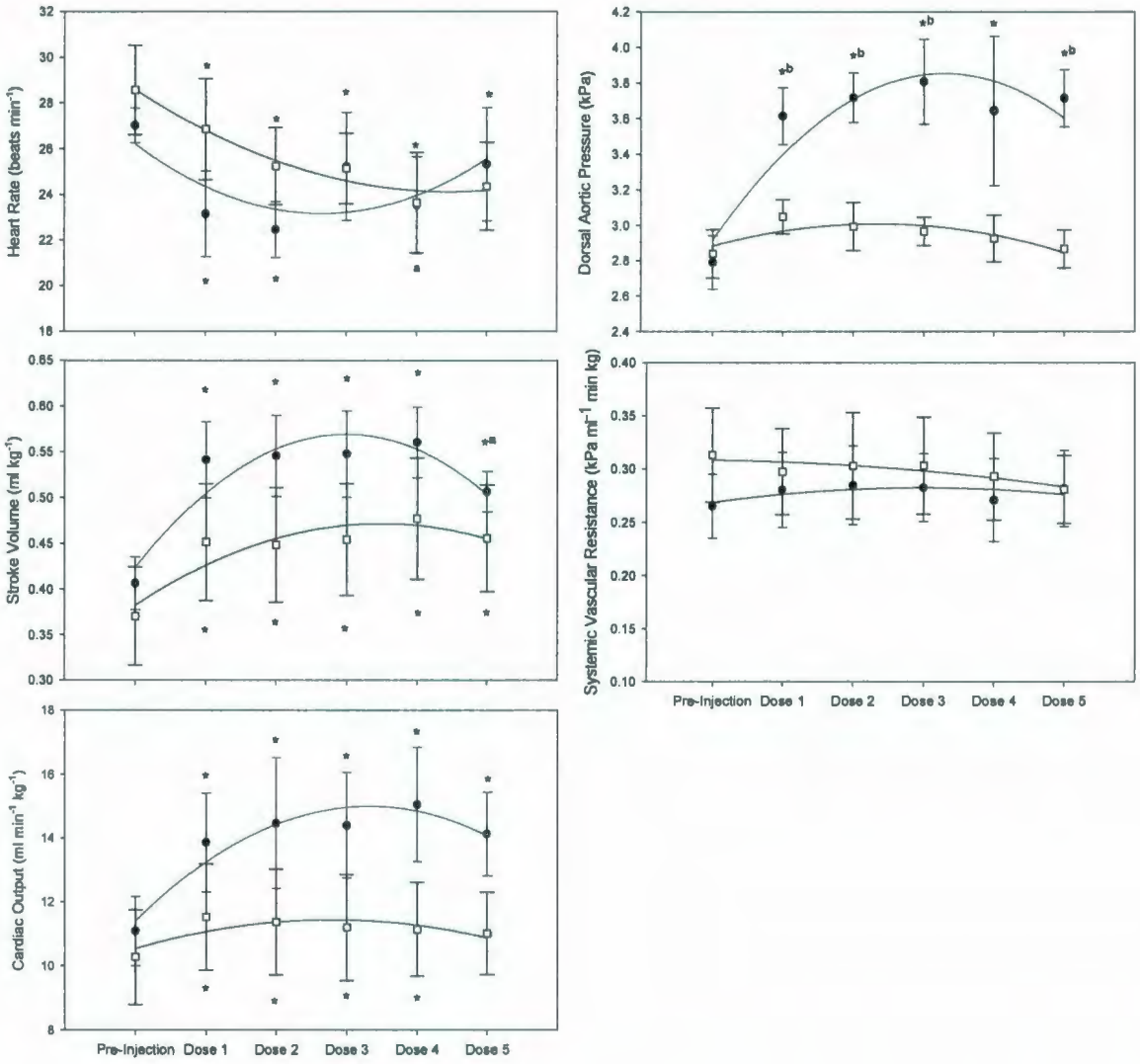

Figure 3.3 Maximum cardiovascular responses of winter flounder to saline administration (□) and increasing doses of catecholamines (●). Saline and catecholamines were injected every 1 h. Catecholamines were administrated in the following order: 0.1 and 0.05 (dose 1), 0.15 and 0.075 (dose 2), 0.2 and 0.1 (dose 3), 0.3 and 0.15 (dose 4) and 0.4 and 0.2 μg kg^{-1} (dose 5) of adrenaline and noradrenaline, respectively. Values are means±s.e.m., n=7. *Values significantly different (P<0.05) from pre-injection values. [a]Different from the previous dosage. [b]Difference between saline-injected fishes and the corresponding catecholamine dosage.

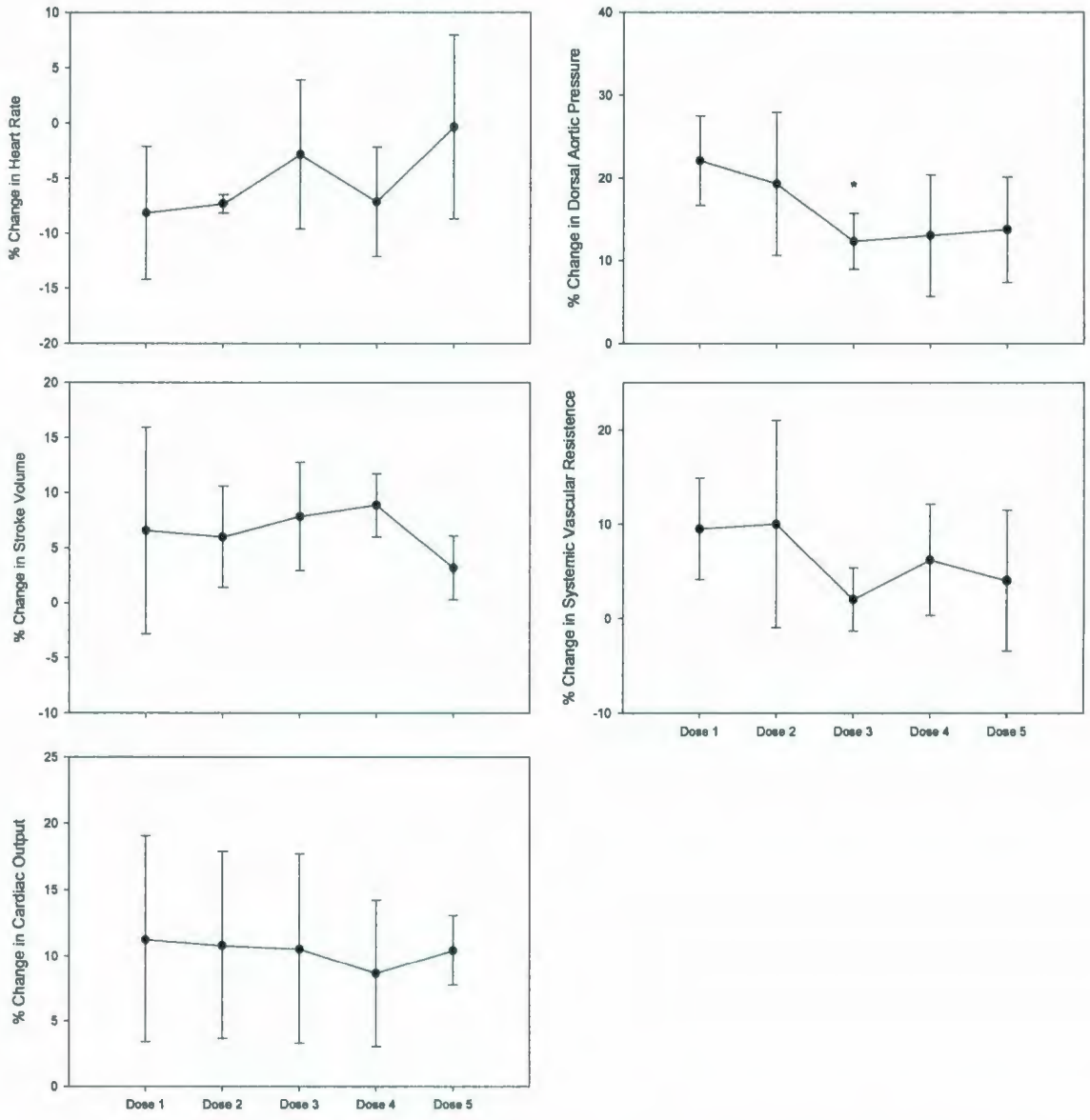

Figure 3.4 Percentage change in winter flounder cardiovascular parameters due to catecholamine administration. Catecholamines were injected every 1 h. Catecholamines were administrated in the following order: 0.1 and 0.05 (dose 1), 0.15 and 0.075 (dose 2), 0.2 and 0.1 (dose 3), 0.3 and 0.15 (dose 4) and 0.4 and 0.2 μg kg^{-1} (dose 5) of adrenaline and noradrenaline, respectively. *Values significantly different (P<0.05) from pre-injection values. Values are means±s.e.m., n=7.

function were mainly an injection artefact. For instance, of the 27% increase in V_S reported after catecholamine injection, only 6% was actually due to the effects of A and NA on this cardiovascular parameter. In a similar fashion, only 10% of the increase in Q could be attributed to the direct effects of the injected catecholamines (Fig. 3.4). In contrast P_{DA} increased by 22% with the injection of A and NA, and this change was measured at the lowest catecholamine dose (0.1 μg kg^{-1} A and 0.05 μg kg^{-1} NA); increasing the dose by fourfold having only a marginal additional influence on P_{DA}.

3.4.2 *In vitro* experiments

3.4.2.1 Cardiac β-adrenoreceptors

The r^2 value for individual CGP-binding curves was always ≥0.86 (range=0.86-0.99; Fig. 3.5*B*), with non-specific binding ranging from 14.6 to 30.9% of total binding (Fig. 3.5*A*). When the binding data were converted into fmol mg^{-1} protein based on punch protein content (28.1±4.4 μg mg^{-1} tissue), the β-adrenoreceptor density (B_{max}) and ligand binding affinity (K_d) for the winter flounder myocardium were determined to be 252.8±45.6 fmol mg^{-1} protein and 1.02±0.11 nM, respectively. Although atenolol (β_1-antagonist) was unable to displace [^{3}H]CGP from the flounder's ventricular β-adrenoreceptors, the β_2-antagonist ICI 118551 decreased [^{3}H]CGP binding beginning at approx. 10^{-7} M; the IC_{50} value for ICI 118551 was 1.91 X 10^{-6} M (Fig. 3.5*C*).

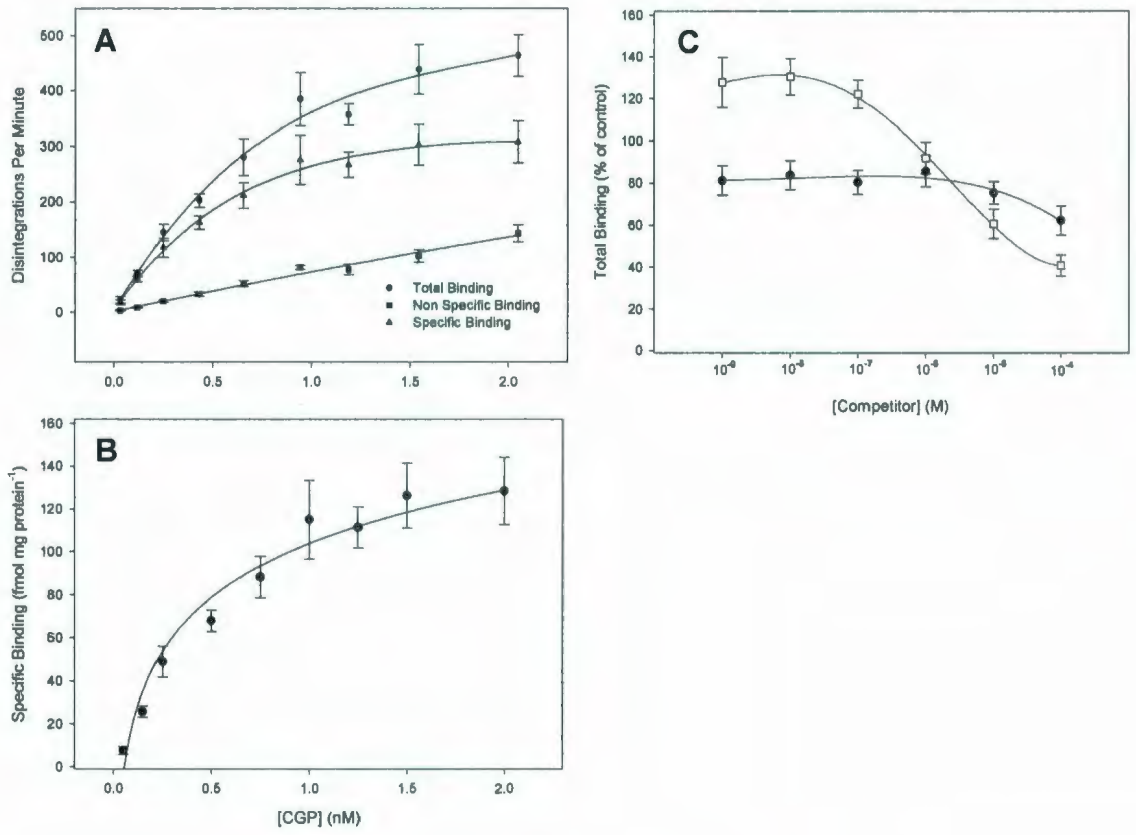

Figure 3.5 (*A*) Total, non-specific and specific binding of [^{3}H]CGP-12177 to ventricular β-adrenoreceptors in the winter flounder; timolol (10^{-5} nM) was used as the competitor (n=8). (*B*) Specific binding of [^{3}H]CGP-12177 to ventricular β-adrenoreceptors in winter flounder (y=36.2 Ln(x)+103.8; r^2=0.963; n=8). (*C*) Comparison of the ability of atenolol (β_1-antagonist; solid circles) and ICI 118551 (β_2-antagonist, open squares) to displace [^{3}H]CGP-12177 from ventricular β-adrenoreceptors in the winter flounder (n=6). ICI 118551 IC_{50} = 1.91 x 10^{-6} M. Values represent means±s.e.m.

3.5 Discussion

3.5.1 Resting cardiac function

Taking into consideration that cardiac function and P_{DA} are sensitive to acclimation temperature (Cech et al., 1976), resting cardiovascular variables in this study (at 8°C) correspond well with previous research on 10°C-acclimated winter flounder. For instance, Joaquim et al. (2004), made the first direct measurements of cardiac function in flatfish, and reported resting V_S and Q values at 10°C of 0.47 ml kg^{-1} and 15.5 ml min^{-1} kg^{-1} (compared with 0.39 ml kg^{-1} and 10.4 ml min^{-1} kg^{-1} in the present study). Furthermore, several authors have reported f_H and P_{DA} values of approx. 35 beats min^{-1} and 3.5 kPa, respectively, at 10°C (Cech et al., 1976; 1977; Joaquim et al., 2004), values very similar to those measured in this study (27.5 beats min^{-1} and 2.9 kPa at 8°C, respectively). By contrast, resting cardiovascular values for the flounder are generally lower than reported for more active species. This is not surprising, however, as significant inter-specific variation exists in cardiac function, and in general, active fishes have higher values for resting Q and V_S than benthic forms. For instance, in the rainbow trout, resting Q can range from 18 to 30.9 ml min^{-1} kg^{-1} and V_S from 0.29 to 0.6 ml kg^{-1} (at ~9–12°C) (Gamperl et al., 1994*e*; Thorarensen et al., 1996; Brodeur et al., 2001). The chinook salmon (*Oncorhynchus tshawytscha*) has resting values for Q of 35.8 ml min^{-1} kg^{-1} and for V_S of 0.63ml kg^{-1} at 9–10°C (Gallaugher et al., 2001), and Q and V_S values of 27.5–35.5 ml min^{-1} kg^{-1} and 0.61 ml kg^{-1} have been reported for the Atlantic salmon (*Salmo salar*) at 9–12°C (Perry and McKendry, 2001; Dunmall and Schreer, 2003). However, the bottom dwelling lingcod (*Ophiodon enlongatus*; Q of 5.9–10.9 ml min^{-1} kg^{-1}; V_S of 0.13–

0.37 ml kg^{-1} at 9–13°C; Stevens et al., 1972; Farrell, 1981) and the sea raven (*H. americanus*; Q of 18.8 ml min^{-1} kg^{-1} and V_S of 0.51 ml kg^{-1} at 6–8°C; Axelsson et al., 1989) have similar resting cardiovascular values as reported here for the flounder.

3.5.2 Neurohormonal control of cardiac function

Atropine induced a 42% increase in f_H, and based on this change in f_H we calculated that the cholinergic contribution to resting heart rate at 8°C was 26.1%. This degree of cholinergic tonus is equivalent to that reported for other teleosts at similar temperatures (see Table 3.2). For instance, the eel pout (*Zoarces viviparous*) has a resting cholinergic tonus of 18.9% (Axelsson et al., 1987) and cholinergic tonus on the cod heart is between 21.3 and 37.7% at 10-12°C (Altimiras et al., 1997; Axelsson, 1988).

Surprisingly, bretylium caused an additional positive chronotropic effect in the winter flounder that lasted for at least 1 h post-injection, and this resulted in a negative resting adrenergic tonus (–11.9%). This finding is in clear contrast to most previous studies on fish which have reported values for resting adrenergic tonus from ~13% for the sea bream (*Sparus aurata*) and cod (Altimiras et al., 1997) to 67.1% for the eel pout (Axelsson et al., 1987) (Table 3.2). Furthermore, the positive chronotropic effect induced by bretylium was not expected since earlier studies suggest that pleuronectids lack myocardial adrenergic innervation. For example, fluorescent histochemical studies in the greenback flounder (*Rhombosolea tapirina*) failed to find adrenergic cardiac nerves, and in isolated heart preparations from this species, stimulation of the vagus nerve had both negative inotropic and chronotropic effects (Donald and Campbell, 1982). In addition, in isolated plaice (*Pleuronectes platessa*) hearts, electrophysiological studies revealed that

Table 3.2 Resting cholinergic and adrenergic tones in different teleost species. [a] Present study (winter flounder); [b] Axelsson et al., 1987 (pollack, ballan wrasse, eel pout and short-spined scorpion); [c] Altimiras et al., 1997 (Mediterranean sea bream and Atlantic cod); [d] Axelsson, 1988 (Atlantic cod).

Species	Temperature °C	Resting Cholinergic Tonus %	Resting Adrenergic Tonus (%)
P. americanus [a]	**8**	**26.1**	**-11.9**
P. pollachius [b]	11-12	19.7	33.2
L. bergylta [b]	11-12	33.9	15.4
Z. viviparus [b]	11-12	18.9	67.1
M. scorpius [b]	11-12	11.1	25.3
S. aurata [c]	16	22.4	12.8
G. morhua [c]	10	21.3	12.9
G. morhua [d]	10-12	37.7	21.1

only cholinergic fibres run in the cardiac branch of the vagus, and that bretylium had no effect on f_H in vagally-stimulated isolated hearts (Cobb and Santer, 1973). However, there are several potential explanations for the increase in f_H seen in the winter flounder following the injection of bretylium. First, unlike other pleuronectids, this species might have cardiac sympathetic innervation, and thus the positive chronotropic effect could be the result of an initial release of catecholamines from sympathetic nerve endings. This bretylium sympathomimetic effect has been demonstrated in both sand flathead (*Platycephalus bassensis*) isolated heart preparations (Donald and Campbell, 1982) and in Atlantic cod *in situ* heart preparations (Axelsson, 1988). Second, it is possible that the flounder has cardiac α-adrenoreceptors, and that basal adrenergic tone has a negative chronotropic influence in this species. In this scenario, bretylium would have increased f_H by inhibiting catecholamine release from adrenergic nerve terminals. Such an inhibitory adrenergic effect, caused by the stimulation of α-adrenoreceptors, has been demonstrated in the hearts of the perch (*Perca fluviatilis*, 15 and 24°C; Tirri and Ripatti, 1982) and eel (*Anguilla anguilla*, 8°C; Peyraud-Waitzenegger et al., 1980). Nonetheless, previous studies have not found any evidence of α-adrenoreceptors in atrial preparations from the rainbow trout and flounder (*P. flesus*) at 8°C (Ask, 1983), and thus, it is unlikely that α-adrenoreceptors were playing a significant role in the regulation of cardiac function in the winter flounder at 8°C. Third, as in mammals (Heissenbuttel and Bigger, 1979; Fallen, 1998), bretylium administration could have caused an initial catecholamine release from peripheral adrenergic nerve terminals into the circulation, elevating f_H. This explanation, however, also seems unlikely as catecholamines are rapidly cleared from the circulation of fishes (Gamperl et al., 1994*b*) and no significant increase in plasma catecholamines

was found in the cod after bretylium injection (Axelsson and Nilsson, 1986). Furthermore, our dose–response curves show that the flounder heart is not very responsive to circulating catecholamines (e.g. see Figs 3.3 and 3.4), and that blocking the flounder's β-adrenoreceptors with atenolol and ICI 118551 did not mitigate the positive chronotropic effect associated with bretylium injection (Fig. 3.2). Unfortunately, at present, we cannot provide a definitive answer as to why bretylium induced a positive chronotropic effect in the flounder heart.

As mentioned above, the β_1 and β_2-adrenergic blockers atenolol and ICI 118551 had no effect on resting cardiovascular function in the winter flounder. This result indicates that neither circulating or endogenous catecholamines support resting cardiac function in this species of flatfish. This result is surprising given that other fish species that lack cardiac innervation (e.g., myxinoids), or whose vagus is composed solely of cholinergic nerves (most elasmobranchs), rely heavily on circulating and endogenous catecholamines for the modulation of cardiac function (Axelsson et al., 1990; Johnsson et al., 1996; Agnisola et al., 2003). However, it is consistent with the findings of other aspects of our investigation into the mechanisms controlling cardiac function in this species. Specifically, I show that the flounder heart is not very sensitive to increases in circulating catecholamines achieved by bolus injections of A and NA (Figs. 3.3 and 3.4), and that β-adrenoreceptors in the flounder heart have a very low affinity (K_d for [^{3}H]CGP 12177 of 1.02 nM).

Intrinsic heart rate in the flounder was calculated to be 40.9 beats min^{-1} at 8°C. This value is similar to the intrinsic f_H reported for the cod (36.6 beats min^{-1}, 10–12°C; Axelsson, 1988), and is in the middle of the range of values reported by Axelsson et al.

(1987) for seven different teleost species at 11-12°C; i.e. from approx. 30 beats min^{-1} for the tadpole fish (*Ranice raninus*) to as high as ~60 beats min^{-1} in the five-bearded rockling (*Ciliata mustela*).

3.5.3 Plasma catecholamine levels following net stress and chasing

The low resting levels of plasma catecholamines found in the winter flounder (A and NA concentrations of 4.6 and 5.1 nM, respectively) are comparable to values reported for other teleosts, and indicate that our fish had recovered fully from surgery. For example, resting levels of A and NA in cod are reported to range from 2.5-4 and 4-5 nM, respectively (Axelsson and Nilsson, 1986; Butler et al., 1989), and circulating catecholamine concentrations in resting rainbow trout are also <5 nM (2.2–2.6 nM for A and 2.7–3.3 nM for NA; Milligan and Wood, 1987; Tang and Boutilier, 1988).

When the flounder was exposed to net stress, plasma A and NA concentrations increased by 3.2-fold and 2.2-fold, respectively. These increases were very similar to those recorded after the flounder was chased to exhaustion (elevations in plasma A levels by 4.5-fold and in NA by 2.7-fold; Table 3.1), but are considerably lower than those recorded for many other teleosts. For example, after 90 s of chasing, plasma A and NA levels measured in the winter flounder were 20.7 and 14 nM, respectively, whereas, using a similar chasing protocol, I. Costa and A.K.G. (unpublished data) found values as high as 188 (A) and 47 nM (NA) in cod and 148 (A) and 149 nM (NA) in capelin (Table 3.3). Our results for the winter flounder are consistent with those for the starry flounder following a 10 min chase to exhaustion (A to ~30 and NA to 37nM; Milligan and Wood, 1987) and for the sea raven (*H. americanus*) after 1 min of air exposure followed by 1

min of chasing (total catecholamine levels ~16 nM; Vijayan and Moon, 1994), and add support to the notion that benthic/sluggish species have low post-stress circulating catecholamine levels. However, I must also caution that this is only a generalization and that exceptions do exist; I. Costa and A.K.G. (unpublished data) found total plasma catecholamine levels of 48 nM for the active swimming Atlantic rainbow smelt (*Osmerus mordax mordax*), a value only approximately one-half of that measured in the benthic dwelling eel pout (*Macrozoarces americanus*; 58 nM A and 41 nM NA; Table 3.2).

Table 3.3 Plasma adrenaline, noradrenaline, total catecholamines concentrations and haematocrit in different teleost species after chasing. A, adrenaline; NA, noradrenaline; CA, total catecholamines; Hct, haematocrit. [a] Present study (winter flounder); [b] Costa and Gamperl, unpublished data (cunner, ocean pout, Atlantic rainbow smelt, capelin, Atlantic cod); [c] Vijayan and Moon, 1994 (sea raven); [d] Tang and Boutilier, 1988 (rainbow trout held in freshwater and seawater); [e] Milligan and Wood, 1987 (rainbow trout and starry flounder).

Species	A (nM)	NA (nM)	Total CA (nM)	Hct (%)	Type of Stress
P. americanus [a]	**21**	**14**	**35**	**21**	**90 s chase**
T. adspersus [b]	80	23	103	35	90 s chase
M. americanus [b]	58	41	99	28	90 s chase
O. mordax mordax [b]	25	26	48	30	90 s chase
M. villosus [b]	148	149	297	28	90 s chase
G. morhua [b]	188	47	235	28	90 s chase
H. americanus [c]	8	8	16	-	1 min air exposure followed by 1 min chase
O. mykiss [d]	179.7	51	230.7	-	10 min chase (freshwater)
O. mykiss [d]	88.1	19.5	107.6	-	10 min chase (seawater)
O. mykiss [e]	~30	~37	67	~40	6 min chase
P. stellatus [e]	~21	~30	51	~15	10 min chase

3.5.4 Catecholamine dose-response curves and cardiac β-adrenoreceptors

The bolus administration of A and NA, at doses (up to 0.4 μg kg^{-1} A and 0.2 μg kg^{-1} NA) that resulted in circulating catecholamine concentrations typical of post-chase levels, did not affect flounder cardiovascular function (Fig. 3.4). This result agrees with recent experiments on cod where *in vivo* cardiac function was only marginally influenced at A doses below 4 μg kg^{-1} (L. H. Petersen and A.K.G., unpublished data), and where *in situ* hearts only showed marginal improvements in maximum pumping capacity and power output even when exposed to 200 nM A (G. J. Lurman, L. H. Petersen, H. O. Portner and A. K.Gamperl, unpublished data). However, it contrasts with the results of Gamperl et al. (1994*d*) for the rainbow trout where the injection of A at doses as low as 0.2 μg kg^{-1} increased Q and V_S by approx. 33%. This diminished adrenergic sensitivity in flounder, as compared with rainbow trout, hearts is clearly not due to a reduced number of cardiac β-adrenoreceptors, as β-adrenoreceptor density in the flounder ventricle (B_{max}, 252.8 fmol mg^{-1} protein) is the highest ever reported for a teleost species, and six- to 12-fold higher than reported for the rainbow trout (23–40 fmol mg^{-1} protein; Gamperl et al., 1994*a*; Olsson et al., 2000). A more likely explanation is that the injected concentrations were not sufficient to stimulate the flounder heart. This conclusion is based on two pieces of evidence: (1) flounder (*P. flesus*) ventricular strips at 10°C respond to 1 μM of A by increasing contractile force by approx. 120% (Lennard and Huddart, 1992); and (2) although the density (B_{max}) of ventricular β-adrenoreceptors is very high compared with other teleosts, their binding affinity for [^{3}H]CGP is 1.02 nM. This latter value is approx. 3–10 times greater than that measured for a variety of other fish species (0.13–0.36 nM; Olsson et al., 2000).

Although unusual, a very low receptor affinity for stress hormones is not unique amongst fishes. For example, gill cortisol receptors in the chub (*Leuciscus cephalus*) have a K_d eightfold higher than those in rainbow trout, probably to compensate for the extremely high levels of cortisol found in the blood of this species (at rest, five- to 10-fold higher than trout; Pottinger et al., 2000). Why the β-adrenoreceptors on the flounder ventricle have such a high K_d is not obvious from the research conducted here (but see below), especially given that resting plasma concentrations of A and NA are low, and typical of those measured in other teleosts. However, it is possible that the high K_d value of flounder heart β-adrenoreceptors is related to this species' life history. The flounder has a benthic and inactive lifestyle (Pereira et al., 1999), a limited aerobic capacity (Duthie, 1982; Lefrançois and Claireaux, 2003; Priede and Holliday, 1980), generally swims intermittently (He, 2003), and appears to find even slow swimming very demanding (Joaquim et al., 2004). Thus, it is reasonable to assume that the flounder's adrenergic and cardiovascular systems are not designed to respond to chasing/exercise, an unlikely situation for the flounder in its natural environment. Interestingly, flounder typically bury themselves several cm deep (i.e. up to 10–15 cm) in soft sediments (sand and mud), and under these conditions gill ventilation may be difficult (Cech et al., 1977; Nonnotte and Kirsch, 1978) and very low oxygen concentrations may be encountered (Fletcher, 1975; Duthie, 1982; Pereira et al., 1999). In experiments using *in situ* heart preparations, Mendonça et al. (2007; Chapter 2) reported that the winter flounder heart has a maximum V_S (2.3 ml g^{-1} ventricle) significantly greater than the cod (1.3) and the Atlantic salmon (1.4), and suggested that this elevated maximum V_S might be important under conditions of severe hypoxia. This hypothesis is supported by recent *in vivo* data

(see Chapter 4) showing that flounder acclimated to 8°C increased V_S by 40% when the water O_2 saturation was lowered from 30 to 20%, and thus it is probable that severe hypoxia is the type of stress/challenge that would promote a release of catecholamines from the chromaffin cells large enough to stimulate flounder myocardial β-adrenoreceptors. In fact, this idea is supported by recent research on Atlantic cod showing that circulating catecholamine levels during severe hypoxia (A ~350 nM and NA ~60 nM at 13% water O_2 saturation; L. H. Petersen and A.K.G., unpublished data) are approximately twice those following exhaustive exercise (A of 188 nM, NA of 47 nM; I. Costa and A.K.G., unpublished data), and that maximal adrenergic stimulation offsets the adverse effects of hypoxia, and the concomitant effects of hyperkalemia and acidosis, on performance of *in situ* trout heart at 10°C (Hanson et al., 2006).

Based on the inability of the β_1-antagonist atenolol to displace [^{3}H]CGP (β-adrenergic antagonist) from flounder's ventricular β-adrenoreceptors, and the IC_{50} value for the β_2-antagonist ICI 118551 (1.91×10^{-6} M), it appears that winter flounder ventricular β-adrenoreceptors are predominantly of the β_2 subtype. This conclusion contrasts with the previously held belief that the flounder heart has equal affinity for A and NA (Ask, 1983), but agrees with Cobb and Santer (1973) who showed that the isolated heart of the flounder *P. platessa* is more sensitive to A than to NA, and with Gamperl et al. (1994*a*) who characterized the ventricular β-adrenoreceptors of the rainbow trout heart as β_2. Nonetheless, the high K_d measured for these receptors (1.02 nM CGP) is difficult to reconcile with previous studies on fish which report K_d values in the range of ~0.1–0.4nM (Gamperl et al., 1994*a*; Olsson et al., 2000). One possible

explanation for the high K_d value measured for β-adrenoreceptors of the flounder heart is that they are functionally uncoupled from adenylate cyclase (Hausdorff et al., 1990).

However, we feel that this is unlikely as resting levels of circulating catecholamines in the flounder are typical of those measured in other teleosts, the repeated injection of catecholamines into rainbow trout does not alter cardiac β-adrenoreceptor affinity (Gamperl et al., 1994*a*) and exposure to β-adrenoreceptor agonists for more than a month only resulted in an increase in the K_d of rainbow trout muscle β-adrenoreceptors of 2-fold (from ~0.2 to 0.5 nM; Lortie et al., 2004). A more probable explanation for the high K_d value reported here for flounder cardiac β-adrenoreceptors is that the flounder heart has a significant population of β_3-adrenoreceptors. These receptors have been found in the hearts of both the rainbow trout (Nickerson et al., 2003) and European eel (Imbrogno et al., 2006), and are activated at higher catecholamines concentrations than β_1/β_2-adrenoreceptors (Gauthier et al., 2007). Furthermore, in trout erythrocytes, β_3-adrenoreceptor binding properties resemble β_2-adrenoreceptors more than β_1 (Nickerson et al., 2003), and consequently the displacement of [^{3}H]CGP by ICI 118551 in the flounder heart (see Fig. 3.5*C*) may be related to the presence of β_2 and/or β_3 subtypes. β_3-receptor stimulation induces negative inotropic effects in most mammals (e.g. see Gauthier et al., 1999; 2007) and in the eel (Imbrogno et al., 2006), and it has been recently shown by Imbrogno et al. (2006) and Angelone et al. (2008) that stimulation of β_3-adrenoreceptors mediates negative inotropy and lusitropy (i.e. their stimulation allows the heart to relax slower) through the induction of endothelial nitric oxide synthase-derived nitric oxide signalling. Whether these receptors also exist in the flounder heart, and what role they might play in mediating cardiac function in this

species, can only be answered through further research. However, it has been suggested that these receptors play a 'protective role' by preventing excessive β_1/β_2 stimulation of the mammalian myocardium (Gauthier et al., 2007; Angelone et al., 2008).

3.6 Perspectives

This study provides valuable new insights into the nervous and humoral control of cardiac function in the winter flounder. For example, our results indicate that neural and humoral adrenergic mechanisms do not support resting cardiac performance in 8°C acclimated flounder, and that increases in plasma catecholamines associated with net stress and chasing are not sufficient to stimulate cardiac β-adrenoreceptors. They also show that although the flounder ventricle is populated with an unusually large number of β-adrenoreceptors, these receptors have a very low binding affinity. These results are not easily explained on the basis of what is known about cardiovascular control/physiology in other fishes, but appear to fit well with the winter flounder's life history and other data on aspects of this species' cardiac function (e.g. Joaquim et al., 2004; Mendonça et al., 2007). Furthermore, they lead to several testable hypotheses. Specifically, we propose that this species' muted cardiac response to catecholamine stimulation is related to the existence of a significant population of myocardial β_3-adrenoreceptors, that circulating catecholamines only reach concentrations capable of stimulating these receptors under conditions such as severe hypoxia (e.g., when flounder are buried in soft sediments), and that β_3-adrenoreceptor stimulation promotes negative inotropism and lusitropism that balances the positive stimulation provided by β_2-receptors.

3.7 Acknowledgements and grants

This work was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery grant to A. Kurt Gamperl. Paula C. Mendonça was supported by a doctoral fellowship SFRH/BD/8082/2002 from the Portuguese Foundation for Science and Technology (FCT) co-financed through POCI 2010 (Programa Operacional Ciência e Inovação) and FSE (Fundo Social Europeu). I am grateful to Dr Trevor Avery for statistical advice, to Isabel Costa for assistance with plasma catecholamine measurements, and to Linda Hanson for help with analysis of the β-adrenoreceptor data.

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4. The Effects of Acute Changes in Temperature and Oxygen Availability on Cardiac Performance in Winter Flounder (*Pseudopleuronectes americanus*)

4.1 Abstract

The winter flounder, *Pseudopleuronectes americanus,* is a benthic coastal species that often encounters environments with low oxygen concentration and fluctuating temperatures. However, studies regarding how flounder cardiovascular function responds to hypoxia and acute temperature fluctuations are scarce, and have relied upon indirect methodologies (i.e., the Fick principle). Thus, I implanted a cannula in the caudal artery of the winter flounder and placed a Transonic® flow probe around its ventral aorta, and: (*1*) exposed 8 and 15°C acclimated flounder to a graded hypoxic challenge (100% to 20% water O_2 saturation); and (*2*) challenged 8°C acclimated fish to an acute increase in temperature to their critical thermal maximum (CTM). The extent of bradycardia in 8°C acclimated fish (decrease in heart rate of 13 beats min^{-1}; 41%) was consistent with that observed for other teleosts, as was this species' CTM (25.8±0.5°C) and its cardiac response to increasing temperature. However, this study also provides further examples of how cardiovascular function is controlled differently in the flounder as compared to other fishes. First, while winter flounder acclimated to 8°C underwent a reflex bradycardia when faced with aquatic hypoxia, the onset of bradycardia occurred earlier than expected for this inactive and hypoxia-tolerant species (60% water O_2 saturation; P_wO_2 ~13 kPa). Second, resting cardiac output was similar in winter flounder acclimated to 8 and 15°C

(approx. 15 ml min^{-1} kg^{-1}), and hypoxic bradycardia was surprisingly absent at 15°C. Finally, systemic vascular resistance decreased more in the flounder than reported for other teleosts when exposed to elevated temperature, and this resulted in a significant (26%) fall in dorsal aortic blood pressure. These are novel findings with regards to the effects of ecologically relevant environmental challenges on fish cardiac function. However, the extent to which some of these differences were influenced by the flounder's behaviour is not known, and should be the focus of future studies.

4.2 Introduction

Fish can encounter water in which the oxygen content has been substantially reduced by biological oxygen demand or anthropogenic activity (Fernandes, 1996; Pereira et al., 1999), and seasonal and/or diurnal fluctuations in temperature (Kramer et al., 1978). Typically, fish respond to aquatic hypoxia by significantly decreasing heart rate (f_H) (Gamperl and Driedzic, 2009). However, the level of hypoxia at which bradycardia is initiated varies greatly between species, and f_H during hypoxia is dependent not only on the water's oxygen partial pressure (P_wO_2) but also on the rate of the decline in water O_2 level and water temperature. For example, during graded hypoxia at 15°C, the rainbow trout (*Oncorhynchus mykiss*) initiates bradycardia at a P_wO_2 of 10.5 kPa (Holeton and Randall, 1967), whereas this value is 6 kPa for the Atlantic cod (*Gadus morhua*, 8-11°C) (McKenzie et al., 2009; Petersen and Gamperl, unpublished data). In Atlantic cod, 6-8 min of exposure to a P_wO_2 of approx. 5 kPa often results in no decrease in f_H (e.g., Fritsche and Nilsson, 1990), whereas fish exposed to the same P_wO_2 during a graded hypoxic challenge show a clear reduction in f_H (McKenzie et al., 2009; Petersen and

Gamperl, submitted). Finally, the PO_2 at which bradycardia was initiated increased from 2.6 to 8 kPa in spangled perch (*Leiopotherapon unicolor*) acclimated to 10 and 30°C (Gehrke and Fielder, 1988) and from <5 to >16 kPa in dogfish (*Scyliorhinus canicula*) at seasonal temperatures of 7 and 17°C (Butler and Taylor, 1975). Much less is known about how stroke volume (V_S) and cardiac output (Q) are modulated during hypoxia, however, the response pattern can be divided into three general categories. In the first response pattern, V_S starts to increase prior to hypoxic bradycardia leading to an initial increase in Q, and V_S is initially able to compensate for hypoxia-induced decreases in f_H before Q eventually falls. In the second pattern, increases in V_S are either concomitant with the onset of bradycardia or begin after the bradycardia is initiated (often near the limit of hypoxia tolerance), however, these increases in V_S are inadequate to compensate for decreases in f_H and Q falls almost continuously (albeit slower than f_H) with the severity of hypoxia. Finally, in some fishes, increases in V_S initiated during hypoxia are able to fully compensate for the drop in f_H with moderate hypoxia (see Gamperl and Driedzic, 2009).

Changes in water temperature also have a dramatic effect on both resting and maximum cardiac function (Farrell et al., 1996; Farrell, 2002; Gollock et al., 2006; Clark et al., 2008; Steinhausen et al., 2008). For example, when cod and salmon (*Oncorhynchus nerka*) are exposed to a progressive increase in temperature, Q increases with a Q_{10} of 2-2.5 until it reaches a plateau corresponding to the fish's optimum temperature and then declines just prior to the fish reaching it's critical thermal maximum (Gollock et al., 2006; Steinhausen et al., 2008). Interestingly, however, it appears that these changes in Q are solely due to changes in f_H. Stroke volume remains largely unchanged as temperature is increased by as much as 15°C. Although there are few data on other fishes, there is some

evidence that this general pattern is not displayed by all species. In the Antarctic fish (*Pagothenia bernacchii*) a progressive temperature increase from 0 to 5°C raised f_H by ~114% (Q_{10}=4.6), and caused a decrease in V_S of approx. 30% (Axelsson et al., 1992). However, this finding was contradicted in a recent study by Franklin et al. (2007) who showed a Q_{10} for Q in this species of 1.62 between -1 and 8°C and that V_S was unchanged by the acute temperature increase.

The winter flounder, *Pseudopleuronectes americanus*, is a coastal flatfish species that can often be found burrowed in the sediment, a situation where gill ventilation may be difficult (Nonnotte and Kirsch 1978), and can experience low O_2 concentrations and considerable fluctuations in seasonal temperatures (from -1.0 to 22°C) (Fletcher, 1975; Duthie, 1982; Moyle and Cech, 1996; Pereira et al., 1999). In addition, a number of aspects of this species' cardiorespiratory physiology are atypical for teleosts, and suggest that heart function is regulated differently in this taxa (flatfishes). For example: 1) this species has extremely large values for resting and maximum V_S when the relative size of the heart is considered (Joaquim et al., 2004; Mendonça et al., 2007); 2) flatfish have low blood haematocrit and haemoglobin levels (Cech et al., 1976; Wood et al., 1979*a*), and relatively low arterial pressures (2.5-3.5 kPa; Cech et al., 1976; 1977; Wood et al., 1979*a*); 3) flatfish lack cardiac adrenergic innervation (Cobb and Santer, 1973; Donald and Campbell, 1982; Ask, 1983); and 4) circulating catecholamines do not appear to support flounder cardiac function at rest or following a severe stressor (Mendonça and Gamperl, 2009). There are some data on winter flounder's cardiovascular response to hypoxia and acute increases in temperature (Cech et al. 1976; 1977); however, these studies are limited in scope, and do not allow for the determination of if/at what PO_2 level

this species exhibits hypoxic bradycardia, or of the extent to which cardiac function is influenced by acute increases in temperature. Further, the results of these experiments are questionable since $\dot{Q}$ values were determined using the Fick equation/principle, and do not take into account that a significant amount of the O_2 consumed (approx. 30%) by flatfish is taken up across skin and that this amount increases when the fish are exposed to hypoxia (Nonnotte and Kirsch, 1978; Steffensen et al., 1981). Hence, we implanted a cannula in the caudal artery and placed a Transonic® flow probe around the ventral aorta of winter flounder to examine the influences of graded hypoxia and a progressive increase in temperature on this species' cardiovascular function.

4.3 Material and Methods

4.3.1 Animals

Ethical approval was obtained from the Animal Care Committee at Memorial University of Newfoundland (protocol # 05-02-KG). Winter flounder (*Pseudopleuronectes americanus*) were caught by divers using a hand net in Conception Bay (Newfoundland) at a depth of 4-6 meters and transported to the Ocean Sciences Centre (OSC, Memorial University). Flounder were acclimated at 8 or 15±1°C for at least 4 weeks prior to experimentation in 1200 L rectangular fiberglass tanks supplied with aerated seawater and natural photoperiod. Fish were fed three times a week with commercial pellets, but fasted for 24 hours prior to surgery.

4.3.2 Surgery

Fish were anaesthetized in seawater containing methane sulfonic acid m-aminobenzoate (MS-222, 0.25 g L^{-1}), and then transferred to a surgical table, where their gills were irrigated with chilled (~4°C) and oxygenated seawater containing 0.1 g L^{-1} MS-222.

4.3.2.1 Implantation of flow probe

The implantation of the blood flow probe was performed as previously described by Crocker et al. (2000) for white sturgeon, with some modifications. In this procedure, the gills and operculum were retracted using umbilical tape (Baxter Healthcare Corporation, Deerfield, IL, USA) which was passed from a hole behind the 4th gill arch into the opercular cavity, the ventral aorta was exposed by a small incision (~0.5 cm) in the isthmus without disrupting the pericardium, and a flow probe (1.5RB, Transonic® Systems; Ithaca, NY, USA) was placed loosely around the vessel. Finally, after verifying the quality of the cardiac output signal, the lead probe was sutured to the eyed side of the fish using 3-0 silk (American Cyanamid Company, Pearl River, NY, USA) at three locations.

4.3.2.2 Cannulation of caudal artery

Cannulation of the caudal artery for the measurement of dorsal aortic pressure was performed as previously described by Cech and Rowell (1976) with modifications. Briefly, a 2 cm long incision was made just below the lateral line at about one third of the animal's length from the tail. The skin and underlying muscle tissue were retracted to

expose the caudal artery which lies between the haemal arches and a heparinised cannula (PE 50, Clay Adams, Parsippany, NJ, USA; 80 cm long, volume 0.2 ml) with indwelling 14 gauge piano wire was then inserted into the vessel. Finally, after removing the indwelling wire, and pushing the cannula approximately 8 cm anteriorly into the artery, the incision was closed with a continuous suture, and the cannula was filled with heparinised saline (181.3 mM NaCl, 5.0 mM KCl, 2.30 mM $CaCl_2$ x $2H_20$, 1.99 mM MgSO4 x $6H_2O$, 2.58 TES acid and 7.33 mM sodium TES base with 100 IU mL^{-1} heparin) and sutured to the fish's dorsal surface at two locations. There was minimal bleeding during cannula placement, and the cannulae were flushed regularly with heparinised saline to prevent clot formation.

Recovery from anaesthesia was initiated after surgery by artificially ventilating the fish with aerated, anaesthetic-free seawater. Once ventilatory activity had returned, the fish was placed in a 45 L cooler supplied with aerated 8 or 15°C seawater and filled with ~5 cm of sand. The flounder were allowed to recover for at least 24h prior to experimentation.

4.3.3 Experimental protocols

4.3.3.1 Cardiac performance during graded hypoxia

To determine how flounder cardiac function is affected by acute hypoxia, two groups of flounder (average mass 0.6±0.03 kg) were acclimated at different temperatures for at least 3 weeks before surgical procedure. One group (n=9) was acclimated to 8°C, while another group (n=8) was acclimated to 15°C (the upper limit of their optimum

thermal range; Pereira et al. 1999). After the recovery period, cardiac output ($\dot{Q}$), heart rate (f_H) and dorsal aortic pressure (P_{DA}) were recorded for 1h before any manipulation of the water oxygen levels. Following the initial recording period, pure nitrogen (N_2) was slowly bubbled into the tank, reducing water O_2 levels from 100 to 20% O_2 saturation (~21 to 4 kPa) over approx. 3.5 hours. After, 30 min at 20% O_2 saturation the system was quickly (within approx. 10 min) reoxygenated to 90% saturation by bubbling pure O_2 into the tank. Water temperature and O_2 content in the tank were continuously measured by pumping water through an external circuit at 50 ml min^{-1} using a peristaltic pump (Masterflex Model 7523–20, Cole-Parmer Instrument Co., Vernon Hills, IL, USA). This circuit was constructed of tubing with extremely low gas permeability (Tygon® Food, Series 6419, Cole-Parmer Instrument Co., Montreal, QC, Canada), and contained a customized flow chamber (D201, WTW Inc., Welheim, Germany) that housed a galvanic oxygen electrode equipped with thermal sensor (Model CellOx 325, WTW Inc.). This oxygen electrode was connected to an oxygen meter (Model Oxi 330i, WTW Inc.) equipped with automatic temperature compensation. The electrode was calibrated at least once a day in an OxiCals—SL calibration vessel (WTW Inc.) containing humidified air. Further, the electrode was briefly rinsed several times during each experiment with distilled water in order to minimize the deposition of residue on the membrane face, which improves membrane stability.

4.3.3.2 Cardiac performance during an acute temperature challenge

A second group of flounder acclimated at 8°C (n=8; average mass 0.54±0.05 kg) was exposed to surgery to study the cardiovascular response to acute elevations in

temperature. Following recovery from surgery, Q, f_H and P_{DA} were recorded for 1h before any temperature manipulation. After the initial recording period, the winter flounder were subjected to an increase in water temperature by 2°C every hour, until their critical thermal maximum (CTM). In most fish studies, CTM is defined as the temperature at which the animal loses equilibrium. However, the flounder is laterally compressed making such observation impossible. Thus, for the purpose of this study, CTM was defined as the temperature at which cardiac activity was reduced dramatically.

4.3.3.3 Instrumentation and data and statistical analyses

Dorsal aortic pressure was measured using a Gould Statham pressure transducer (Model P23 ID, Oxnard, CA, USA) that was calibrated daily against a static water column, where zero pressure (0 kPa) was set equal to the water level in the experimental cooler. Cardiac output was monitored by connecting the flow probe to a small animal blood flow meter (Model T206, Transonic® Systems Inc., Ithaca, NY, USA), and the pressure and flow signals were amplified and filtered using a Model MP100A-CE data acquisition system (BIOPAC Systems Inc., Santa Barbara, CA, USA). The acquired signals (recorded at 20 Hz) were then stored and analyzed using Acq*knowledge* Software (BIOPAC Systems Inc., Santa Barbara, CA, USA) installed on a 300 MHz Toshiba laptop computer.

Cardiovascular function was continuously monitored throughout the experiment by measuring cardiac output (Q, ml min^{-1} kg^{-1}) and dorsal aortic pressure (P_{DA}, kPa). Heart rate (f_H, beats min^{-1}) was calculated by measuring the number of systolic peaks during 20-30 second intervals. Mass specific stroke volume (V_S, ml kg^{-1}), was calculated

as: V_S=cardiac output (ml $min^{-1}kg^{-1}$)/heart rate (beats min^{-1}). Systemic vascular resistance (R_{sys}, kPa ml^{-1} min kg) was calculated as: dorsal aortic pressure (kPa)/cardiac output (ml min^{-1} kg^{-1}).

In the graded hypoxia experiment, a univariate general linear model was used to test for significant differences between the 8°C and the 15°C groups during normoxia (100% O_2 saturation, ~21 kPa). Contrasts were performed to assess significant differences within groups; specifically, between normoxia and each O_2 saturation level. Similarly, in the temperature challenge study, contrasts were used to detect differences between the control (8°C) and each 2°C temperature increment. All values in the text, and figures and tables, are expressed as means±standard error of the mean (s.e.m.). All statistical analyses were performed using SPSS Software for Windows version 15.0 (SPSS Inc., Chicago, IL, USA) and $P<0.05$ was used as the level of statistical significance.

4.4 Results

4.4.1 Graded hypoxia

Winter flounder acclimated to 8°C maintained f_H and Q at approximately 31 beats min^{-1} and 15 ml min^{-1} kg^{-1} until 60% air saturation (P_wO_2 ~13 kPa). However, as water O_2 levels were reduced from 60 to 20% air saturation, these two variables fell by 41 and 15%, respectively (Fig. 4.1). In contrast, while V_S was unchanged down to 30% saturation, it increased from 0.51±0.05 ml kg^{-1} to 0.74±0.07 ml kg^{-1} at 20% air saturation (P=0.08). No changes in P_{DA} or R_{sys} were observed at 8°C, with values averaging 2.7 kPa and 0.22 kPa ml^{-1} kg^{-1} min^{-1}, respectively. Reoxygenation quickly returned most

parameters to pre-hypoxic levels at 8°C. Nonetheless, f_H was significantly above pre-hypoxic levels at 80 and 90% air saturation (~38 beats min^{-1}), and P_{DA} was significantly reduced once an O_2 saturation level of 50% was reached (approx. 2.2 vs. 2.7 kPa at 100% air saturation).

At normoxic levels, f_H and V_S were 60% higher and 40% lower in 15°C acclimated flounder as compared to 8°C acclimated fish (51.6±2.4 vs. 32±2.6 beats min^{-1} and 0.28±0.02 ml kg^{-1} vs. 0.51±0.05 ml kg^{-1}, respectively); the net result was that Q did not increase between 8 and 15°C. P_{DA} and R_{sys} were also not affected by acclimation to the higher temperature (Fig. 4.1). Surprisingly, heart function in the winter flounder was not affected by graded hypoxia at 15°C, with the exception of slight increases in P_{DA} (0.2 kPa) and R_{sys} (0.1 kPa ml^{-1} min kg) at 80 and 70% air saturation. During reoxygenation, f_H increased significantly once the water O_2 level reached 40% air saturation; averaging 59.4 beats min^{-1} as compared to 51.6±2.4 beats min^{-1} prior to hypoxia. Cardiac output was also elevated between 40% and 80% water O_2 concentration (to approx. 19 ml min^{-1} kg^{-1}), but was not significantly different from pre-hypoxic levels at 90% O_2 saturation.

4.4.2 Acute temperature increase

Heart rate was the most sensitive variable to the acute temperature increase, with significant effects observed by 10°C. Whereas significant changes in the other variables were not observed until 14-16°C. During the acute temperature challenge (Fig. 4.2), f_H increased by approx. 2.0-fold between 8°C and 18°C (from 36.1±2.6 beats min^{-1} to 73.2±4.5 beats min^{-1}). Thereafter, it plateaued at approx. 70 beats min^{-1} between 20 and 24°C, before falling by 10 beats min^{-1} at 26°C. Cardiac output increased by 2.6-fold

(Q_{10}=2.0) between 8 and 22°C (to 31.1±3.5 ml min^{-1} kg^{-1}), but declined with further increases in temperature (Q 23.6±5.9 ml min^{-1} kg^{-1} at 26°C). In contrast to values for f_H and Q, V_S did not change significantly and P_{DA} and R_{sys} decreased gradually as temperature was increased from 8 to 26°C. Values for these latter two parameters falling by 40 and 72%, respectively, by 26°C. The mean CTM for the winter flounder was 25.8 ± 0.5°C.

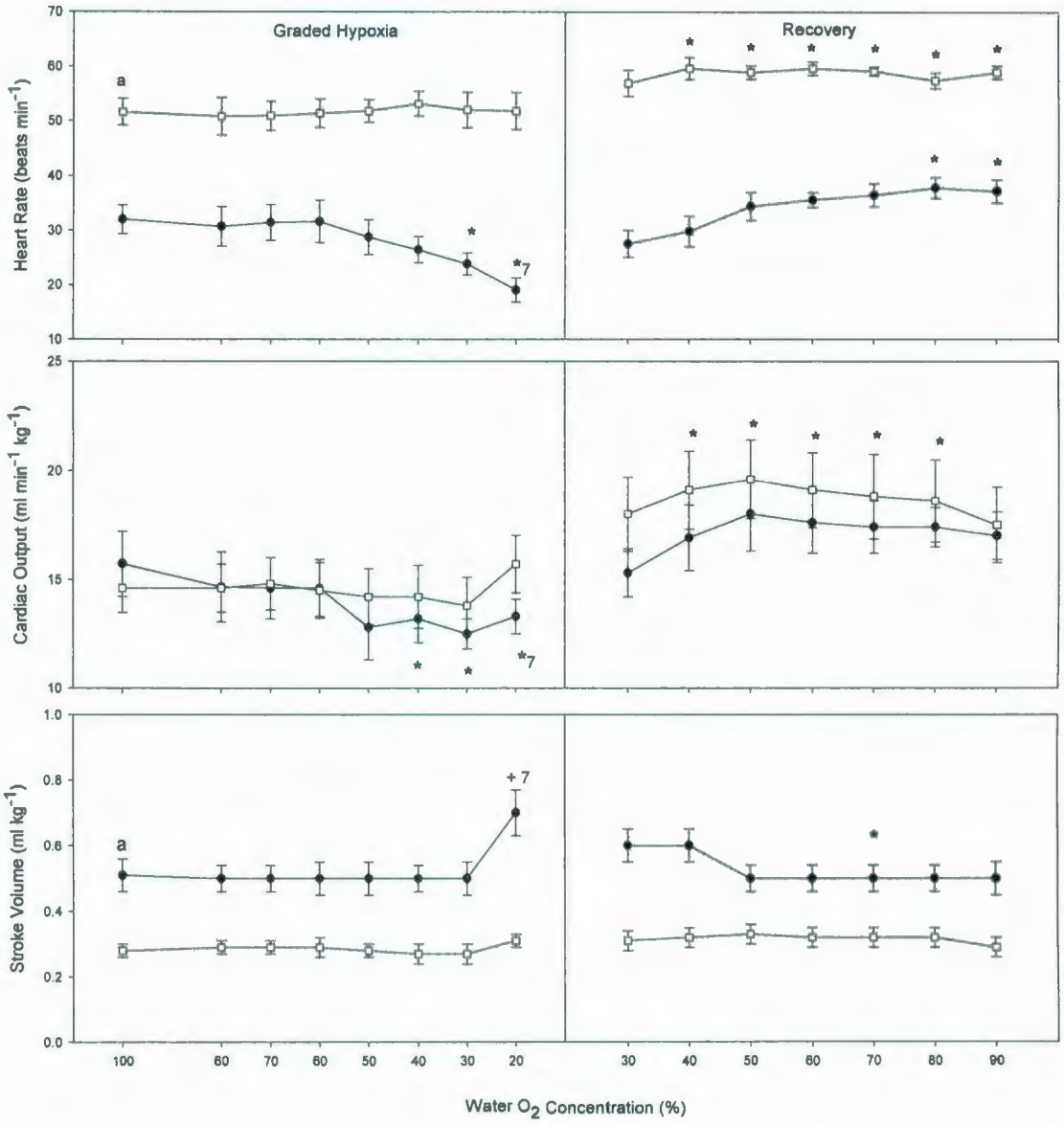

Figure 4.1 continues on next page.

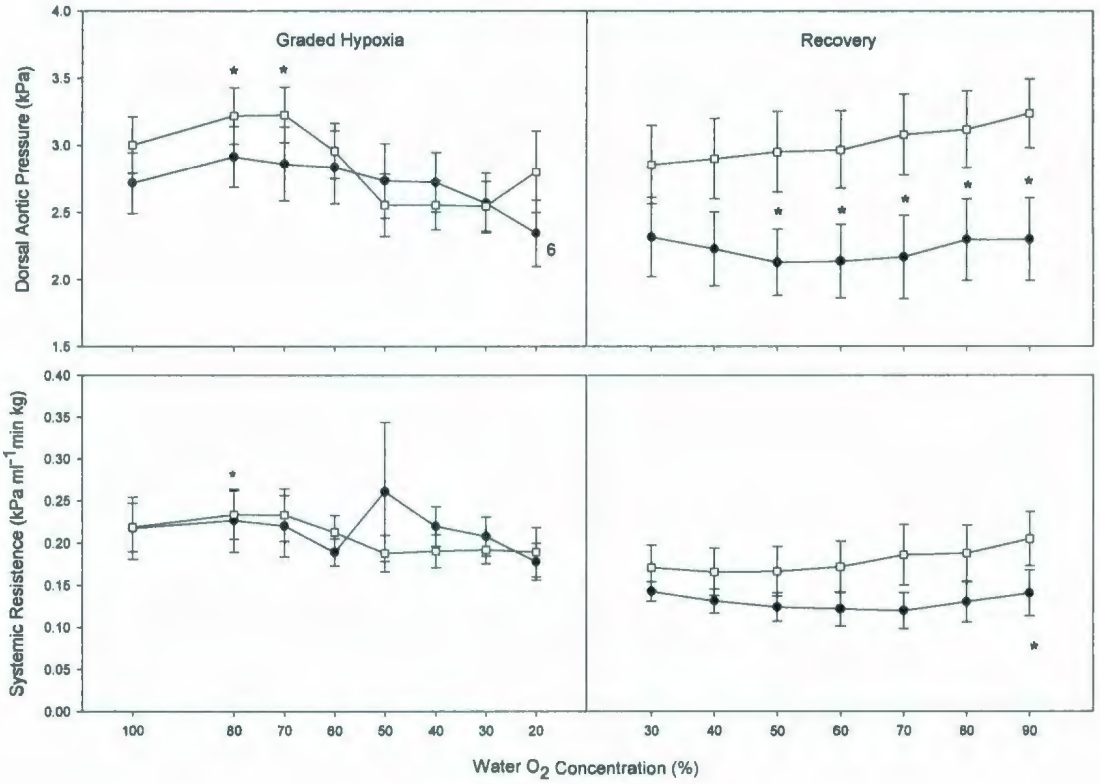

Figure 4.1 Effect of graded hypoxia on the cardiac performance of winter flounder acclimated to 8°C (●) and 15°C (□). Values represent means±s.e.m; n=8-9 except dorsal aortic pressure at 8°C (n=7), systemic resistance (n=5-6) at 8°C and when numbers appear next to data point. * and $^{+}$ indicate values that were significantly different from normoxia at P<0.05 and <0.10, respectively. ^{a} indicates significant differences between the 8 and 15°C groups at normoxia. Note: the time-frames of the graded hypoxia and recovery periods were different. The duration of the graded hypoxia was approx. 3.5 hours while the recovery period lasted approx. 10 min.

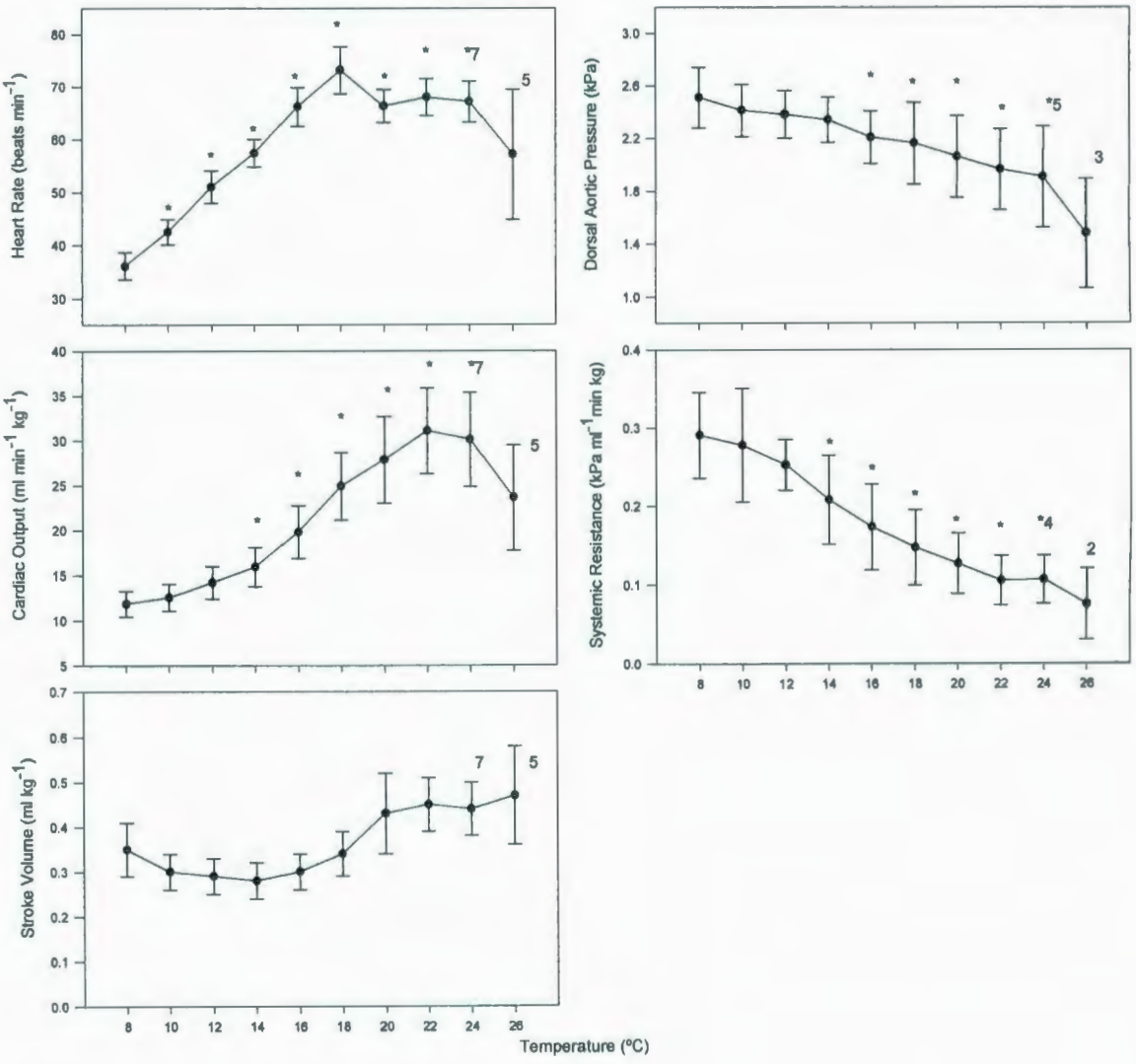

Figure 4.2 Effect of an acute temperature increase on the cardiac performance of 8°C winter flounder. Values represent means±s.e.m; n=8 except dorsal aortic pressure (n=6), systemic resistance (n=5) and when numbers appear next to a data point. * indicates values that were significantly different (P<0.05) from 8°C.

4.5 Discussion

4.5.1 Cardiac performance during graded hypoxia at 8°C

The values I found for cardiovascular variables in 8°C, normoxic winter flounder correspond well with previous *in vivo* studies on this species. For example, at 10°C, resting V_S and Q were 0.47 ml kg^{-1} and 15.5 ml min^{-1} kg^{-1}, respectively (Joaquim et al., 2004), as compared with 0.51±0.05 ml kg^{-1} and 15.7±1.5 ml min^{-1} kg^{-1} in the present study. Further, I measured a f_H of 32±2.6 beats min^{-1} and a P_{DA} of 2.7±0.23 kPa (8°C), whereas previous studies recorded f_H and P_{DA} values of 34-35 beats min^{-1} and 3.7 kPa, respectively, at 10°C (Cech et al., 1976; 1977; Joaquim et al. 2004).

Similar to the majority of water-breathing fishes (e.g., Holeton and Randall, 1967; Fritsche and Nilsson, 1989; Gehrke and Fielder, 1988; Rantin et al., 1993), winter flounder acclimated to 8°C underwent a reflex bradycardia when faced with aquatic hypoxia. However, the onset of bradycardia occurred earlier than expected (60% water air saturation; P_wO_2 ~13 kPa) given the low water temperature, the low resting/routine metabolic rate reported for flatfishes (e.g., Priede and Holliday, 1980; Duthie, 1982) and that, in general, inactive and hypoxia-tolerant species tend to initiate bradycardia later during a graded hypoxic challenge as compared with more active species. For instance, Furimsky et al. (2003) reported that the smallmouth bass, an active predator which inhabits deep and cold waters initiates bradycardia at a water O_2 saturation level of around 60% saturation (12 kPa) at 22°C, whereas the onset of the bradycardia for the largemouth bass, a less active predator found in shallower and warmer waters, is about 6 kPa. The erythrinids *Hoplias lacerda* and *Hoplias malabaricus* (25°C) initiate bradycardia

at 4.8 kPa and 2.7 kPa, respectively, which is not surprising considering that *H. lacerda* is found in well oxygenated rivers while *H. malabaricus* occurs in stagnant hypoxic waters (Rantin et al., 1993). Finally, the onset of bradycardia in the active and migratory dourado (*Salminus maxillosus*) and the rainbow trout (an active species normally found in well oxygenated waters) was observed at ~14 kPa (at 25°C; de Salvo Souza et al., 2001) and 10-17 kPa (at 10-12°C; Gamperl et al., 1994; Holeton and Randall, 1967), respectively, while in the benthopelagic Atlantic cod (at 8°C; McKenzie et al., 2009; Petersen and Gamperl, unpubl.) bradycardia is initiated at ~5-6 kPa. Further, flatfish can consume a significant amount of oxygen through their skin (~30% under normoxic conditions; Nonnotte and Kirsch, 1978; Steffensen et al., 1981), and oxygenated blood returning from the skin would elevate the oxygen content of central venous blood. This should make the flounder heart less dependent on blood returning from the major tissues for its oxygen supply, and thus enable it to maintain f_H until P_wO_2 reached relatively low levels.

Furimsky et al. (2003) suggested that the difference in the onset of bradycardia between largemouth and smallmouth bass was related to an enhanced haemoglobin-O_2 binding affinity in the former species. However, haemoglobin-O_2 affinity is unlikely to explain why bradycardia is initiated in the flounder at relatively high water PO_2. This is because the P_{50} value (PO_2 at which 50% of haemoglobin is saturated) for a closely related species, the starry flounder (*Platichthys stellatus*) is $\leq$2 kPa (Watters and Smith, 1973; Milligan and Wood, 1987); a value considerably lower than for species such as the rainbow trout (~3 kPa; Weber et al., 1976; Milligan and Wood, 1987) or Atlantic cod (~4-7 kPa; Karpov and Novikov, 1980; Gollock et al., 2006; Petersen and Gamperl, unpublished data), and even lower than that measured in the largemouth bass (2.7 kPa;

Furimsky et al., 2003). However, the winter flounder does have a reduced blood oxygen carrying capacity due to low haematocrit and haemoglobin concentration (approx. 21%; Mendonça and Gamperl, 2009 and 2.4-3.5 g %; Cech et al., 1976), and this may explain the high P_wO_2 at which bradycardia occurred.

In the winter flounder, V_S remained constant as the water O_2 level was lowered from approx. 21-4 kPa, but increased by 37% when water O_2 was lowered to 2.6 kPa (20% saturation). The net result was that Q only fell slightly, and was still 85% of that measured under normoxia at the end of hypoxic challenge (Fig. 4.1). A relatively unchanged Q when exposed to hypoxia has also been observed for the dogfish (Butler and Taylor, 1971), and sturgeon (*Acipenser naccarii*; Agnisola et al., 1999) (at least down to a PO_2 of 5 kPa). The flounder recovered quickly from hypoxia, with all cardiac variables having returned to, or being only slightly above, pre-hypoxic levels by the end of the experiment. The response of flounder cardiac variables to reoxygenation is very similar to that observed for the sculpin (*Myoxocephalus scorpius*; MacCormack and Driedzic, 2004) and armoured catfishes (*Glyptoperichthyes gibbceps* and *Liposarcus pardalis*; MacCormack et al., 2003). In contrast to cardiac variables, P_{DA} and R_{sys} decreased during recovery, and were significantly lower as compared with pre-hypoxic levels at 90% air saturation (Fig. 4.1). The decreases in these variables were likely associated with the hyperaemia which often follows tissue O_2 deprivation (Clark et al., 2008; Walker, 1991), and would have had the additional advantage of decreasing the workload on the heart as it recovered from exposure to severe hypoxia.

4.5.2 Cardiac performance during graded hypoxia at 15°C

Heart rate was 19 beats min^{-1} higher in winter flounder acclimated to 15°C as compared to 8°C (51.6±2.4 vs. 32±2.6 beats min^{-1}; Q_{10}=1.98). This temperature-dependent increase corresponds well with values reported for the winter flounder by Cech et al. (1976) where fish acclimatized to 15°C had a f_H 11 beats min^{-1} higher as compared to 10°C acclimatized individuals (f_H in this group 35 beats min^{-1}). In addition, it compares well with that measured for the spangled perch (where f_H increased from approx. 12 to 40 beats min^{-1} from 5 to 15°C; Gehrke and Fielder, 1988), the dogfish, where a 10°C rise in acclimation temperature from 7 to 17°C promoted an increase in f_H from 19.5 to 40.8 beats min^{-1} (Butler and Taylor, 1975), and rainbow trout acclimated to 5 (f_H=30 beats min^{-1}) and 20°C (f_H=80 beats min^{-1}) (Wood et al. 1979*b*). Although f_H increased by 60% when winter flounder were acclimated to 15 vs. 8°C, I found that Q did not change because V_S fell by approx. 40% (from 0.51±0.05 to 0.28±0.02 ml kg^{-1}; Fig. 4.1). This decrease in V_S with increased acclimation temperature is in contrast to the findings of Joaquim et al. (2004) and Cech et al. (1976). Joaquim et al. (2004) found that V_S was unchanged with an increase in acclimation temperature from 4 to 10°C, whereas Cech et al. (1976) reported that V_S (calculated using the Fick principle) was 0.55, 0.68 and 0.9 ml kg^{-1} at 5, 10 and 15°C respectively. Further, both these studies showed that Q increased with acclimation temperature, as would be expected based on data from other teleosts (Farrell et al., 1996; Gamperl and Farrell, 2004). At present, I cannot explain the discrepancy between studies. Nonetheless, V_S was maintained, and Q increased by 50%, when flounder from the same population were exposed an acute increase in temperature from 8 to 15°C (Fig. 4.2). This latter data clearly demonstrates that the flounder I used

were capable of increasing Q when challenged with elevated temperatures.

In the winter flounder, it was also surprising that hypoxic bradycardia was absent at 15°C, as fish subjected to warmer acclimation temperatures typically initiate bradycardia earlier. For example, the onset of bradycardia occurred at a water O_2 level of approx. 12 kPa in dogfish acclimated to 12°C, while at 7°C it was initiated at approx. 9 kPa (Butler and Taylor, 1975). Similarly, in the spangled perch, bradycardia was initiated at 8.5 kPa vs. 4.5 kPa O_2 when acclimated to 35°C and 5°C, respectively (Gehrke and Fielder, 1988). This relationship between water temperature and the onset of bradycardia makes sense given the inverse relationship between water O_2 content and temperature, and that increases in temperature are concomitant with a decrease in haemoglobin-O_2 affinity (e.g., Watters and Smith, 1973; Perry and Reid, 1994; Gollock et al., 2006). However, this is not the first time that the lack of bradycardia has been reported in flounder when exposed to hypoxic conditions. Cech et al. (1976) showed that f_H was unchanged when 10°C acclimated flounder were exposed to water with a PO_2 of 8.5 kPa.

Flatfish lack adrenergic cardiac innervation (Cobb and Santer, 1973; Donald and Campbell, 1982; Ask, 1983) and cholinergic tone on the heart increases (see Sureau et al., 1989), not decreases, with temperature as has been shown for teleosts such as the rainbow trout (e.g., Wood et al., 1979*b*). Thus, it is possible that these differences in cardiac nervous control were responsible for the inconsistency in the response of the flounder versus the dogfish and spangled perch to graded hypoxia; the hypothesis being that a higher cholinergic tone in flounder at 15°C precludes increases in vagal tone from mediating a decrease in f_H in response to lowered water oxygen levels. This explanation is unlikely, however, as elasmobranchs also lack cardiac adrenergic innervation, and Taylor

et al. (1977) showed that cholinergic tone on the heart increases in the dogfish with temperature. At present we have no clear explanation as to why there was no bradycardia in 15°C-acclimated winter flounder down to water oxygen levels of 20% air saturation (4.5 kPa). However, there are a number of possibilities. First, non-adrenergic non-cholinergic (NANC) factor(s) might be important for supporting f_H in the flounder at higher temperatures. This hypothesis would fit with the suggestion of Altimiras et al. (1997) that the contribution of NANC to f_H regulation increases during environmental challenges. However, the proposed effect of NANC on f_H in flounder at 15°C would have to be opposite to that previously reported for fish exposed to hypoxia. For example, in the eel (*Anguilla anguilla*), Atlantic cod and tambaqui (*Colossoma macropomum*) f_H decreases during progressive hypoxia even after the abolition of reflex bradycardia through atropinisation or vagotomy (Peyraud-Waitzenegger and Soulier, 1989; Sundin et al., 2000; McKenzie et al., 2009). Second, the lack of a decrease in f_H at this higher temperature could be related to some, as yet unexplained, ability of the founder heart to avoid myocardial dysfunction. For example, MacCormack and Driedzic (2002) demonstrated that ventricle strips of the yellowtail flounder (*Limanda ferruginea*) show a transient increase in force development when subjected to anoxia (i.e. N_2 gassing). Further, Rantin et al. (1995) showed that changes in the electrocardiogram (ECG) of *Cyprinus carpio* were decidedly different as compared to three other tropical fish species. In *C. carpio*, the direction of the ECG reversed from + to – with the onset of severe hypoxia, as compared to no change, in *Piaractus mesopotamicus*, or a – to + transition in *H. malabaricus* and *H. lacerdae*, and there was only minimal change in the amplitude of the T-wave of *C. carpio* with graded hypoxia as opposed to a 1.8- to 4-fold increase in

this parameter in the other three species. Third, Cech, et al. (1977) suggested that the lack of hypoxic bradycardia (down to 8.5 kPa) seen in winter flounder at 10°C was in part related to the high energetic costs of increasing ventilation when this species is buried in several cm of sediment (where flounder are frequently found; Fletcher, 1975). In other words, it is less expensive to elevate circulatory flow to maintain an effective O_2 supply when buried at high temperatures. Finally, Behrens et al. (2007) recently showed that buried sandeels (*Ammodytes tobianus*) gradually move towards the sediment surface as water O_2 levels decline, and that their heads emerge from the sediment at a PO_2 of approx. 8.5 kPa. Indeed, if the flounder displayed a similar behaviour (i.e., either partially or fully emerging from the sand when exposed to graded hypoxia at 15°C), this might explain why there was no bradycardia at this temperature. Branchial and cutaneous gas exchange would have been improved by moving to the sand's surface, and thus, it is likely that both the PO_2 and O_2 content of blood entering the heart would have increased. Given the fact that the flounder in which Cech et al. (1977) failed to see a hypoxia-induced bradycardia were not provided with a substrate in which to bury, and the accumulating evidence that bradycardia serves to protect cardiac performance, not improve branchial gas exchange (Perry and Desforges, 2006; Farrell, 2007), this latter explanation is quite plausible.

4.5.3 Cardiac performance during an acute temperature challenge

When the winter flounder were exposed to a progressive increase in temperature, f_H and $\dot{Q}$ increased gradually until they peaked at approx. 18 and 22°C, respectively. The peak of these variables was then followed by a plateau close to maximum levels (f_H

approx. 67 beats min^{-1}; Q approx. 30 ml $min^{-1}kg^{-1}$), and a rapid decline just prior to the flounder's CTM (25.8±0.51°C). In contrast, V_S remained constant during the entire experiment at approx. 0.37 ml kg^{-1} (Fig. 4.2). Cech et al. (1976) also reported an increase in f_H and Q, but no change in V_S, when winter flounder were acutely exposed to a 5°C increase in temperature, and the pattern of change in Q reported here is similar to that observed for the starry flounder when warmed from 12 to 21°C (Watters and Smith, 1973): although it should be noted that Q and V_S were calculated using the Fick principle in both studies. Further, my results are in general agreement with those reported for Chinook salmon (*Oncorhynchus tshawytscha*; Clark et al., 2008), sockeye salmon (*Oncorhynchus nerka*; Steinhausen et al. 2008), lingcod (*Ophiodon elongates*; Stevens et al., 1972) and Atlantic cod (Gollock et al., 2006) exposed to a similar range of temperatures, and to the Antarctic fish (*Pagothenia borchgrevinki*) when warmed from -1 to 8°C (Franklin et al., 2007).

Collectively, these data indicate that *in vivo* changes in Q when fish are exposed to acute elevations in temperature are normally mediated solely by increases in f_H. Why resting fish do not increase V_S when acutely exposed to increases in temperature is a hotly debated topic in the literature, and there are numerous explanations (see Gollock et al., 2007; Steinhausen et al., 2008 and Clark et al., 2008): (1) cardiac contractility decreases with increasing contraction frequency (due to the negative force-frequency relationship) and temperature, and leads to an decrease in end-systolic volume; (2) cardiac filling time is negatively correlated with heart rate and temperature, and high temperatures results in a reduced cardiac filling pressure (both of which reduce end-diastolic volume and V_S); (3) deleterious changes in blood chemistry (decreased pH, low venous PO_2, and

hyperkalemia) have a negative impact on cardiac contractility; and (4) central cardiac control maintains $\dot{V}_S$ constant as temperature increases. However, to what extent, if at all, the first three mechanisms influence $\dot{V}_S$ during an acute thermal challenge is unclear. For example, exposure of resting sockeye salmon to a progressive temperature challenge from of 10-25°C only results in slight reductions in plasma pH and venous PO_2 and elevations in K^+, and only at 25°C (Steinhausen et al., 2008). These authors also showed that salmon swimming at 1.35 body lengths s^{-1} (75% of their critical swimming speed) maintained $\dot{V}_S$ at nearly twice that of resting fish (approx. 0.7 vs. 0.4 ml kg^{-1}) between 15 and 25°C, despite the fact that: (*1*) P_{DA} (and thus cardiac afterload) is significantly greater in swimming fish (Randall and Daxboeck, 1982), (*2*) f_H was generally 15 beats min^{-1} higher in swimming salmon, and (*3*) the effects of temperature on blood chemistry were exacerbated by the exercise regimen. Sandblom and Axelsson (2007) and Clark et al. (2008) showed that cardiac filling pressure is maintained in salmonids at temperatures between 10 and 20°C, and that it actually increases greatly when Chinook salmon are warmed from 21 to 25°C (Clark et al., 2008). Flatfish appear to be able to maintain cardiac contractility quite well in the face of oxygen limitation and acidosis (MacCormack and Driedzic, 2002; Gesser and Poupa, 1979). Finally, exposure of rainbow trout to 25°C results in very large increases in circulating catecholamines (adrenaline and noradrenaline reaching 200 and 100 nM, respectively; Currie et al. 2008), and Hanson et al. (2006) and Hanson and Farrell (2007) show that similar levels of catecholamines (500 nM adrenaline) can completely ameliorate the negative effect of acidosis, hypoxemia and hyperkalemia on *in situ* maximum $\dot{V}_S$ in this species at both 10 and 18°C. Interestingly, Gamperl et al. (2008) showed that 10°C acclimated rainbow trout

can maintain maximum $\dot{Q}$ at 24°C as f_H is progressively cut in half using the pharmacological agent zatebradine. This finding highlights the tremendous plasticity in how fish cardiorespiratory physiology responds to environmental challenges, and suggests that V_S is regulated at a constant level during an acute thermal challenge. Why fish use tachycardia to meet the increased cardiac pumping requirements associated with elevated temperature is unknown, but Steinhausen et al. (2008) observed a high degree of cardiorespiratory synchrony at high temperatures, and suggested that efferent vagal burst activity can entrain f_H.

When exposed to a temperature increase from 8-26°C, P_{DA} and R_{sys} were largely unchanged until 14-16°C, but then gradually fell as temperature was increased further; these two variables decreasing by 20 and 72% by the time they reached their CTM (Fig. 4.2). The significant decrease in R_{sys} with elevated temperature is consistent with the data of Clark et al. (2008) for Chinook salmon. However the fall in flounder R_{sys} was much greater that measured for the Chinook salmon (approx. 50%). This resulted in P_{DA} falling by 20% in the flounder, whereas it increases slightly in both Chinook salmon (Clark et al., 2008) and rainbow trout (Heath and Hughes, 1973). This decrease in R_{sys} is consistent with systemic vasodilation, and Clark et al. (2008) suggested that this vasodilation reflects the recruitment of vascular beds such that tissue O_2 diffusion distances are reduced. It is possible that an even greater dilation/recruitment of vascular beds is required to meet the tissue oxygen demands of the flounder given its lower maximum Q (31.1±4.8 ml min^{-1} kg^{-1} at 22°C) and haematocrit (~21%, Cech et al., 1977) as compared with the Chinook salmon (56.3 ml min^{-1} kg^{-1} and 35%, respectively; Clark et al., 2008). However, flatfish are also reported to have extremely low rates of active and standard

metabolism (Wood et al., 1979*a*; Priede and Holliday, 1980; Duthie, 1982), and thus there may be another explanation for the greatly reduced R_{sys} in the flounder. This species has a relative ventricular mass (RVM) approx. 30-40% lower than in salmonids, and a maximum Q that is much more sensitive to output pressure (cardiac afterload; Mendonça et al., 2007). Given that V_S (in ml kg^{-1}) was similar (approx. 0.4-0.5 ml kg^{-1}) in the flounder, sockeye salmon, and chinook salmon prior to their respective CTMs (present study; Steinhausen et al., 2008; Clark et al., 2008), it is likely that the large decrease in R_{sys} and modest drop in P_{DA} were required to protect V_S at high temperatures.

The CTM of flounder I measured was 25.8±0.51°C. This value is comparable to those of chinook and sockeye salmon (approx. 25°C; Clark et al., 2008; Steinhausen et al., 2008), but approx. 3.5°C greater than that of the cod (22.2±0.2°C; Gollock et al., 2006). The difference in the CTM of the cod vs. flounder and salmon is not surprising given the life history of these three groups. The salmon experience rapid and substantial increases in water temperature during their spawning migration (Brett 1971; Lee et al., 2003) and the winter flounder, is a coastal species which is usually found burrowed in the sediments, where considerable temperatures fluctuations (-1.0 to >22°C) can be encountered (Fletcher, 1975; Duthie, 1982; Moyle and Cech, 1996; Pereira et al., 1999). In contrast, the cod is a bathypelagic species which is not normally found in water temperatures greater than 10°C off the Atlantic coast (Jean, 1964; Rose and Leggett, 1988) and shows a strong thermoregulatory behavioural response (Claireaux et al., 1995).

4.6 Conclusions

My research provides novel information on the interactive effects of acclimation temperature and graded hypoxia, and a progressive increase to the flounder's CTM, on cardiovascular function in winter flounder. My results show that while the flounder has a typical response to progressive hypoxia at 8°C (albeit with a higher than expected P_wO_2 for the onset of bradycardia), no acclimation effect on Q is observed in this species between 8 and 15°C and a hypoxia-induced bradycardia is absent at the latter temperature. Further, I show that while the response of Q, f_H and V_S to a progressive temperature increase was typical to that shown for other teleost species, R_{sys} decreases more in the flounder and this results in a fall in P_{DA}. Overall, my results demonstrate that cardiovascular function is controlled differently in the flounder when challenged with ecologically-relevant environmental perturbations. However, to what extent some of the reported changes were physiological vs. behavioural (e.g., due to the flounder being buried vs. non-buried) is not clear, and should be clarified in future experiments.

4.7 Acknowledgments and grants

The authors thank S.K. Killen for his comments on earlier versions of this paper. This work was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery grant to A. Kurt Gamperl. Paula C. Mendonça was supported by a doctoral fellowship SFRH/BD/8082/2002 from the Portuguese Foundation for Science and Technology (FCT) co-financed through POCI 2010 (Programa Operacional Ciência e Inovação) and FSE (Fundo Social Europeu).

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5. Thesis Summary

5.1 Summary

This doctoral thesis investigated the functional relationships between maximum cardiac performance, heart morphology, and the adrenergic capacity of the winter flounder (*Pleuronectes americanus*) heart, and examined the effects of hypoxia and temperature on the cardiovascular physiology of this species. In addressing these issues, this thesis provides novel information on flounder cardiovascular function relative to other teleost species, and data which can potentially be used as indices of environmental stress, and possibly for making inferences about habitat quality.

This thesis started by generating *in situ* Starling and power output curves to determine the maximum pumping capacity of the flounder heart. This was the first time that an *in situ* heart preparation was developed for any flatfish species and, in fact, proved to be technically difficult due to the laterally compressed body morphology and rigid pericardium of this species. However, my results showed that the winter flounder have an enhanced pumping capacity relative to other fish species, with a maximum V_S g^{-1} ventricle that is the highest ever reported for teleosts. This finding was surprising considering the benthic and inactive lifestyle of this species, and taking into account that the flounder heart appears to be unable to deliver blood at high pressures. A subsequent experiment, where *in vitro* pressure-volume curves were used to examine heart chamber compliance, further clarified the mechanisms responsible for the enhanced pumping capacity. Overall, the results presented in Chapter 2, show that the flounder has a high

volume, low pressure, cardiovascular system that is based on an increased cardiac sensitivity to filling pressure, compliant atria and ventricles, and a large compliant bulbus which assists with the delivery of a large V_S at pressures which do not become limiting. The inability of the flounder heart to deal with high-pressures is likely related to their lifestyle. Flounder are typically buried in, or in contact with, the sediment (Fletcher, 1975; Pereira et al., 1999) and, consequently, are rarely exposed to situations that would demand a high-pressure cardiovascular design (e.g., intense exercise). In fact, winter flounder are poor swimmers (Joaquim et al., 2004). However, the need for a such a large V_S and comparable maximum Q to other teleost species (e.g., salmonids, cod) is unresolved as V_S did not approach maximum *in situ* levels when the flounder was exposed to either acute hypoxia or increases in temperature (see Chapter 4). Clearly this area will require additional research, as will the determination of the factor(s) that allow for easy ventricular and atrial expansion, yet the development of a substantial amount of contractile force. With regard to flounder myocardial function, performing single cardiac myocyte length-tension curves (see Cazorla et al., 2000; Shiels et al., 2006) to examine if flounder cardiac cells possess unique physiological adaptations may be particularly insightful. In fact, I performed such experiments when I briefly visited Dr. Shiels' lab in 2006. Although technical difficulties (e.g., getting flounder myocytes to adhere to the carbon fibres; visualization of flounder myocyte banding for the calculation of sarcomere length) precluding me from acquiring a robust data set, these preliminary experiments indeed suggested that the winter flounder myocytes are smaller and could be stretched less than the trout myocytes (Shiels et al. 2006).

In Chapter 3, I report that after exhaustive exercise (chasing), plasma adrenaline and noradrenaline concentrations were considerably lower than those recorded for many other teleost species (see Table 3.3, Chapter 3) and that such concentrations do not affect flounder cardiovascular function. This finding was contrary to the assumption that adrenergic control of the flounder heart would be primarily *via* circulating catecholamines due to the absence of adrenergic cardiac innervation (see Fig. 3.2), and led me to quantify and characterize this species' ventricular β-adrenoreceptors to better understand how they relate to, and mediate, the effects of catecholamines on the heart. This study showed that while β-adrenoreceptor density in the flounder ventricle is the highest ever reported for a teleost species, binding affinity for [^{3}H]CGP-12177 is very low. Based on these results, I suggested that the amount of plasma catecholamines released after chasing (exhaustive exercise) is not sufficient to stimulate the heart in this species, and that a type of stressor that flounder typically encounters (e.g., hypoxia) would be required to elevate circulating catecholamines to levels that could stimulate β-adrenoreceptors. Further, given that the reported β-adrenoreceptor binding characteristics (high B_{max} and K_d) are typically associated with β_3-adrenoreceptor subtypes, I proposed that the flounder heart possesses a significant amount of these adrenoreceptors and that their role is to prevent excessive stimulation by β_2-adrenoreceptors during severe hypoxia. However, these ideas are unsubstantiated at this point, and will require: (*1*) a careful examination of catecholamine levels following a number of ecologically relevant stressors; (*2*) a detailed pharmacological characterization of the flounder's cardiac β-adrenoreceptors to confirm that β_3-adrenoreceptors are present in significant numbers; and (*3*) *in vitro* and/or *in situ*

examinations of the effects of catecholamines and β_3-adrenoreceptor agonists and antagonists on flounder cardiac function and myocardial contractility.

The winter flounder is a coastal flatfish species that can often be found buried in the sediment (a situation where gill ventilation may be difficult) and experiences low oxygen concentrations (hypoxia) and considerable fluctuations in seasonal temperatures (from -1.0 to 22°C). These life history traits, and the unique aspects of their cardiac physiology revealed in Chapters 2 and 3, naturally led me to investigate how these two environmental factors influence cardiovascular function in this species. In these experiments, flounder acclimated to 8 and 15°C were exposed to a graded hypoxic challenge (100% to 20% water O_2 saturation) and 8°C acclimated fish were exposed to an acute increase in temperature to the flounder's critical thermal maximum (CTM). These experiments show that during environmental perturbations, cardiovascular function is also controlled differently in this species as compared to other teleosts. First, the onset of bradycardia in 8°C acclimated flounder occurs earlier than expected for this inactive and hypoxia-tolerant species (60% water O_2 saturation; P_wO_2 ~13 kPa), resting $\dot{Q}$ in 8 and 15°C acclimated fish does not differ, and hypoxic bradycardia was absent in 15°C acclimated flounder. The mechanisms/factors mediating these unusual cardiac responses to hypoxia and temperature may be physiological. However, it is also possible that they are primarily or partially due to the behaviour of the flounder when exposed to these environmental challenges. For example, it was not possible to verify when the flounder were buried (or not) in the sediment, which could affect both branchial and cutaneous gas exchange and, consequently, affect their cardiovascular responses (e.g., the lack of hypoxic bradycardia at 15°C might be related to the flounder emerging from the sediment

and thus an improvement in cutaneous gas exchange). Conversely, the characteristics/limitations of the flounder cardiovascular system may influence this species' behaviour (e.g., when faced with predators the flounder may chose to bury instead of showing a locomotor response since the heart it is not capable of supporting sustained intense exercise). Through the use of an integrated approach, where behaviour and physiology are assessed simultaneously, one would be able to more effectively evaluate the flounder's responses to natural and semi-natural conditions. Such research is clearly needed before this species' biology is accurately described, and its potential use as a model of environmental stress/quality can be evaluated.

5.2 References

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