THERMAL ACCLIMATION, CARDIAC PERFORMANCE, AND ADRENERGIC SENSITIVITY IN RAINBOW TROUT, (*Oncorhynchus mykiss*)

by

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Thermal acclimation, cardiac performance and adrenergic sensitivity in rainbow trout (Oncorhynchus mykiss).

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ABSTRACT

Changes in temperature are of particular importance to fishes whose body temperatures largely reflect ambient temperature. Fish respond to temperature change by either avoiding, conforming or adapting to the existing conditions. Rainbow trout adapt and remain active despite large seasonal fluctuations in ambient temperature. Since aerobic performance is dependent upon cardiovascular function, this thesis examines the effect of acclimation temperature on swimming activity and cardiac function in rainbow trout experimentally-acclimated to either cold (8°C) or warm (18°C) temperatures.

While maximum prolonged swimming speed (U_{crit}) was lower at cold-temperatures, the effect of temperature upon U_{crit} was marginally reduced with cold-acclimation. In contrast, intrinsic cardiac performance, determined using *in situ* working heart preparations (WHP), was significantly improved by temperature acclimation.

Cardiac performance *in vivo* is likely affected by circulating catecholamines which increase as U_{crit} is approached and have positive inotropic and chronotropic effects. Accordingly, I examined the effects of acclimation temperature on the adrenergic responsiveness of WHPs and *in vitro* ventricular strip preparations (VSP). In both preparations, the sensitivity to adrenaline increased 5-10 fold with cold-acclimation. Furthermore, the magnitude of the inotropic response at maximally-stimulating levels of adrenaline was significantly greater in cold-acclimated WHPs.

In an attempt to explain the observed shift in adrenergic sensitivity following acclimation, I performed radioligand binding studies of homogenates and isolated sarcolemmal (SL) fractions from ventricles of

warm- and cold-acclimated trout. The cell surface adrenoceptor population was significantly greater in cold-acclimated heart tissue. Additionally, biochemical analysis of some of the components involved in transduction of receptor occupation revealed basal cAMP production to be greater in cold-acclimated trout heart. Moreover, the magnitude and sensitivity of cAMP production in response to adrenergic stimulation was greater in cold-acclimated tissue, reflecting both the greater SL density and heightened basal activity. These changes in both adrenoceptor population and biochemical cascade likely affect the influx of calcium into the cytosol and thus modulate cardiac contractility.

Cardiac tissue has an obligatory requirement for SL Ca^{2+} influx which, in the mammalian myocardium, triggers the release of calcium from the sarcoplasmic reticulum (SR) to initiate contraction. To examine the potential importance of the SR in fishes and the possible impact of temperature acclimation on this, ryanodine, a noted blocker of SR Ca^{2+} release in mammals, was used and found to have no effect on contractility in either *in situ* WHPs or *in vitro* VSPs at physiologically-realistic pacing regimes at either warm or cold acclimation temperature. This finding suggests that the sarcolemma plays the primary role in supply of Ca^{2+} to the contractile element in the fish heart.

Therefore, this thesis demonstrates temperature to have a significant impact on swimming and cardiac performance and that temperature acclimation significantly modifies intrinsic cardiac activity and the heart's responsiveness to adrenaline. These modifications likely increase the ability of the heart to supply working muscle despite reduced ambient temperatures.

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CHAPTER I

GENERAL INTRODUCTION

This thesis describes a series of studies which examine the effect of acclimation temperature on the adrenergic sensitivity of the heart of the rainbow trout. The studies were conducted at a number of organizational levels, ranging from intact fish to the subcellular which, together, provide insights into the biochemical correlates underlying a physiological response.

Thermal Effects

Biochemical, metabolic and other physiological functions depend on the rate at which chemical reactions can occur. Decreases in temperature decrease reaction rates and, consequently, maximum activities of biological systems. This is of particular importance in aquatic poikilotherms whose body temperatures are largely governed by the ambient temperature of their surroundings. It is therefore obvious that seasonal variations in ambient temperature present a major challenge to the physical condition and survival of aquatic poikilotherms. To meet this challenge, aquatic poikilotherms use a number of strategies. Many, if able, will avoid temperature change through either horizontal or vertical migration to warmer or cooler waters. Often simple migration, however, is not sufficient to meet the challenge of a changing water temperature and thus a number of other strategies have evolved.

The effect of temperature on a physiological or biochemical process is often expressed by a Q_{10} value which describes the effect a 10°C change would have on the rate of that process. It is calculated as

$$Q_{10} = (k_1/k_2)^{10/(t_1-t_2)}$$

where t_1 and t_2 are temperatures and k_1 and k_2 are the rates determined at t_1 and t_2. In the absence of mechanisms designed to counteract the effect of temperature change, a 10°C decrease in temperature will typically halve the maximal rate of a particular process (Q_{10}=2.0). Many poikilotherms, in fact, do not compensate for temperature change and, as a result, become relatively inactive during winter months. Other poikilotherms respond to decreasing temperature by reducing metabolic output to levels below those produced by the decline in temperature itself. These animals in essence "under-compensate" (Precht, 1958; Prosser, 1969) and enter a period of dormancy during winter months. Other poikilotherms, however, undergo physiological and biochemical modifications such that the effect of temperature is either partially ($1.0<Q_{10}<2.0$) or completely ($Q_{10}=1.0$) compensated (Precht, 1958; Prosser, 1969), thereby permitting levels of activity which otherwise would not be possible. Partial compensation is the most common acclimatory response to temperature change found in fishes, including rainbow trout (*Oncorhynchus mykiss*), and provides a general focus for this Thesis.

In fishes (like rainbow trout) that remain active in winter months, systems and system components that determine swimming capacity must undergo at least partial compensation for the fish to eat and avoid being eaten. One of the most widely used measures of overall swimming capability is maximal prolonged swimming speed (U_{crit}). U_{crit} is a measure of the aerobic capacity for swimming measured by stepwise increases in speed, each step having a duration of 15-30 minutes (Beamish, 1978). Thus U_{crit} defines the ability of a fish to maintain swimming for this period. Acute change in ambient temperature generally produces a Q_{10} for U_{crit} of 1.5-1.8 (Beamish, 1978; Rome *et al.*, 1985), indicating

that intrinsic mechanisms exist to partially compensate for temperature change. Furthermore, U_{crit} is increased with acclimation, particularly at cold temperatures, and the Q_{10} is reduced to 1.3-1.5 (Rome *et al.*, 1985; Sisson and Sidell, 1987; Kolok, 1992). Maximum sustained swimming speed is the product of a complex interplay of neural, mechanical, respiratory and cardiovascular components, many of which are highly temperature sensitive. The physiological processes that limit prolonged swimming speed in fishes are not known in detail and are not the focus of this thesis. However, one of the more important elements underlying prolonged aerobic activity in mammals is cardiac performance and resulting delivery of oxygenated blood to working muscle (Saltin and Stenberg, 1964; Rowell, 1971; Savard *et al.*, 1987). Myocardial activity must play a crucial (but not necessarily limiting) role in maintaining aerobic swimming. Indeed, aerobic swimming speed has been found to be linearly related to cardiac output (Kiceniuk and Jones, 1977). If this relationship is fundamental in describing maximum aerobic swimming capacity, then the effect of temperature, both acute and chronic, on maximum cardiac performance should be mirrored by similar effects on U_{crit} and thus Q_{10} values should be closely matched. *In situ* analysis of trout hearts operating at acclimation temperature and under conditions approximating *in vivo* ventral aortic pressures, however, indicates the Q_{10} of maximal cardiac performance (Graham and Farrell, 1989) to be greater than that found for U_{crit}. This suggests that the performance of the muscle tissues used for swimming is not compromised by a decrease in temperature to the same extent as the ability of the heart to deliver blood to those tissues and thus the aerobic activities of cardiac and skeletal muscle are not well-matched.

This is probably not the case, however, for two reasons. Firstly, the preparation used by Graham and Farrell (1989) did not have an intact

pericardium. The pericardium is important in cardiac and power output generation (Farrell *et al.*, 1988b), making extrapolation to the situation *in vivo* difficult at best. Secondly, as U_{crit} is approached, the effect of catecholamines on both cardiac and swimming performance likely becomes increasingly important and could have a major impact on the matching of U_{crit} and cardiac performance.

In vivo heart performance is subject to an adrenergic input derived from both circulating levels and sympathetic release of catecholamines (Randall, 1970; Laurent *et al.*, 1983; Farrell, 1984). The rainbow trout heart is stimulated by adrenaline and, to a lesser extent, noradrenaline (Ask *et al.*, 1980, 1981; Farrell *et al.*, 1986). Phenylephrine, a potent alpha-adrenergic antagonist, has no apparent effect on inotropy or chronotropy (Farrell *et al.*, 1986) and the trout heart, as in most other fishes (Falck *et al.*, 1966; Farrell, 1984; Holmgren, 1977), is considered to be beta-adrenergically driven.

Circulating catecholamine levels increase 6-15-fold during intense exercise (Butler *et al.*, 1986; Primmett *et al.*, 1986; Milligan *et al.*, 1989) but the relative proportions of adrenaline and noradrenaline vary with acclimation temperature. In summer-acclimated trout, adrenaline and noradrenaline levels are nearly equal during heavy exercise but in winter-acclimated trout adrenaline levels are 4-fold greater than noradrenaline (Milligan *et al.*, 1989). Given the greater potency of adrenaline as an agonist in heart tissue (Ask *et al.*, 1981; Farrell *et al.*, 1986), cardiac stimulation may be greater at cold temperatures and may partially offset the depressant effect of cold temperatures on maximum cardiac performance and, perhaps, U_{crit}.

Some indication of the potential importance of catecholamines in improving *in vivo* cardiac performance at cold temperatures comes from *in situ* experiments which examined the effect of acclimation temperature on the ability of adrenaline to stimulate the heart (Graham and Farrell, 1989). Both the sensitivity

(estimated from the concentration producing one-half maximal response [EC_{50}]) and relative increases in inotropy and chronotropy were greater in hearts from cold- (5°C) than warm-acclimated (15°C) trout. As noted previously, however, the pericardium was disrupted in this preparation and the applicability of these results to the situation *in vivo* may be limited.

Matching of cardiac and swimming performance, and associated Q_{10} values, thus likely has an acclimatory component related to adrenergic stimulation. Indeed the Q_{10} of *in situ* maximum cardiac output in cold- and warm-acclimated trout hearts tested at acclimation temperature was significantly reduced after application of maximally stimulating levels of adrenaline (Graham and Farrell, 1989). Given that these studies again employed a preparation with a disrupted pericardium, the first objective of this Thesis was to examine the effect of acclimation temperature on cardiac performance and adrenergic sensitivity using a preparation having an intact pericardium, and the possible effect this might have on prolonged swimming speed.

The shift in adrenergic sensitivity in the hearts of fishes with acclimation to different environmental temperatures is not limited to rainbow trout but has been reported in a number of species and with a variety of responses. For example, in the perch (*Perca fluviatilis*), adrenaline induces a bradycardia which is greater at low (15°C) than at high (24°C) temperatures (Tirri and Ripatti, 1982). Adrenaline in the carp produces different effects dependent upon temperature, with bradycardia observed over 1-8°C and tachycardia from 9-20°C (Laffont and Labatt, 1966). Given it's widespread occurrence, modification of adrenergic responsiveness may be one of the primary mechanisms used to compensate for the effects of a temperature change.

To date, however, no investigation has been undertaken to determine the biochemical correlates of the reported shifts in adrenergic responsiveness that

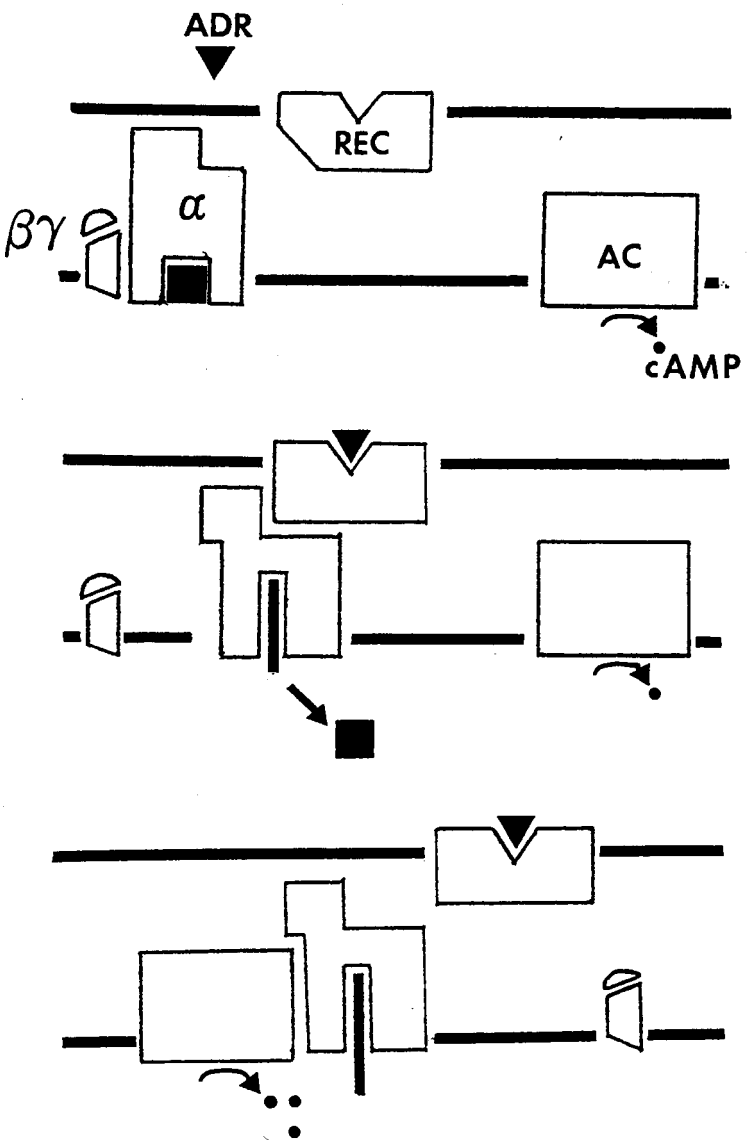
occur with thermal acclimation in fish hearts. This then forms the second major component of my Thesis.

Adrenaline and Transmembrane Signaling

Beta-adrenergic actions are brought about through a series of dynamic interactions between membrane-bound receptors, enzymes and ion channels. Beta-adrenergic receptors are a class of surface membrane proteins which act as one link in the translation of binding of an extracellular compound into generation of an intracellular event (Figure 1.1).

Catecholamine-occupation of a surface beta-adrenoceptor promotes a conformational change allowing the hormone-receptor complex to interact with stimulatory guanine nucleotide binding protein (G_s; Pfeuffer, 1977; Ross and Gilman, 1977; Schwinn *et al.*, 1992), one of a family of intramembrane GTP-binding proteins (Dohlman *et al.*, 1987; Birnbaumer, 1990). G proteins are trimeric proteins consisting of alpha, beta and gamma subunits (Kuhn, 1980; Northup *et al.*, 1980). The alpha subunit contains a GTPase enzyme which, under non-stimulated conditions, has a GDP molecule bound to it. Interaction with the hormone-receptor complex initiates a conformational change in the G protein such that the affinity of the alpha subunit for GTP is much greater than that for GDP and GTP becomes bound to the subunit (Susanni *et al.*, 1992). The conformational change induced by the interaction with the hormone occupied receptor promotes dissociation of the beta and gamma subunits, which are thought to inhibit the alpha subunit (Schwinn *et al.*, 1992; Susanni *et al.*, 1992) from interacting with another membrane-bound enzyme, adenylate cyclase. The alpha subunit, free of inhibition by beta and gamma subunits, and having a bound GTP molecule, interacts with membrane-bound adenylate cyclase (Neer and

Figure 1.1. The beta-adrenergic signaling pathway. Abbreviations are for adrenaline (ADR), adrenoceptor (REC), stimulatory G protein (alpha, beta and gamma subunits), adenylate cyclase (AC) and cyclic AMP (cAMP). A general description of events are in the text.


ADR
REC
βγ
α
AC
cAMP

Clapham, 1988).

Stimulation of adenylate cyclase increases the rate of conversion of ATP to cyclic AMP (cAMP). Cyclic AMP serves as a second messenger which binds to cAMP-dependent kinase (protein kinase A; PK A), inducing dissociation of regulatory subunits and removing inhibition of the enzyme. PK A in the heart phosphorylates, amongst other targets (Tada *et al.*, 1975; Ray and England, 1976; Lindemann *et al.*, 1983; Solaro, 1986), the L-type calcium channels responsible for calcium influx across the sarcolemma (Osterrieder *et al.*, 1982; Rinaldi *et al.*, 1982; Fedulova *et al.*, 1985). The activated G_s protein can also interact directly with the L-type calcium channel (Yatani *et al.*, 1987; Brown and Birnbaumer, 1988; Mattera *et al.*, 1989). Both of these events increase the probability of the channel opening (Reuter and Scholz, 1977; Osterrieder *et al.*, 1982; Yatani *et al.*, 1987), thereby increasing Ca^{2+} influx across the sarcolemma and, in conjunction with stimulation of other subcellular elements, producing a positive inotropy.

The amount of interaction between adrenoceptor, G_s protein and adenylate cyclase indicates the potential for significant amplification of cAMP generation and thus the stoichiometry of the interrelationships becomes very important. The stoichiometry is presently unknown but some evidence exists which indicates that the stoichiometry is not equivalent. Stimulation of adenylate cyclase is maximized at concentrations below those required to occupy all beta-adrenoceptors (Venter, 1979). Furthermore, in S49 lymphoma cells, quantitation with antibodies and selective radioligands indicate there to be ~100,000 G_s proteins per cell but only ~1000 beta-adrenoceptors and adenylate cyclase enzymes per cell (Ransnas and Insel, 1988; Alousi *et al.*, 1991). It appears that the catecholamine-bound receptor interacts with a number of G_s proteins which, likewise, may activate a number of effectors (adenylate cyclase). None of these experiments have yet been conducted on fish tissues. The shifts in cardiac response to adrenaline could potentially

result from alteration in the overall content of any of the signalling components (adrenoceptor, G_s, adenylate cyclase) or in properties which determine their stoichiometric relationships. Obviously the *in vivo* and *in situ* observed shifts in cardiac response to adrenaline could be brought about through modifications of some or all of the components involved in transmembrane-signaling. As mentioned previously, these have yet to be detailed in fishes.

Alterations in the sensitivity of cardiac tissue to adrenaline could also occur by modification of events responsible for attenuating adrenergic stimulation. Availability of the adrenoceptor for occupation by catecholamine can be altered in a number of ways. On a short-term basis, availability can be reduced by phosphorylation by beta-adrenergic receptor kinase (BARK). BARK is specific for the hormone-occupied receptor form (Benovic *et al.*, 1986) and causes the receptor to be sequestered away from the cell surface into a membrane-associated compartment (Harden, 1983; Benovic *et al.*, 1986), thus preventing interaction with G_s protein (homologous desensitization). Depression of adrenergic stimulation can also be achieved through an autoregulatory pathway as PK A is able to phosphorylate the adrenoceptor (Benovic *et al.*, 1985). If cAMP levels remain high for a relatively long period of time, PK A phosphorylation of the receptor also promotes intracellular sequestration and possible adrenoceptor degradation (heterologous desensitization). Again, all research to date has been conducted on mammalian tissues and there is no information on the mechanisms of short- and long-term desensitization in trout, although desensitization has been observed (Graham and Farrell, 1989; Keen and Farrell, *unpub. obs.*).

Alteration and attenuation of adrenergic sensitivity can also be brought about through activation, in the instance of the L-type channel, of processes responsible for channel dephosphorylation. A number of protein specific

phosphatases (PPases) have been identified in mammalian cardiac tissues and at least two, PPase 1 and PPase 2a, appear to be restricted to the sarcolemma (Kameyama *et al.*, 1986). Intracellular injection of the catalytic subunit of PPase 1 or PPase 2a reverses stimulation of calcium current in voltage-clamped myocytes produced by either the catalytic subunit of adenylate cyclase, PK A or isoproterenol (Kameyama *et al.*, 1985, 1986; Hescheler *et al.*, 1987). Differences in quantities or activity rates of these enzymes could also have an effect on the sensitivity of myocardial tissue to adrenergic stimulation.

I therefore examined the effect of thermal acclimation on some of the components involved in adrenergic signal transduction, namely adrenoceptor, G_s protein and adenylate cyclase, in an attempt to discover some of the biochemical correlates of observed shifts in adrenergic sensitivity.

Adrenaline in mammals promotes phosphorylation of the L-type channel and increases transarcolemmal calcium influx. If this influx is of sufficient magnitude, an increased influx can ultimately produce a positive inotropy. Although not detailed in fishes, similar events are likely to occur. However, while the consequence of an increased calcium influx (increased force production) is likely not different in mammals and lower vertebrates, differences may exist in the mechanisms, due to differences in excitation-contraction (E-C) coupling, by which it occurs. A preliminary examination of E-C coupling in the trout myocardium and the possible effects of acclimation temperature on this process comprises the final component of my Thesis.

Excitation-Contraction Coupling

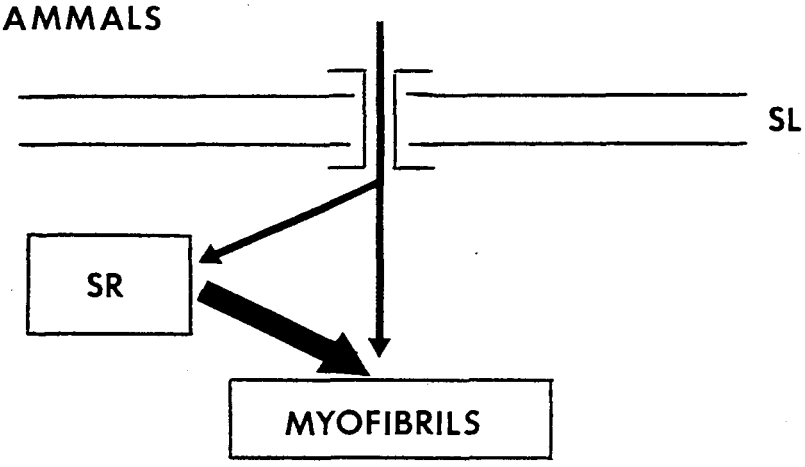
E-C coupling in cardiac tissue is the process whereby an action potential excites the sarcolemma (SL) in such a way that intracellular conditions are

changed to a state in which contraction is favored. Two primary models of E-C coupling in cardiac tissue exist and are, in general, characteristic of mammals and lower vertebrates, respectively (Figure 1.2). In the mammalian model, the calcium that activates the myofilaments is derived from two sources. The mammalian myocyte is organized such that invaginations of the SL, transverse or t-tubules, are closely apposed to the terminal cisternae of the sarcoplasmic reticulum (SR; Sommer and Jennings, 1986) in which SR calcium release channels are situated. In the transverse tubule system, depolarization of the SL initiates an increase in cytosolic calcium levels via an influx through sarcolemmal, voltage-dependent, L-type calcium channels (Wier and Isenberg, 1982; Tsien, 1983; Beuckelmann and Wier, 1988) and, possibly, sarcolemmal sodium-calcium exchange (Mullins, 1979; Bers *et al.*, 1988; Leblanc and Hume, 1990). This influx of calcium plays a relatively minor role in activating the myofilaments but is central to promoting the release of a much greater quantity of calcium stored in the SR. Calcium released from the SR serves as the primary source of calcium which activates the contractile elements (Fabiato and Fabiato, 1975). Species and age-related differences do exist, however, in the relative importance of SR- and SL-mediated calcium supply in this process (Fabiato and Fabiato, 1978a; Bers, 1989).

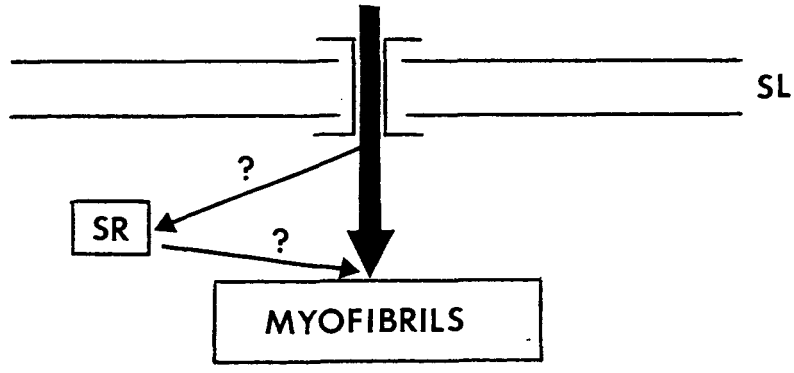
In the hearts of lower vertebrates, the dependence on SR calcium stores in muscle contraction appears to be much reduced. Sarcoplasmic reticulum content in lower vertebrates, most notably the frog and fishes, is greatly reduced on a cell volume basis (Santer, 1974, 1985). Additionally, the transverse tubule system is absent in both frogs and fishes and invaginations are restricted to much shallower caveolae (Page and Niedergerke, 1972; Santer, 1974). In frog, a variety of electrophysiological and pharmacological evidence indicates the magnitude of calcium influx to be sufficient to support full force generation (Morad and

Figure 1.2. The relative roles of the sarcolemma and sarcoplasmic reticulum in excitation-contraction coupling in higher and lower vertebrates. The relative contribution each makes to contractility is indicated by arrow size. See text for details.

MAMMALS



FROG (TROUT ?)



Orkand, 1971; Morad and Goldman, 1973; Klitzner and Morad, 1983). Evidence in fishes is scant in comparison and is largely limited to the contractile response of ventricular strips to agents known to inhibit release of Ca^{2+} from the SR in mammals, most notably ryanodine (Gesser and Hoglund, 1988; El-Sayed and Gesser, 1989). The observations that ventricular strips are insensitive to ryanodine under ionic and thermal conditions approximating those found *in vivo* suggests a minimal role for the SR in E-C coupling. Furthermore, estimates of L-type calcium channel density in trout heart, assessed by dihydropyridine-binding in enriched SL preparations, indicate a density 3-4 times higher than found in mammals (Tibbits *et al.*, 1990). Taken together, these results are consistent with a greater role for extracellularly-derived calcium in direct activation of the myofilaments in the trout heart than is found in mammals.

Adrenergic stimulation of calcium influx across the SL will promote an increase in force generation in the models for both higher and lower vertebrates by, in large part, increasing the amount of calcium available to the contractile element. While these models do not predict quantitative differences in inotropic development, they do suggest qualitative differences in the manner by which it is achieved. In mammals, adrenergic stimulation of calcium influx serves to increase the trigger and subsequent release of SR calcium stores, thus acting indirectly to increase force development. In the trout heart, however, the adrenergically-stimulated increase in calcium influx across the SL should be directly related to the increase in contractile force.

In fishes ambient temperature may have an effect on the roles of the SL and SR in E-C coupling. The rapid cooling contracture experiments of Bers (Shattock and Bers, 1987; Bers, 1987, 1989; Bers and Bridge, 1989), coupled with the recent electrophysiological studies by Sitsapesan *et al.* (1991) suggest the ryanodine-sensitive calcium release channel of the SR in mammals to have a high thermal

dependence. This raises the possibility that previous observations of a lack of an effect of ryanodine on force development in fish ventricular strips was due to the experimental temperatures used. At low temperatures the SR calcium release channels might be in the open configuration and ryanodine would thus be ineffective in reducing contractile force (Tibbits *et al.*, 1991). Experimental work using tuna atrial strips (Keen *et al.* 1992) provides support for this possibility. Tuna normally occupy warm waters (exceeding 25°C) and it was found that application of 10 *u*M ryanodine to tuna atrial strips at 25°C depressed contractility by about 30%. If the thermal dependency of the ryanodine release channel is similar in fish and mammals, seasonal variations in ambient temperature might affect the relative roles of the SL and SR in E-C coupling in the trout myocardium, and thus the route(s) by which adrenergic stimulation of contractility is achieved. In the final Chapter of my Thesis I examined the effect of temperature on the response of the trout heart to ryanodine, and thus the role of the SR in E-C coupling.

Objectives

The objectives of this Thesis are as follows:

1. To determine if the thermal dependence of maximum aerobic swimming capacity and cardiac performance are similar in trout (Chapter II).

2. To determine if beta-adrenergic sensitivity of the trout heart is altered by acclimation temperature (Chapter III).

3. To determine if the change in beta-adrenergic sensitivity of the trout heart that occurs with thermal acclimation is correlated with alteration of components involved in the beta-adrenergic signal transduction pathway (Chapter III and IV).

and,

4. To examine the role of the SR in myocardial E-C coupling in the trout and the effect of temperature on this role (Chapter V).

CHAPTER II

THE EFFECT OF THERMAL ACCLIMATION ON MAXIMAL PROLONGED SWIMMING SPEED AND IN SITU CARDIAC PERFORMANCE

A. Introduction

The pumping capacity of the heart (and consequent perfusion of working tissues) is critical for prolonged aerobic activity in mammals (Rowell, 1971; Savard *et al.*, 1987). Prolonged aerobic activity in fishes, i.e., swimming speed, and cardiac output are closely related in rainbow trout (Kiceniuk and Jones, 1977). If a close relationship between cardiac performance and swimming speed is a fundamental property of aerobic swimming capacity in trout, the relationship should be maintained under all environmental conditions routinely encountered by trout and particularly evident at maximal performance levels.

In poikilotherms such as trout, the ambient conditions fluctuate and can change dramatically over both long and short periods of time. Temperature provides a prime example of this and changes occur both acutely (excursions through thermoclines) and chronically (seasonal changes). In order to assess the robustness of the relationship between cardiac and swimming capacities, I compared maximal performance of the heart with that achieved by the intact trout under conditions where the heart probably operates maximally, i.e., at U_{crit}. Using rainbow trout acclimated to warm (18°C) and cold (8°C) temperatures, heart performance was examined using an *in situ* perfused working heart preparation (WHP; Farrell *et al.*, 1988b).

The WHP can generate power outputs similar to those found *in vivo* (Kiceniuk and Jones, 1977; Farrell *et al.*, 1988b). The matching of U_{crit} and

maximal heart performance was estimated through comparison of the Q_{10}'s of U_{crit} and *in situ* power output of acclimated animals or hearts tested at acclimation temperatures. Furthermore, the capacity for acclimation-induced mechanisms to improve performance beyond that of intrinsic compensations to temperature change was determined through comparison of the Q_{10} of U_{crit}s determined after acute temperature transition with that of fish swimming at acclimation temperature.

B. Materials and Methods

Origin and maintenance of rainbow trout. Yearling rainbow trout of undetermined sex were purchased from a local supplier (West Creek Trout Farms, Aldergrove, B.C.) and held indoors in 2000 L fibreglass tanks receiving aerated, dechlorinated tap water. Fish were separated into warm (18°C) and cold (8°C) tanks. Warm water temperature was maintained via a countercurrent heat exchanger of local construction whereas cold temperatures were maintained using a Min-O-Cool cooling unit (Frigid Units Inc., Blissfield, Michigan). In neither instance did temperature vary by more than 1°C from the desired set point. Fish were held at the experimental temperature for 3 weeks under a 12:12 light:dark photoperiod. Fish were fed daily *ad libitum* with a commercial trout pellet. After acclimation, fish in each acclimation tank were separated into two groups for either swimming trials or *in situ* WHPs.

Swimming Trials. Fish used in swimming trials were lightly anesthetized and tagged using small colored beads sewn into the skin immediately behind the dorsal fin. Swim experiments were conducted on individual fish using a Brett-style swimming respirometer (Gehrke *et al.*, 1988). Water volume was 120 L and

water speed was generated by a centrifugal pump whose motor frequency was controlled by a variable speed drive unit. Water speeds (up to 1.07 $m \cdot sec^{-1}$) at selected motor frequencies were verified using an impeller connected to a precalibrated frequency counter. Water speed was linearly related to motor frequency at water temperatures of both 8 and 18°C. Calibration curves for the two temperatures were linear and no significant ($p > 0.05$) difference between either the slope or intercept was found. The results were subsequently pooled and the equation relating water speed to motor frequency was determined to be

$$\text{Water Speed} = (0.0536\ m \cdot sec^{-1} \cdot Hz^{-1})\ \text{Freq} + 0.002$$

where water speed is in $m \cdot sec^{-1}$ and motor frequency of the centrifugal pump (Freq) is in Hz ($r^2 = 0.997$).

No means of ensuring a constant supply of 8 or 18°C water was available and thus, once the desired water temperature was established in the swim tunnel, flow of water into the swim tunnel was stopped. To prevent problems with oxygen depletion, oxygen tension for both warm- and cold-water trials was maintained above air-saturated levels by introduction of a 20 second pulse of 100% oxygen every ten minutes. Oxygen levels above air-saturated levels have been previously demonstrated to have no effect on swimming performance (Davis *et al.*, 1963). The flow profile of the water was disturbed each time for a period of approximately 30 seconds as a result. Initial and final temperatures were monitored for each experiment. Temperature in any individual trial did not vary by more than 2°C and the mean value was used in subsequent calculations as the trial temperature. Mean temperature values ranged from 6.9-9.1 and 17.7-19.8°C.

Fish were placed in the swimming chamber which held water at the acclimation temperature of that particular fish (~8 or 18°C). Fish were allowed to

become accustomed to the chamber for a period of 2 hours during which water speed was set at 0.22 $m \cdot sec^{-1}$. This period of time was considered sufficient to eliminate the effects of handling stress on aerobic swimming performance (Glova and McInerney, 1977). After this period, the water speed was increased by 0.08 $m \cdot sec^{-1}$ every 20 min. Fish readily swam against the opposing current and were prevented from resting at the back of the chamber by means of an electrified screen. At the completion of each 20 min period, the water speed was increased by an additional 0.08 $m \cdot sec^{-1}$ and was maintained at the new speed for 20 min or until the fish became fatigued and was unable to swim against the imposed current. The water speed and point in time within the 20 min exercise period at which the fish became fatigued was noted and used in the calculation of U_{crit} (see below). Water speed was then reduced to 0.22 $m \cdot sec^{-1}$ and the fish allowed to recover for 15 minutes prior to return to the indoor holding tank. Comparison of U_{crit} values from warm- and cold-acclimated fish swum at their acclimation temperature and calculation of Q_{10} from mean values provided estimates of the chronic effect of temperature acclimation.

Following completion of trials with each group (4 days) at their acclimation temperature (chronic trials), acute effects were examined by retesting each fish at the opposite temperature (cold-acclimated at 18°C, warm-acclimated at 8°C) in the same order as initially tested.

Once each fish had completed this second swim trial, morphometric descriptors (body weight, fork length, width, depth) were determined. These measurements were used to correct water speed for the blocking effects of the fish on water flow in the swimming chamber. Blocking effects were corrected according to the following equation (Gehrke *et al.*, 1988):

$$V_c = V_m (1+E_s)$$

where V_c is the corrected water flow velocity, V_m is the measured water flow velocity determined in the absence of a fish in the swim chamber and E_s is the proportional error due to the solid blocking effect of the fish. Assuming the cross-sectional area and length of a fish to be best represented by an elliptical cylinder, E_s, as previously described by Bell and Terhune (1970) is:

$$E_s = TL\,(A_o \cdot A_t^{-1})^{1.5}$$

where T is 0.8; L=0.5 x (fish length · fish thickness^{-1}), A_o is the cross-sectional area of the fish (described by 0.25 · fish depth · fish width) and A_t is the cross-sectional area of the cylindrical test section.

Maximum prolonged swimming speed (U_{crit}) was calculated, after correction for blocking effects, in both absolute (cm·sec^{-1}) and relative (normalized for body length [BL]) units, using the formulation of Brett (1964) as:

$$U_{crit} = U_i + [(t_i/t_{ii}) \times U_{ii}]$$

where U_i=highest speed (in cm·sec^{-1} or BL·sec^{-1}) maintained for the full 20 minutes, U_{ii}=the speed increment (0.08 m·sec^{-1} or 8.2/BL in BL·sec^{-1}), t_i=the length of time in minutes that the fish was able to swim against the water speed which produced fatigue, and t_{ii}=the time increment between changes in water speed (20 minutes).

The relationship between a change in swimming speed as a function of temperature (Q_{10}) was calculated for each fish as described in the General Introduction.

In situ Working Perfused Heart Preparations. In situ WHPs were performed as previously described (Farrell *et al.*, 1988b). In brief, fish were anesthetized (MS222, 1:5000 w/v) and transferred to an operating sling and the gills superfused with a chilled, buffered and aerated 1:10,000 (w/v) MS222 solution. Fish were injected with 75 USP of sodium heparin in saline via caudal vessels. Input and output cannulae were constructed of stainless steel from chromatography columns (I.D. 1.9 mm [input] and 1.5 mm [output]). The input cannula was introduced into the sinus venosus via a hepatic vein and the output cannula inserted into the ventral aorta to a point confluent with the bulbus arteriosus. Silk ligatures were used to prevent backflow into remaining hepatic veins. Ductus Cuvier were ligated, thereby crushing the cardiac branches of the vagus nerve; heart rate was maintained by the sino-atrial pacemaker rhythm. The pericardium was not disturbed and the heart received saline (described below) at a constant pressure throughout the surgery once the input cannula was inserted.

Following surgery, the fish were transferred to, and immersed in, a saline-filled bath and the input cannula was switched from the temporary surgical reservoir to a constant pressure head delivering perfusion saline to the heart. Both immersion bath and perfusion saline reservoirs were water-jacketed and temperature maintained by a Lauda cooling unit (Brinkmann Instruments, Rexdale, Ont) set at the acclimation temperature (8 or 18°C) of the fish in use.

Cardiac output (in $mL \cdot min^{-1}$) was measured by an electromagnetic flow probe in the output line (Zepeda Instruments, Seattle, Washington) while filling (P_i) and output (P_o; afterload) pressures were measured using Micron pressure transducers (Narco Life Sciences, Houston, Texas). Filling pressure and afterload were referenced to the immersion bath level. Afterload was initially set at a physiologically-relevant 50 cmH_2O and filling pressure was varied to produce a basal cardiac output of ~10-12 (8°C) or ~18-20 $mL \cdot min^{-1} \cdot kg$ body wt^{-1} (18°C)

approximating *in vivo* resting conditions (Kiceniuk and Jones, 1977). Pressure and flow signals were amplified and displayed on a chart recorder (Model 2400; Gould, Cleveland, Ohio). Input pressures were routinely negative, indicative of a well-functioning heart with an intact pericardium (Farrell *et al.*, 1988b). Signals were also fed into an Apple II+ microcomputer via an analog-to-digital interface for subsequent analysis (Farrell and Bruce, 1987).

Maximal power output determinations. Maximal myocardial power outputs were determined through stepwise increases in filling and output pressures. Stepwise increases in filling pressure produced increases in cardiac output (flow). Filling pressure was increased until no further improvement in flow was observed. At this point the heart was maximally volume-loaded. Maximum power output was subsequently generated through stepwise increases in afterload under the volume-loaded condition. Signals were recorded after each increase in afterload until maximal power output was achieved. Power output ($mW \cdot g^{-1}$ ventricular mass) was calculated from the following:

$$\text{Power output} = [\text{Flow} \times (P_o - P_i) \times a]/\text{ventricular mass}$$

where flow is in $mL \cdot min^{-1}$, P_o and P_i are in cmH_2O, ventricular mass is in grams and a = 0.00162 $mW \cdot min \cdot mL^{-1} \cdot cm^{-1}$ and is a conversion to mW.

A Q_{10} estimate for maximal power output was calculated from the mean maximal power output of hearts from each acclimation group using the equation given previously.

Salines. Bath and perfusion salines were of the same composition and contained the following (in mM): NaCl, 124.1; KCl, 3.1; $CaCl_2$, 2.5; $MgSO_4$, 0.9; dextrose,

5.0. Salines were buffered with 20 mM N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES, sodium salt and free acid combinations) and gassed with 100% O_2. Saline pH was 7.90 and 7.74 at 8 and 18°C, respectively, and simulate *in vivo* blood values at these temperatures (Howell *et al.*, 1970; Randall and Cameron, 1973; Railo *et al.*, 1985). Adrenaline (5 nM) was added to all salines used in WHP experiments. This level approximates *in vivo* resting levels (Milligan *et al.*, 1989) and, *in situ*, both maintains a tonic cardiac stimulation and reduces deterioration of the preparation (Graham and Farrell, 1989).

Chemicals. All chemicals used were reagent grade and were purchased from Sigma Chemical (St. Louis, MO).

Statistical analysis. Appropriate non-parametric statistical comparisons were made using Mann-Whitney U or Spearman rank tests employing a significance level of 0.05.

C. Results

Morphometric data for acclimated fish used in swimming speed experiments are presented in Table 2.1. No significant differences were found between the two groups in any of the described variables. One fish from each acclimation group was excluded from the data sets as they did not complete the full regime of swimming trials. Morphometric data for the fish used in WHP determinations of maximum cardiac performance are presented in Table 2.2. Ventricular mass, as a percentage of body mass, was significantly greater ($p<0.05$) in cold- than warm-acclimated trout. An increase in relative ventricular mass with acclimation to cold

Table 2.1. Morphometric data from warm- (18°C) and cold-acclimated (8°C) rainbow trout used in swimming experiments. Measurements were made at the conclusion of all swim trials. Condition factor was calculated as [(Weight)/(Length)^3] x 100. Mann-Whitney U-tests revealed no significant differences ($p > 0.05$) between acclimation groups in any of the measured or derived variables. Values are mean $\pm$ SEM. N = 9 in all groups.

Acclimation Group	Length (cm)	Width (mm)	Depth (mm)	Weight (g)	Condition Factor
Warm	34.9$\pm$0.5	38.1$\pm$1.2	70.6$\pm$2.0	468$\pm$24	1.10$\pm$0.05
Cold	34.1$\pm$0.6	36.4$\pm$0.6	66.8$\pm$0.9	450$\pm$24	1.13$\pm$0.03

Table 2.2. Morphometric data of the cold- and warm-acclimated rainbow trout used in WHP determinations of maximum cardiac performance. Relative ventricular mass, as a function of body mass, (V/B) was calculated from (ventricular mass/body mass) x 100. Significant differences ($p<0.05$) in V/B between acclimation groups are indicated by paired lower case letters. Values are mean$\pm$SEM; N = 14 for both acclimation groups.

Acclimation Group	Vent Mass (g)	Body Mass (g)	(V/B) x 100
Cold (8°C)	0.42$\pm$0.03	430$\pm$18[a]	0.097$\pm$0.001[b]
Warm (18°C)	0.49$\pm$0.03	567$\pm$35[a]	0.087$\pm$0.002[b]

temperatures has been previously demonstrated (Farrell et al., 1988a) and indicated the three week acclimation period (as a minimum) to be sufficient to stimulate acclimatory responses.

Temperature acclimation was investigated using both swimming performance and *in situ* cardiac performance trials conducted at acclimation temperature. Q_{10} estimates of chronic temperature exposure on maximal performance were found to be different between the swimming trials and *in situ* WHP. A Q_{10} value of 1.35, calculated from mean U_{crit} estimates (in $BL \cdot sec^{-1}$) of fish swum at their acclimation temperature, was determined for the effect of chronic temperature exposure on swimming performance. A somewhat higher Q_{10} value of 1.44 was calculated for maximal power generation of the *in situ* WHP.

Heart rate, cardiac output and power development were found to be significantly greater in hearts from warm-acclimated individuals (Table 2.3). Maximum heart rate demonstrated the greatest temperature dependency, a Q_{10} of 1.52 under both volume and pressure-loaded conditions. Maximum stroke volume, however, was significantly reduced with acclimation to warm temperatures. As a result, maximum cardiac output has a Q_{10} of 1.26 and 1.32, under volume- and pressure-loaded conditions, respectively. The largest temperature effect was observed in maximal power output, or the ability to perform work. Under volume-loaded conditions, i.e., only a resting afterload, a Q_{10} value of 1.31 was calculated. Under conditions approximating maximal swimming performance (afterload of 70-80 cmH_2O) however, the Q_{10}, as previously mentioned, increased to 1.44.

Acute swimming trials revealed prolonged swimming performance to be significantly ($p<0.05$) increased by an increase in water temperature, independent of acclimation group (Table 2.4). No significant differences in swimming ability were found between the warm- and cold-acclimated trout when

Table 2.3. Effect of acclimation temperature on in situ maximal cardiac performance from fish acclimated to either 8 or 18°C. Q_{10} values were calculated from the mean of each acclimation group. Variables are in the following units: heart rate (f_H; beats·min^{-1}), stroke volume (SV; mL·kg^{-1} body weight), cardiac output (Q; mL·min^{-1}·kg^{-1} body weight) and power (mW·g^{-1} ventricular weight). Values are mean ± SEM. Significant differences ($p < 0.05$) are indicated by paired lower case letters.

Condition\ Parameter	Cold-Acclimated	Warm-Acclimated	Q_{10}
Volume-Loaded			
f_H	51.9±1.4[a]	78.8±1.0[a]	1.52
SV	0.96±0.03[b]	0.80±0.02[b]	0.83
Q	49.73±1.04[c]	62.87±1.57[c]	1.26
Power	4.42±0.14[d]	5.80±0.18[d]	1.31
Pressure-Loaded			
f_H	51.4±1.4[e]	78.2±1.0[e]	1.52
SV	0.92±0.03[f]	0.79±0.02[f]	0.86
Q	46.89±0.88[g]	61.68±1.54[g]	1.32
Power	6.12±0.15[h]	8.81±0.22[h]	1.44

Table 2.4. U_{crit} and Q_{10} values for warm- (18°C) and cold-acclimated (8°C) trout. Significant differences ($p<0.05$) between groups, as revealed by Mann-Whitney U-tests, are indicated by matched superscripted lower case letters. Values are mean ± SEM. N=9 in all groups.

Acclimation Group	Trial Water Condition	U_{crit} (cm·sec^{-1})	U_{crit} (BL·sec^{-1})	Q_{10}
Warm	Warm	96.2±2.4^{ae}	2.76±0.08^{b}	1.31±0.02^{f}
Warm	Cold	72.3±1.2^{a}	2.08±0.05^{b}	
Cold	Cold	69.2±2.7^{c}	2.04±0.10^{d}	1.22±0.01^{f}
Cold	Warm	86.1±2.9^{ce}	2.54±0.11^{d}	

tested at either 8°C or 18°C (Table 2.4). Acute Q_{10} estimates, however, were significantly different between warm- and cold-acclimated individuals (Table 2.4). The temperature dependence of swimming performance (Q_{10}) in cold-acclimated individuals was significantly lower ($p < 0.05$) than found in warm-acclimated trout.

D. Discussion

The relative health of an individual fish can have a major impact on performance (Butler and Milleman, 1971; Beamish, 1978). The well-being of the fish in this study was estimated through a "condition factor" (Table 2.1), defined as the ratio of body weight to the cube of body length (Carlander, 1950). The suitability of the relationship as an index of general health has not been rigorously tested. The index was applied by Reimers (1963) to overwintering survival of hatchery-reared trout and he concluded 0.7 to represent a threshold value below which physical condition was poor. Condition factor values in this study averaged 1.13 and 1.10 for cold- and warm-acclimated fish, respectively, (Table 2.1) and compare favorably with other values from both hatchery-reared (Jones, 1971) and wild trout (Fessler and Wagner, 1969).

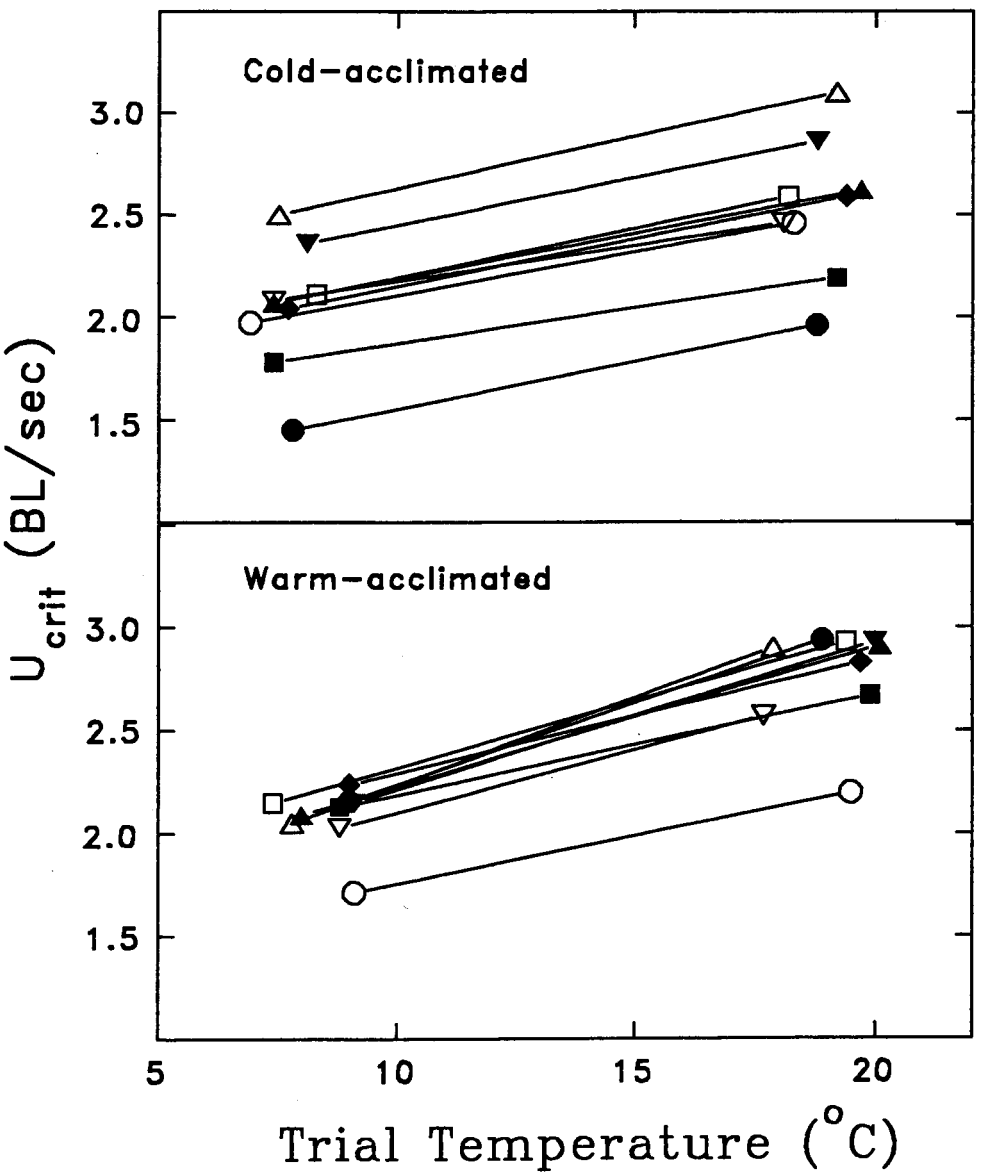
Published reports of maximum aerobic swimming speed in rainbow trout vary as a function of temperature and body size. Maximum U_{crit} values for cold-acclimated (8°C) trout in this study were similar to other studies performed in this lab (Farrell *et al.*, 1990; 1991) under with similar water temperatures. The general estimate of 2 BL·sec^{-1} at 8°C is also in general agreement with that found by Kiceniuk and Jones (1971; 10°C; 2 BL·sec^{-1}) and Schneider and Connors (1982; 10°C, 3.15 BL·sec^{-1}) for trout of similar size.

U_{crit} at warm temperatures was significantly ($p < 0.05$) higher than at cold temperatures in both warm- and cold-acclimated fish. This is unlike the results of

Schneider and Connors (1982) who found no significant effect of acute temperature change upon U_{crit} (Q_{10}=1.0) on fish acclimated to 10°C and tested at 10, 15 and 20°C. No obvious reasons exist to account for the different responses to acute temperature change found between the two studies as similar equipment, animal size and work regimes were used; differences in stocks, water conditions, or both, are possible sources of variability.

Prior to these experiments, it was expected that thermal acclimation would be manifested as higher swimming speeds for acclimated groups at their acclimation temperature than at non-acclimated temperatures. Thus I predicted cold-acclimated fish would swim better at cold temperatures than warm-acclimated fish and *vice versa*. Comparisons of group U_{crit}s, however, did not reveal a significant difference between groups ($p>0.05$) at either trial temperature. Q_{10}s calculated for individual fish, on the other hand, demonstrated U_{crit} to be significantly ($p<0.05$) less affected by acute temperature change in cold-acclimated than in warm-acclimated trout. This apparent dichotomy results from an intraspecific difference in the amount of variability associated with swimming speed and Q_{10} estimates. While a relatively broad range of U_{crit} values is found in warm- and cold-acclimated fish groups, the effect of an acute temperature change is remarkably consistent (Figure 2.1) for individual fish and the relative rank of an individual within an acclimation group (in terms of U_{crit}) is maintained ($p<0.05$; Spearman rank test). A similar maintenance of rank order of U_{crit} has been observed in U_{crit} values for largemouth bass (*Micropterus salmoides*) after an acute temperature change (Kolok, 1992). Therefore, the inability to find differences in U_{crit} between groups at a particular trial temperature despite differences being found in Q_{10} stems from differences in the amount of intraspecific variability associated with swimming capacity and that associated with a response to an imposed temperature change on that swimming

Figure 2.1. Effect of acute temperature transition on rank order of U_{crit} in cold- and warm-acclimated trout. The rank of fish in terms of U_{crit} was not significantly different (i.e., rank order was maintained) in both groups when tested at warm and cold temperatures. Points are for individual fish and solid lines join U_{crit} values determined at warm and cold temperatures.


Cold-acclimated
Warm-acclimated
3.0
2.5
2.0
1.5
U_{crit} (BL/sec)
5
10
15
20
Trial Temperature (°C)

capacity. This lack of variability in Q_{10} estimates also indicates Q_{10} to be a better index of adaptational differences than simple comparisons of mean group swimming ability.

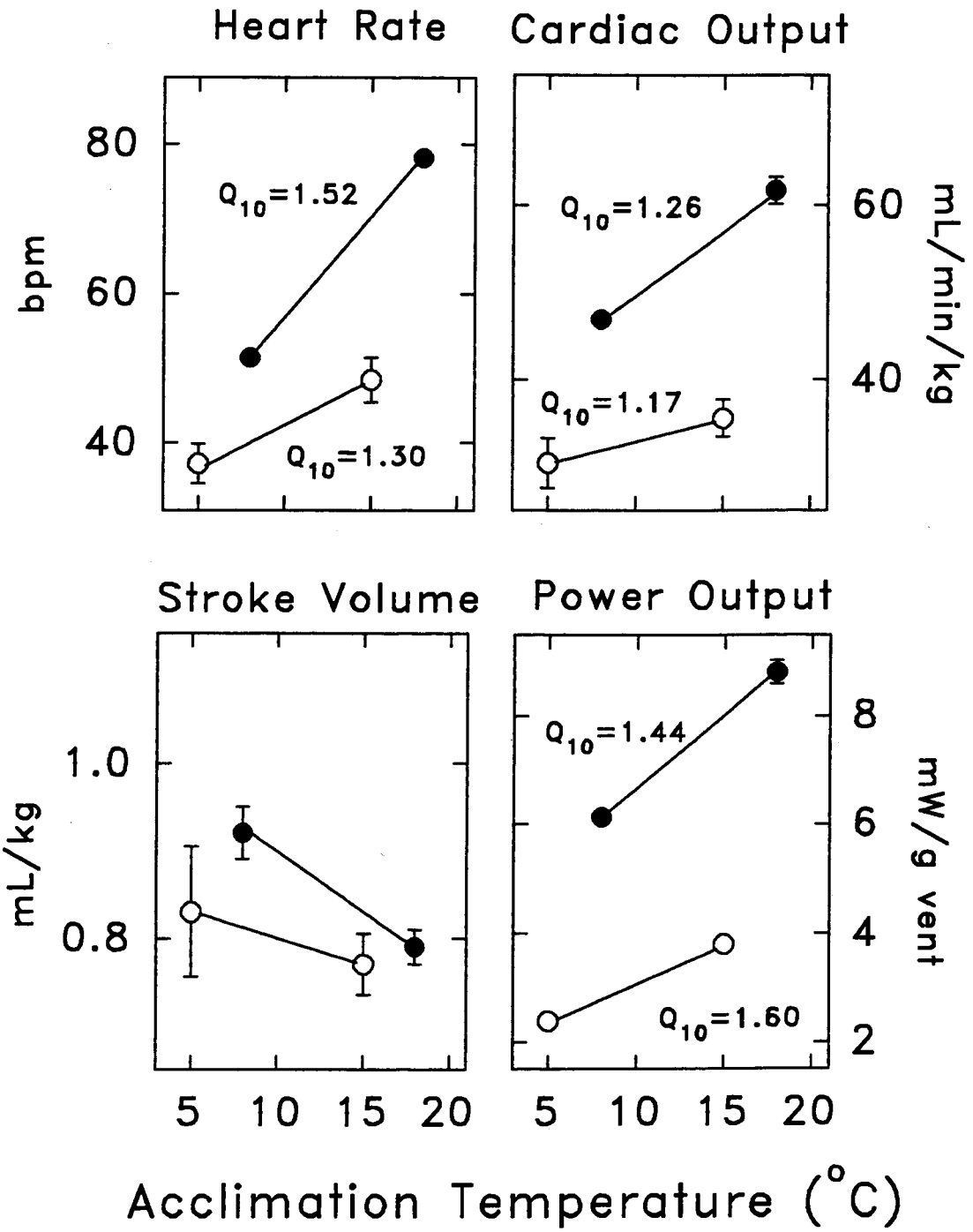
The thermal sensitivity of swimming performance of a particular fish to an acute temperature change (Q_{10}) was thus found to depend upon the chronic thermal history of that fish and indicated that acclimatory adjustments had occurred. Acclimation-induced improvements in U_{crit} were found in this study but appear to be restricted to cold water fishes. Although the data are limited at best and provide no indication of variation, the chronic Q_{10} of U_{crit} in trout was similar to that following acute temperature change in warm-acclimated trout (Q_{10} values of 1.35 vs 1.31). Only in cold-acclimated trout did acclimation provide a degree of thermal independence (Q_{10} values of 1.35 vs 1.22), indicating that some form of compensation or modification had occurred. The reasons underlying an absence of acclimation-induced changes in U_{crit} in warm-acclimated trout are not known but may be related to species limitations in thermal tolerance. The warm water temperature used in this study (18°C) approaches the upper limit of water temperatures normally encountered by this species (Scott and Crossman, 1973) and may preclude expression of acclimatory adjustments.

The effect of acclimation temperature on cardiac performance was assessed in this study using a WHP which, despite the invasiveness of the technique, can provide important insights into the mechanisms regulating cardiac function *in vivo*. One of the more important considerations of the technique is the loss of a coronary supply to the heart which could compromise cardiac performance. In fact coronary ligation reduces U_{crit} in a related species by 35% (Farrell and Steffensen, 1987), presumably due to an impairment of cardiac performance. The absence of a coronary circulation in this study, however, was not considered to significantly affect myocardial performance. The trout heart is composed of two

layers, an inner spongy layer (60-70% of ventricular mass) which derives an oxygen supply from venous blood, and an outer compact layer which receives the coronary circulation. The coronaries in this preparation were not perfused and the saline was gassed with 100% O_2 to provide a sufficiently high oxygen gradient to meet O_2 requirements of compact as well as spongy layers. That these demands were met was indicated through comparison of performance capacities of this preparation with *in vivo* estimates (not shown). Maximal power generated by these preparations were comparable to those found *in vivo* (Kiceniuk and Jones, 1977) and this would not have been possible if a significant portion of the heart was anaerobic.

This preparation, as previously mentioned, retains a functioning pericardium, the importance of which is clearly demonstrated by comparison of maximal cardiac and power outputs achieved in this study (at 8 and 18°C) with those obtained by Graham and Farrell (1989; 5 and 15°C; Figure 2.2). Ignoring differences related to the disparity in temperatures (3°C) used in the two studies, maximum cardiac output was 63% greater at the cold test temperature in the present study while maximum power output was improved by a remarkable 157%. At warm trial temperatures, an intact pericardium permitted attainment of 77% and 132% greater peak cardiac and power outputs, respectively (Figure 2.2). In addition to aiding in venous return (Farrell *et al.*, 1988b), the pericardium limits the extent to which the chambers can expand, thus influencing stretch-related influences on both contractility (Starling effects) and chronotropism. Although the presence of an intact pericardium appeared to have no effect on stroke volume, heart rate was significantly greater but likely does not account for the majority of the increase in cardiac and power outputs. Heart rate was 40% higher in the present study at cold temperature and 65% higher at warm temperature than found by Graham and Farrell (1989). Even assuming the effect

Figure 2.2. Comparison of maximum in situ cardiac performance in preparations in preparations having either an open (open symbols) or closed (closed symbols) pericardium. Open pericardium data are derived from Graham and Farrell (1989).


Heart Rate
Cardiac Output
bpm
80
60
40
Q_{10}=1.52
Q_{10}=1.30
Q_{10}=1.26
Q_{10}=1.17
60
40
mL/min/kg
Stroke Volume
Power Output
mL/kg
1.0
0.8
Q_{10}=1.44
Q_{10}=1.60
8
6
4
2
mW/g vent
5 10 15 20
5 10 15 20
Acclimation Temperature (°C)

of acute temperature change on heart rate to have a Q_{10} of 2.0 and this to be consistent over a temperature range of 5-18°C, temperature corrected values for the results of Graham and Farrell (1989) produce heart rates of 45.8 and 59.5 beats per minute at 8 and 18°C, respectively. The temperature corrected increases in heart rate as a function of an intact pericardium are thus reduced to 13% (8°C) and 32% (18°C), and do not account for all of the improvement in cardiac and power output, even after similar correction for temperature, that were observed. The mechanism(s) by which the pericardium produces this improvement in the WHP have yet to be determined.

Compensatory adjustments were found in cardiac performance although a division between cold and warm water acclimation capacities could not be made. Acute temperature change exerts its primary effect on cardiac performance *in vivo* through effects on heart rate. A Q_{10} of ~2.0 for both resting cardiac output and heart rate have been found following a 10°C temperature change in flounder (Cech *et al.*, 1976) and dogfish (Butler and Taylor, 1975) and in perfused sea raven hearts (Graham and Farrell, 1985). In rainbow trout at rest, a Q_{10} of 2.6 for cardiac output was observed by Barron et al. (1987) over a temperature range of 6-18°C.

Temperature acclimation apparently reduces the thermal dependency of heart rate as a Q_{10} of 1.52 was found in the present study for *in situ* preparations operating at acclimation temperature. This partial compensation in heart rate was accompanied by an increase in stroke volume (Table 2.2) which reduced the Q_{10} of cardiac output to 1.32. This result is like that of Graham and Farrell (1989), using the disrupted-pericardium WHP, who found stroke volume in warm-acclimated trout hearts to be either equivalent to or less than that found in hearts from cold-acclimated trout. The importance of the pericardium in acclimation is suggested in examination of heart rate. In the present study, the

Q_{10} of 1.52 for heart rate in warm- and cold-acclimated WHP's under pressure-loaded conditions is significantly lower than the Q_{10} of 1.76 found by Graham and Farrell (1989) in their disrupted-pericardium WHP. The importance of the pericardium in reducing the effect of temperature at maximum performance levels was also observed in comparison of Q_{10}'s for maximum power output. Graham and Farrell (1989), calculated the Q_{10} of *in situ* maximal power output of trout heart to be 2.22 under pressure-loaded conditions which differs substantially from the estimate of 1.44 determined in this study. Again, this underscores the importance of an intact pericardium in the ability of the heart to work against an imposed resistance.

If U_{crit} is inherently linked to the ability of the heart to supply working tissues with oxygenated blood, swimming and cardiac performances should remain matched, independent of acclimation temperature. Consequently, determination of a chronic Q_{10} value for both system and whole animal performance should be near equivalent. This was indirectly assessed in this study using Q_{10} values derived from maximal cardiac and aerobic swimming capacities of thermally-acclimated fish tested at acclimation temperature. A Q_{10} value of 1.35 was calculated for maximum prolonged swimming speed and compared favorably with the Q_{10} of 1.32 determined for maximum cardiac output under pressure-loaded conditions. The effect of temperature on maximum power output, i.e., the ability of the heart to pump and do work, was also similar to the effects observed on swimming speed. At U_{crit}, ventral aortic blood pressures approximate 70-80 cmH_2O (Kiceniuk and Jones, 1971; Farrell, 1984) and under pressure-loaded conditions, the Q_{10} of 1.44 for maximum power outputs was similar to the Q_{10} of 1.35 determined for U_{crit}. Therefore, it appears that maximal swimming and cardiac performance are reasonably well matched at the two acclimation temperatures. Power output appears, however, to be more severely affected by

temperature change and may have to work harder at cold temperatures to maintain the same level of cardiac output to meet the demands of working tissues. Maximum cardiac performance as measured using the WHP in these experiments, however, likely underestimates the capacity of the heart to work under conditions approximating *in vivo*. As the swimming trout approaches U_{crit}, *in vivo* catecholamine levels begin to rise and likely augment both skeletal and cardiac muscle performance. Consequently, the relationship between swimming and cardiac performance *in vivo* may be an even closer one than would seem to be evidenced in this study. Support for this has been provided by Graham and Farrell (1989) using a similar WHP who found that the Q_{10} of maximal power output (under pressure-loaded conditions) of hearts acclimated to 5 and 15°C was substantially reduced by maximal stimulatory levels of adrenaline.

In summary, the data for the WHPs are qualitatively very similar to earlier work, but qualitatively are a better representation of the *in vivo* conditions. They also show that the matching of intrinsic cardiovascular function with swimming capacity, assessed through comparison of chronic Q_{10} values for *in situ* maximal cardiac output and *in vivo* swimming performance (U_{crit}), was found to be a close one. It is likely, however, that additional factors operate *in vivo* which provide an even better matching of swimming and myocardial activities. Catecholamines, having a major impact on both inotropy and chronotropy, are the most likely of these and, given *in vivo* levels during exercise and *in vitro* potency, adrenaline the most obvious. Furthermore, maximal circulating levels of adrenaline differ in warm- and cold-acclimated fish and may be partially responsible for differences in the temperature dependency of U_{crit}. Accordingly, in the next Chapter, the influence of adrenaline on cardiac performance was assessed as a function of acclimation temperature.

CHAPTER III

THERMAL MODIFICATION OF ADRENERGIC SENSITIVITY IN CARDIAC TISSUE: IN SITU AND IN VITRO ANALYSIS

A. Introduction

As described in the General Introduction, thermal acclimation induces a shift in sensitivity to beta-adrenergic agonists in the rainbow trout heart and in the ratio of circulating levels of adrenaline and noradrenaline. Graham and Farrell (1989) using an *in situ* heart preparation witnessed a 10-fold shift in adrenergic responsiveness in relation to changes in heart rate, stroke volume, cardiac output and mass specific power output. However, the pericardium in their perfused heart preparation was not intact and thus extrapolation to an intact fish are limited. The data presented in the previous Chapter indicate that the presence of an intact pericardium has a significant effect on cardiac and power output capabilities of the heart preparation and the preparation likely better represents the situation *in vivo*. Furthermore, an intact pericardium limits the extent to which the atrium can fill and thus has an effect on stroke volume. The first objective of this study was therefore to re-examine the adrenergic sensitivity of trout hearts from cold- and warm-acclimated individuals using the improved *in situ* heart preparation which leaves the pericardium intact (Farrell *et al.*, 1988b) and determine if previously observed shifts in sensitivity observed with an open pericardium (Graham and Farrell, 1989) are corroborated.

Many of the previous studies utilizing live animals and isolated heart preparations have not determined whether or not the changes in sensitivity are a function of a shift in sensitivity as a result of acclimation or a consequence of a temperature effect on the properties of the heart itself. This problem is difficult

to resolve *in situ* as inotropy is influenced by the chronotropic changes produced by different temperatures. The problem can be resolved, however, by using paced ventricular strips and examining inotropic response to adrenergic agonists in the absence of rate changes. Thus, the second objective was to measure adrenergic sensitivity at warm and cold temperatures in ventricular strips from trout acclimated to warm or cold temperatures. In this manner the direct influence of temperature on sensitivity could be assessed and distinguished from effects due to thermal acclimation.

Lastly, thermally-induced shifts in the adrenergic sensitivity of cardiac performance in fishes must be correlated with alterations in some cellular component(s). The number of sites for potential modification are extensive and include modifications in the number of receptors, the affinity for agonist or the transduction of the signal (hormone occupation of receptor) into an intracellular response (cAMP production, phosphorylation of proteins, etc.). These have yet to be detailed in cardiac tissue of fishes. Thus, given that acclimatory alterations in adrenergic sensitivity do occur, I was interested in looking at some of the potential sites that might be altered over the course of acclimation and focussed on adrenoceptor density and affinity as a first step. Accordingly, I examined the binding of the radioligand (-)-(^{125}I)iodocyanopindolol (ICYP), a non-selective, hydrophobic beta-antagonist, to crude homogenates and isolated membrane fractions from ventricles of warm- and cold-acclimated trout in order to quantify both total and surface myocyte beta-adrenoceptor densities.

B. Methods and Materials

Stock origin and maintenance was as described in Chapter II. Fish used in this particular study ranged from 295-670 g.

In situ working heart preparation (WHP). Surgical procedures, methods of data acquisition and salines used were as described in Chapter II.

Adrenaline "dose-response" trials. As previously mentioned in Chapter II, the saline used during surgery in the WHP series was supplemented with 5 nM adrenaline in order to reduce time-dependent preparation deterioration. I was concerned that this concentration might have stimulatory effects in warm- and cold-acclimated fish, particularly in preparations from cold-acclimated fish (Graham and Farrell, 1989), and thus might attenuate the response to further additions. Consequently, after stabilization at basal levels, the saline was switched to one containing 0.5 nM adrenaline. Cardiac output was then increased 1.5-2 fold to approximate a working condition but not one of *extremis*. This performance level of the heart represented 40-50% of maximum power output. After stabilization, adrenaline was added to the perfusate and dose-response assessments made over an adrenaline concentration range of 0.5 nM to 50 *u*M. The heart was not seriously affected by this procedure as concentrations below 5 nM reduced myocardial power output by less than 10% prior to observance of adrenergic stimulation.

An examination of acute temperature change was not performed on *in situ* WHPs due to the relatively high thermal inertia of a nearly intact fish and the logistics of rapidly equilibrating bath and perfusate saline temperatures.

Ventricular strip preparations (VSP). Ventricular strips were set up as previously described (Keen *et al.*, 1992). To summarize, trout were stunned by a sharp blow to the head and the heart quickly excised and placed in ice-cooled saline. Ventricular strips were dissected using two parallel razor blades. Thin (5-0) silk

threads were tied to both ends of the strip which was mounted in a saline-filled, O_2-aerated and water-jacketed organ bath (20 mL volume). Bath temperature was initially either 8°C (cold) or 18°C (warm), depending upon the holding temperature of each particular fish. One end was attached to a fixed post and the other to a Metrigram isometric force transducer (Gould, Cleveland, OH). Signals from the force transducer were suitably amplified and displayed on a chart recorder (Model 3400; Gould, Cleveland, OH). Strips were electrically paced by a Grass SD9 Student stimulator delivering current via flattened platinum electrodes positioned on either side of the strip. The voltage (10.3 V $\pm$ 0.6) was 1.25 times the threshold required to produce a contraction and pulse duration was 10 msec. Muscle strips were stretched until active force (generated force) reached a peak and were allowed to equilibrate for 1 hr at a basal stimulation rate of 0.2 Hz. Bath saline was then replaced and, following an equilibration period, the adrenaline trials initiated. Saline used in VSP experiments was of the same composition (excepting adrenaline) as that described in Chapter II for WHP studies. Control strips were run in parallel with trial strips. Changes in peak force generation (as a percentage of pre-trial values) in control strips were subtracted from trial results to account for the slow, modest deterioration of the preparations.

Adrenaline (in saline) was added to ventricular strips in a cumulative dose-dependent manner to generate a concentration range of 10 nM to 100 uM. Trials for both acclimation groups were performed at a stimulation rate of 0.2 Hz. After completion of a dose-response trial at one temperature, a new strip was hung and the trial repeated at the opposite trial temperature (either 8 or 18°C.)

Homogenization and sarcolemma (SL) isolation procedures. Homogenization and isolation procedures were conducted as previously described (Tibbits *et al.*, 1990)

with minor modifications. For each isolation, 13-15 fish were killed by a sharp blow to the head and the ventricles removed and placed in an ice-cold buffered homogenization medium consisting of 280 mM sucrose and 20 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES, pH 7.7 at 21°C). After removal of fat and connective tissue, ventricles were minced with scissors in 10 volumes of ice-cold homogenization medium. Homogenization was completed by 3x3 sec bursts of a Tissumizer (Tekmar, Cincinnati, OH) on a setting of 40. The homogenate was then passed through two layers of stainless steel mesh (no. 28 and 40). A 1 mL aliquot of the filtered homogenate was removed at this point for use in marker and radioligand binding analyses (described below).

Enriched sarcolemmal fractions were prepared from the remainder of the homogenate. Isolation procedures, as during homogenization steps, were conducted either on ice or under refrigerated (4°C) conditions. Contractile proteins were solubilized through a 10 min incubation with KCl (100 mM) and $Na_4P_2O_7$ (25 mM). The suspension was then centrifuged at 180,000 g (Ti50.2 rotor) for 1 hr. The supernatant was subsequently discarded and the pellet resuspended in fresh homogenization medium. All resuspensions were conducted using a motor-driven Teflon pestle (10 reps) at low speed. The suspension was re-centrifuged at 2000 g for 10 min. The supernatant was then retained (pellet discarded) and re-centrifuged at 180,000 g (Ti50.2 rotor) for 1 hr. The resulting pellet was resuspended in 5 mL of 45% (w/v) sucrose and a discontinuous sucrose gradient constructed through sequential addition of 5 mL each of 32, 30 and 28% sucrose to the suspension. The gradient was completed by addition of a top layer of 5-7 mL of 8% sucrose and then centrifuged at 122,000 g (SW27 or SW28 rotor) for 15-16 hr. The gradient was separated into four fractions designated F1 to F4 in terms of increasing density. Five mL of "loading" medium (560 mM NaCl; 80 mM TES; pH 7.7 at 21°C) were added to each fraction and

tubes incubated for 1 hr on ice. Following this, 5 mL of 280 mM NaCl + 40 mM TES were added and a 30 min incubation conducted. Tubes were then brought to a final volume of 28.5 mL with 140 mM NaCl + 20 mM TES and centrifuged for 1 hr at 180,000 g (Ti50.2 rotor). Pellets from each fraction were resuspended in 1.0-1.5 mL of 140 mM NaCl + 10 mM TES. Aliquots were frozen in liquid nitrogen for subsequent marker and radioligand binding analyses.

Protein and marker analysis. Protein concentrations for homogenates and isolated fractions were determined by the method of Bradford (1974) with bovine serum albumin as a protein standard. Sarcolemmal enrichment was estimated through the activity of two sarcolemmal enzymes, potassium-stimulated *p*-nitrophenylphosphatase (K^+-*p*NPPase, EC 3.1.3.16) and adenylate cyclase (EC 6.6.1.1), in both crude homogenates and isolated fractions.

K^+-*p*NPPase activity was determined by the method of Heller and Hanahan (1972) with minor modifications. In brief, total phosphatase activity was determined through addition of 10-100 *u*g protein to tubes containing 200 *u*M ethyleneglycol-bis-(beta-aminoethyl ether)-N,N'-tetraacetic acid (EGTA), 1 mM $MgCl_2$, 20 mM TES (pH 7.6 at 37°C), 5 mM *p*-nitrophenylphosphate and 50 mM KCl (final volume of 1 mL). Non-K^+-stimulated phosphatase activity was measured through equimolar substitution of NaCl for KCl in a separate series of tubes. All assays were performed in triplicate. Sample dilutions, when needed, were made using 140 mM NaCl + 20 mM TES (pH 7.6 at 37°C). Test tubes were incubated for 20 minutes at 37°C and the reaction terminated through addition of 2 mL of 0.1 M NaOH. Absorbance was measured at 415 nM on a Novaspec spectrophotometer (LKB Biochrom, Cambridge, UK) and values compared with those obtained using *p*-nitrophenol standards. K^+-*p*NPPase activity was calculated from the difference between total and non-K^+-stimulated phosphatase

activities.

Adenylate cyclase activity was quantified as described by White and Zenser (1971) with minor modifications. In a series of test tubes, 20-300 *u*g of protein was added to a reaction medium containing the following components: 1 mM 3-isobutyl-1-methyl xanthine (IBMX), 10 mM $MgCl_2$, 25 mM TES (pH 7.10 at 22°C), 10 mM phosphocreatine, 500 U/mL creatine phosphokinase, 0.1 % bovine serum albumin, 500 *u*M ATP and 0.53 *u*M ^{32}P-ATP (25 Ci·mmole^{-1}). Final reaction volume was 150 *u*L. Tubes were incubated with gentle agitation at room temperature (22°C) for 30 minutes. Reaction was terminated through the addition of 100 *u*L of 100 mM disodium ethylenediamine tetraacetate (EDTA, pH 7.6 at 22°C) and 100 *u*M cAMP and immediately placed on ice. A 50 *u*L recovery standard of 0.333 *u*M ^{3}H-cAMP (30 Ci·mmole^{-1}) was added to each tube. Following this, 2.70 mL of 0.05 M imidazole (pH 7.6 at 22°C) were then added and contents immediately applied to pre-washed alumina columns for separation of ^{32}P-cAMP from other adenylates. Columns were prepared as follows: 1 cm of glass wool was packed into 5 mL polypropylene disposable syringes. 1.0-1.2 g of neutral alumina was then added and columns washed through passage of 10 mL of 1.0 M and 20 mL of 0.05 M imidazole buffer (pH 7.6 at 22°C). After application of test tube contents to columns, an additional 15 mL of 0.05 M imidizole buffer was applied. The eluent was collected and 3 x 1 mL aliquots were used for determination of radioactivity. Ten mL of scintillation cocktail were added to each 1 mL aliquot and ^{3}H and ^{32}P radioactivity determined through standard scintillation counting techniques. Blanks were conducted by incubation in the presence of EDTA. Recovery from columns, estimated from ^{3}H-cAMP counts, typically exceeded 95%. ^{3}H-cAMP and ^{32}P-ATP counting standards were run during each determination.

The enrichment index (EI) of isolated fractions over crude homogenate was

determined through the following:

$$EI = (Sp\ Act_{Fi})/(Sp\ Act_{HMG})$$

where specific activities (Sp Act) of either K^+-*p*NPPase (in *u*moles·mg protein^{-1}·hr^{-1}) or adenylate cyclase (in nmoles·mg protein^{-1}·hr^{-1}) from isolated fractions and crude homogenate are indicated by subscripted F_i, where $_i$=fraction number (F1, F2, F3 or F4), and HMG. The percentage of SL recovered (Rec) from crude homogenate was calculated from:

$$Rec = \{[(Sp\ Act_{Fi})(Prot_{Fi})]/[(Sp\ Act_{HMG})(Prot_{HMG})]\} \times 100$$

where Prot = total protein and is calculated from the product of the protein concentration and total volume of either the crude homogenate or each isolated fraction.

Radioligand binding studies. In order to assess the densities of surface and intracellular beta adrenoceptor pools, radioligand binding studies were conducted using the hydrophobic, non-selective beta-antagonists (-)-(^{125}I)iodocyanopindolol and propranolol. Crude homogenate adrenoceptor binding was used to determine total receptor density while the most highly enriched sarcolemmal fraction (F2) was used to quantify surface adrenoceptor density. Intracellular compartmentalization of beta adrenoceptors was calculated from the difference between total and surface adrenoceptor populations. Ten point binding assays were performed in duplicate. Total binding in homogenate (100-300 *u*g protein per assay) or F2 (20-50 *u*g protein per assay) fraction was determined in a reaction medium containing 10 mM $MgCl_2$, 100 *u*M GTP, 20

mM TES (pH 7.77 at room temperature [21-22°C]), and 20-250 pM ICYP. Non-specific binding was assayed by addition of 0.2-2.5 *u*M propranolol to a second series of reaction tubes (ICYP:propranolol ratio of 10,000:1 in all instances). Final reaction volume was 300 *u*L. Binding was carried out at room temperature in a shaking water bath for 3 hrs (see results and Figure 3.3). After incubation, 2.2 mL of ice-cold buffer (10 mM $MgCl_2$, 20 mM TES) were added to each tube (total volume 2.5 mL) and a 1 mL aliquot of this applied to a Whatman GF/C filter under vacuum filtration. The filter was immediately rinsed with 3 x 4 mL of ice-cold buffer and put in a scintillation vial. This was then repeated with a second aliquot (two determinations per assay tube). After allowing the filters to dry, 10 mL of scintillation cocktail were added to each vial and radioactivity determined through standard liquid scintillation techniques. Specific binding is defined as total binding in the absence of competing ligand (propranolol) minus the amount bound in the presence of propranolol. The density of binding sites and affinity for ICYP was quantified through Scatchard plot analysis (Scatchard, 1949). Assays were performed in duplicate and the K_d and B_{max} calculated from the mean of the two determinations.

Chemicals. All chemicals other than radioactive compounds were reagent grade and purchased from either Sigma (St. Louis, MO) or BDH (Toronto, Ontario) Chemical Companys. Radiolabelled ^{125}I-ICYP and ^{3}H-cAMP were purchased from Amersham (Oakville, Ont) while ^{32}P-ATP was purchased from ICN (Costa Mesa, CA).

Statistical Evaluation. Statistical comparisons were made non-parametrically using Mann-Whitney U-tests and a significance level of 0.05.

C. Results

Morphometrics. Morphometric data for cold- and warm-acclimated trout used in this study are presented in Table 3.1. Ventricular mass, expressed as a percentage of body mass, was significantly ($p<0.05$) higher in cold-acclimated fish. An increase in relative ventricular mass with acclimation to cold temperatures has been previously demonstrated (Farrell *et al.*, 1988a) and indicated the 3 week (minimum) holding period was sufficient to stimulate acclimatory responses.

Dose-response curves - Sensitivity. In situ WHPs and *in vitro* VSPs clearly demonstrated adrenergic sensitivity to be highly temperature-dependent. *In situ* hearts from cold-acclimated fish, tested at 8°C, were significantly ($p<0.05$) more sensitive to adrenaline than hearts from warm-acclimated trout in trials conducted at 18°C (Figure 3.1). Stroke volume, heart rate, cardiac output and myocardial power output in cold-acclimated hearts were, on average, 10-fold more sensitive to positive beta-adrenergic stimulation than were warm-acclimated hearts (Table 3.2).

Tension development in experiments using *in vitro* ventricular strip preparations followed a similar pattern (Table 3.2; Figure 3.2). Strips from cold-acclimated hearts tested at cold temperatures had a 4.7 fold greater inotropic sensitivity ($p<0.05$), as estimated from EC_{50} values, than warm-acclimated hearts tested at warm temperatures. Importantly, the differences in responsiveness of ventricular strips to adrenaline were not a consequence of the incubation temperature *per se* but were instead a function of acclimation temperature (Figure 3.2). This was clearly indicated upon testing strips at non-acclimated temperatures. No significant ($p>0.05$) differences were found in EC_{50} estimates between cold-acclimated strips tested at 8 and 18°C. Similar results were found in

Table 3.1. Morphometric data of the cold- and warm-acclimated rainbow trout used in WHP (N=5 [cold] or 6 [warm]), VSP (N=6 in each group) and RLB (N=52 [cold] or 58 [warm]) studies. Relative ventricular mass, as a function of body mass, (V/B) was calculated from (ventricular mass/body mass) x 100. Significant differences ($p<0.05$) in V/B between acclimation groups are indicated by paired lower case letters.

Acclimation Group	Series	Vent Mass (g)	Body Mass (g)	(V/B) x 100
Cold (8°C)	WHP	0.46+0.03[a]	477+34	0.097+0.003[b]
	VSP	0.44+0.03	434+37	0.101+0.002[c]
	RLB	0.44+0.01	458+08	0.097+0.002[d]
Warm (18°C)	WHP	0.37+0.02[a]	456+30	0.081+0.002[b]
	VSP	0.45+0.06	488+56	0.091+0.002[c]
	RLB	0.43+0.01	474+06	0.090+0.000[d]

Figure 3.1. Sensitivity of mass specific myocardial power output of in situ WHP's from cold- (8°C; closed symbols) and warm- (18°C; open symbols) acclimated trout hearts as a function of applied adrenaline. Power output is expressed as a percentage of maximum and was calculated as

$$(Power_{dose}-Power_{min})/(Power_{max}-Power_{min}) \times 100$$

Values are mean$\pm$SEM unless error bars are smaller than symbol radius. N is 5 for cold- and 6 for warm-acclimated preparations, respectively.

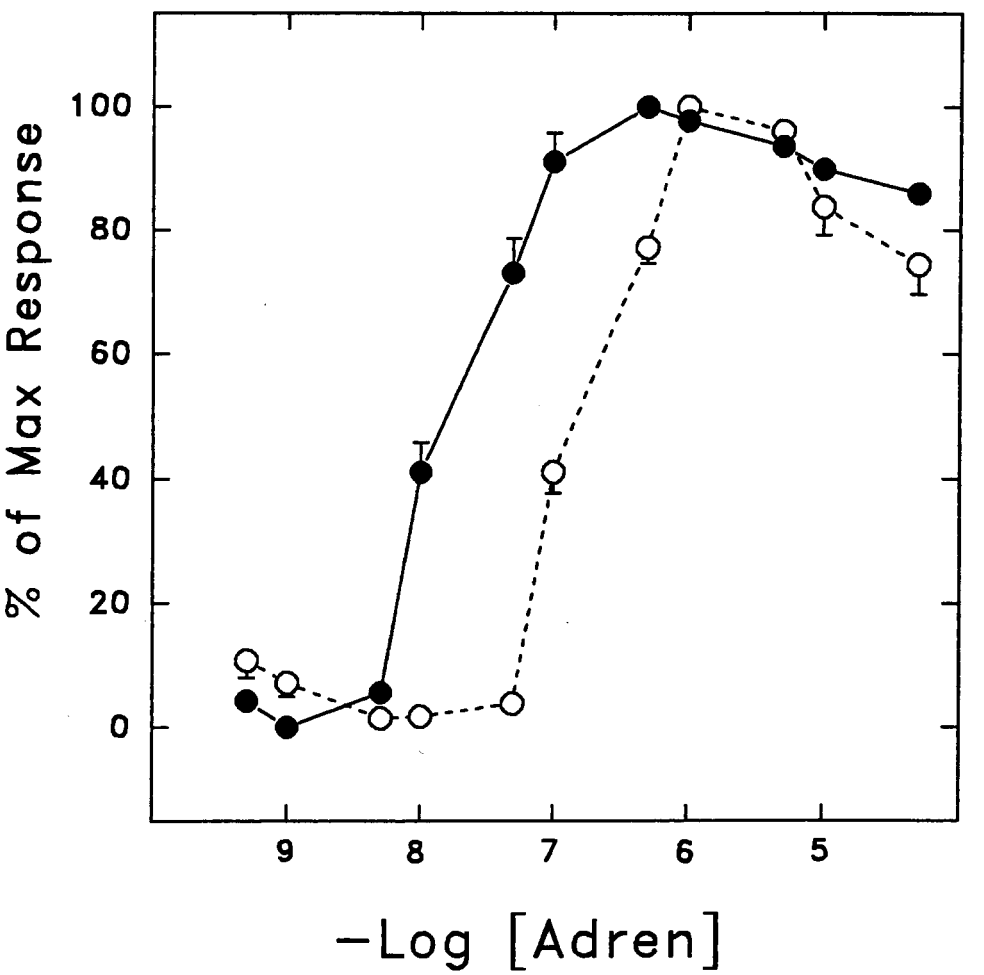

% of Max Response
100
80
60
40
20
0
9
8
7
6
5
−Log [Adren]

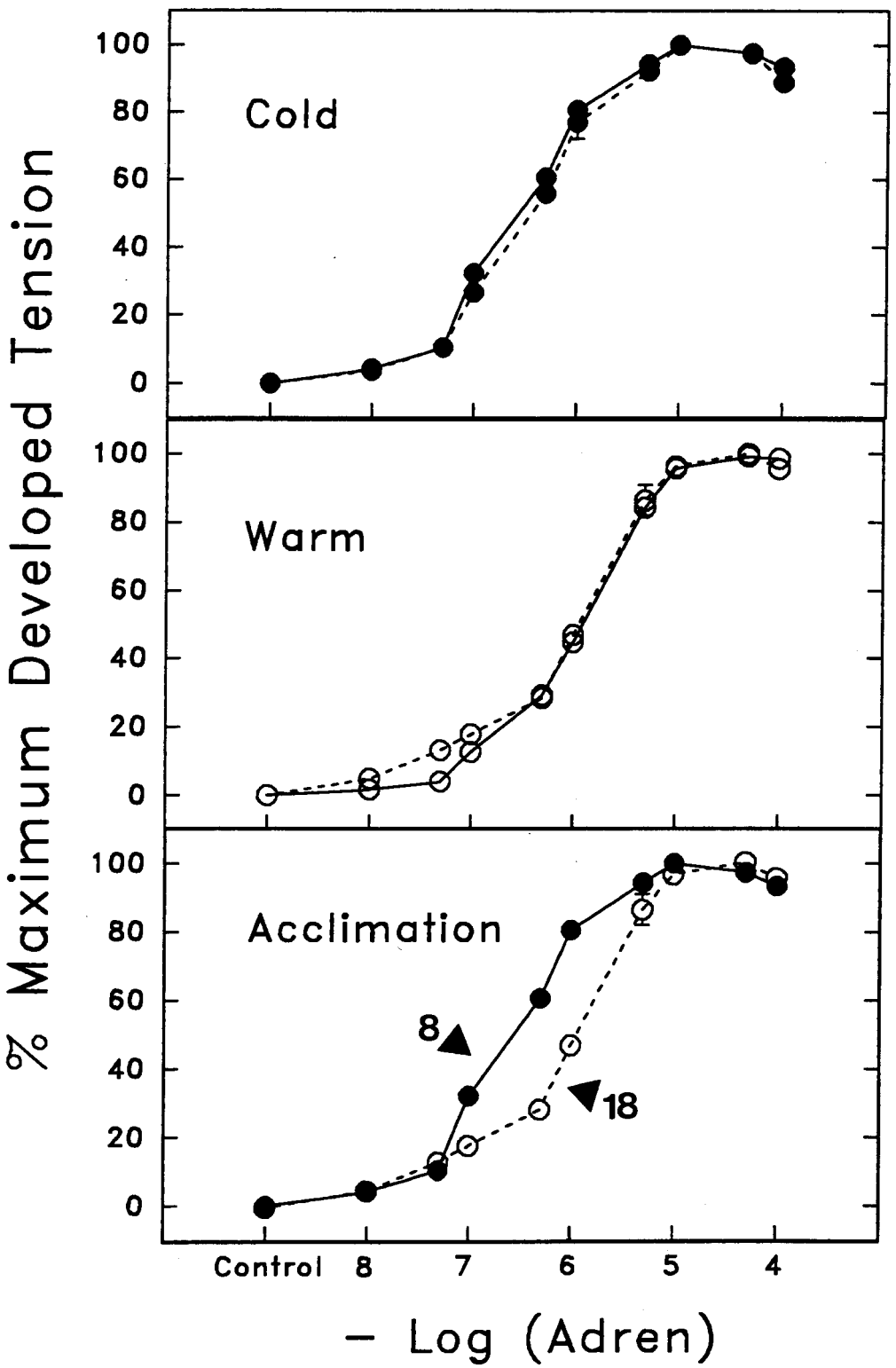
Table 3.2. Effect of acclimation temperature and trial temperature on the sensitivity (EC_{50}) and stimulatory scope of in situ **WHPs and** in vitro **VSPs to applied adrenaline.** Scope for each variable is defined as the fold increase of the maximum response over the minimum response. Values are mean±SEM. The N value for each group is as follows: *in situ* - cold (5), warm (6); *in vitro* - cold (6), warm (6). Statistically significant ($p<0.05$) differences are indicated by paired superscripted letters.

Acclimation Temperature	Trial Temperature	Parameter	EC_{50}	Scope
In situ WHP				
Cold	8 °C	SV	7.826^{a} (±0.08)	1.23^{b} (±0.04)
		Q	7.71^{c} (±0.06)	1.37^{d} (±0.02)
		Power	7.71^{e} (±0.06)	1.37^{f} (±0.03)
		f_H	7.65^{g} (±0.10)	1.14 (±0.03)
Warm	18 °C	SV	6.81^{a} (±0.10)	1.13^{b} (±0.02)
		Q	6.79^{c} (±0.04)	1.26^{d} (±0.02)
		Power	6.78^{e} (±0.04)	1.27^{f} (±0.02)
		f_H	6.93^{g} (±0.07)	1.14 (±0.02)
In vitro VSP				
Cold	8 °C	Tension	6.57^{h} (±0.06)	2.55 (±0.33)
	18 °C	Tension	6.45 (±0.03)	2.57 (±0.24)
Warm	8 °C	Tension	5.92 (±0.03)	3.08 (±0.28)
	18 °C	Tension	5.96^{h} (±0.03)	2.94 (±0.26)

Figure 3.2. Effect of trial temperature on twitch tension in ventricular strips from warm- and cold-acclimated trout. Incubation temperatures are indicated by solid (8°C) and broken (18°C) lines. The percent developed tension was calculated as

$$(Tension_{dose}-Tension_{min})/(Tension_{max}-Tension_{min}) \times 100$$

Acclimation temperatures are indicated by the text in upper and middle panels. The bottom panel indicates the difference in adrenergic sensitivity between cold- and warm-acclimated ventricular strips when tested at acclimation temperature. N=6 for each point and values are the mean$\pm$SEM unless error bar is smaller than symbol radius.


% Maximum Developed Tension
100
80
60
40
20
0
Cold
Warm
Acclimation
8
18
Control
8
7
6
5
4
– Log (Adren)

trials using warm-acclimated strips at the two trial temperatures (Table 3.2; Figure 3.2).

Dose-response curves - Scope. Changes in scope, defined for each parameter as the maximum increase as a function of the minimum estimate, as a function of acclimation temperature were different in WHP and VSP experimental series (Table 3.2). *In situ*, the maximal scope of the adrenergically-mediated increase in cardiac performance was significantly greater in cold-acclimated trout hearts ($p<0.05$) in almost all instances (Table 3.2). The scope for improving stroke volume, and as a result, cardiac output and power output were all greater in hearts from cold-acclimated fish while no significant difference ($p>0.05$) was observed in the scope of heart rate change between the two acclimation groups. Therefore, in addition to a shift in sensitivity, cold-acclimated hearts achieved a relatively greater increase in cardiac performance with adrenergic stimulation than found in warm-acclimated hearts.

In ventricular strips, however, the scope for inotropic increase was not correlated with either acclimation or incubation temperature (Table 3.2). In all instances, maximum twitch tension was 2-3 fold higher than found prior to adrenaline exposure.

Sarcolemmal isolations. Summaries of sarcolemmal isolations made using ventricles from cold- and warm-acclimated trout are presented in Table 3.3a and 3.3b. The lightest fraction collect from the discontinuous sucrose gradient, F1, typically possessed little or no detectable protein and was therefore discarded. The mostly highly enriched fraction, as assessed from K^+-*p*NPPase and adenylate cyclase activities, was F2. No significant differences ($p>0.05$) in recovery of F2 fraction or in yield and K^+-*p*NPPase activities of homogenates and F2 fractions

Table 3.3a. Summary of sarcolemmal isolations from ventricles of trout acclimated to 8°C. Units are as follows: Yield in mg protein·g^{-1} wet wt; specific activity either in *u*moles·mg protein^{-1}·hr^{-1} (K^+-*p*NPPase) or nmoles·mg protein^{-1}·hr^{-1} (adenylate cyclase); enrichment in fold over homogenate; recovery in % of homogenate.

	Isolation No.				
	$B8_1$	$B8_2$	$B8_3$	$B8_4$	Mean±SEM
Yield					
HMG	101.7	112.0	99.0	93.5	101.6±4.5
F2	0.62	0.43	0.53	0.59	0.54±0.05
Specific Activity					
K^+-*p*NPPase					
HMG	0.34	0.20	0.26	0.23	0.26±0.03
F2	2.82	1.97	2.02	1.57	2.10±0.30
Adenylate cyclase					
HMG	0.67	0.52	0.57	0.58	0.59±0.04
F2	5.51	4.47	4.67	3.60	4.56±0.45
F2 Enrichment					
K^+-*p*NPPase	8.4	9.8	7.7	6.9	8.2±0.7
Adenylate cyclase	8.2	8.6	8.2	6.2	7.8±0.6
F2 Recovery					
K^+-*p*NPPase	5.1	3.8	4.1	4.3	4.3±0.3
Adenylate cyclase	5.0	3.3	4.4	3.9	4.2±0.4

Table 3.3b. Summary of sarcolemmal isolations from ventricles of trout acclimated to 18°C. Units are as follows: Yield in mg protein·g^{-1} wet wt; specific activity either in μmoles·mg protein^{-1}·hr^{-1} (K^+-*p*NPPase) or nmoles·mg protein^{-1}·hr^{-1} (adenylate cyclase); enrichment in fold over homogenate; recovery in % of homogenate.

	Isolation No.				
	$B18_1$	$B18_2$	$B18_3$	$B18_4$	Mean±SEM
Yield					
HMG	97.91	88.55	107.28	87.02	95.19±5.42
F2	0.57	0.49	0.61	0.51	0.55±0.03
Specific Activity					
K^+-*p*NPPase					
HMG	0.27	0.34	0.25	0.24	0.28±0.03
F2	2.19	2.64	2.10	2.13	2.27±0.15
Adenylate cyclase					
HMG	0.39	0.42	0.29	0.35	0.36±0.03
F2	2.69	3.11	2.29	3.08	2.79±0.22
F2 Enrichment					
K^+-*p*NPPase	8.0	7.8	8.4	9.0	8.3±0.3
Adenylate cyclase	6.9	7.4	7.9	8.8	7.8±0.5
F2 Recovery					
K^+-*p*NPPase	4.6	4.3	4.8	5.3	4.8±0.2
Adenylate cyclase	4.0	4.1	4.5	5.2	4.5±0.3

were found in isolations conducted on warm- and cold-acclimated ventricular tissues. Mean homogenate yield (all isolations) was 98.37±3.28 mg protein·g^{-1} wet weight and K^+-*p*NPPase activity was 0.27±0.02 *u*moles·mg protein^{-1}·hr^{-1}. Mean sarcolemmal (F2) recovery was 4.5±0.2% and mean yield was 0.54±0.02 mg SL·g^{-1} wet weight. K^+-*p*NPPase activity averaged 2.18±0.15 *u*moles·mg SL protein^{-1}·hr^{-1}. Mean enrichment of this fraction was 8.3±0.3 (K^+-*p*NPPase) and 7.8±0.3 (adenylate cyclase). These parameters are in general agreement with previous isolations conducted on ventricles from winter-acclimated trout (Tibbits *et al.*, 1990) although K^+-*p*NPPase activities of homogenate and F2 are somewhat depressed. The underlying reasons for the reduced enzyme activity are not apparent. Interestingly, adenylate cyclase activity was significantly ($p<0.05$) higher in both homogenate and F2 fractions from cold-acclimated cardiac tissue (Table 3.3a,b). This is a consequence of a significantly higher basal adenylate cyclase activity in cold-acclimated fish hearts (Keen *et al.*, in prep.) but does not affect the use of the enzyme as an indicator of enrichment.

Radioligand binding studies. Pilot studies were performed to determine the time required to reach equilibrium between radioligand and receptor. Studies were conducted on both crude homogenates and isolated F2 fractions using 3 concentrations (25, 125, 250 pM) of radioligand (Figure 3.3). These concentrations spanned the K_d of ICYP for trout heart beta adrenoceptors. Equilibrium binding was slower in crude homogenate samples than in F2 fractions. Homogenates reached equilibrium with the radioligand after ~2 hrs as opposed to the ~1 hr required for F2 binding (data not shown). In order to ensure equilibrium achievement, subsequent studies employed a 3 hr incubation period.

Saturation binding curves established saturable and non-saturable radioligand binding components (Figure 3.4). Specific binding, calculated from

Figure 3.3. Equilibrium binding time course determination of homogenates from cold-acclimated ventricular tissue of rainbow trout. ICYP binding to homogenate is expressed as counts per minute. Equilibrium was followed over a range of ICYP concentrations (25, 125 and 250 pM) which spans the K_d of ICYP for receptor in these preparations. Values are the mean+SEM of three trials, unless error bar is smaller than symbol radius.

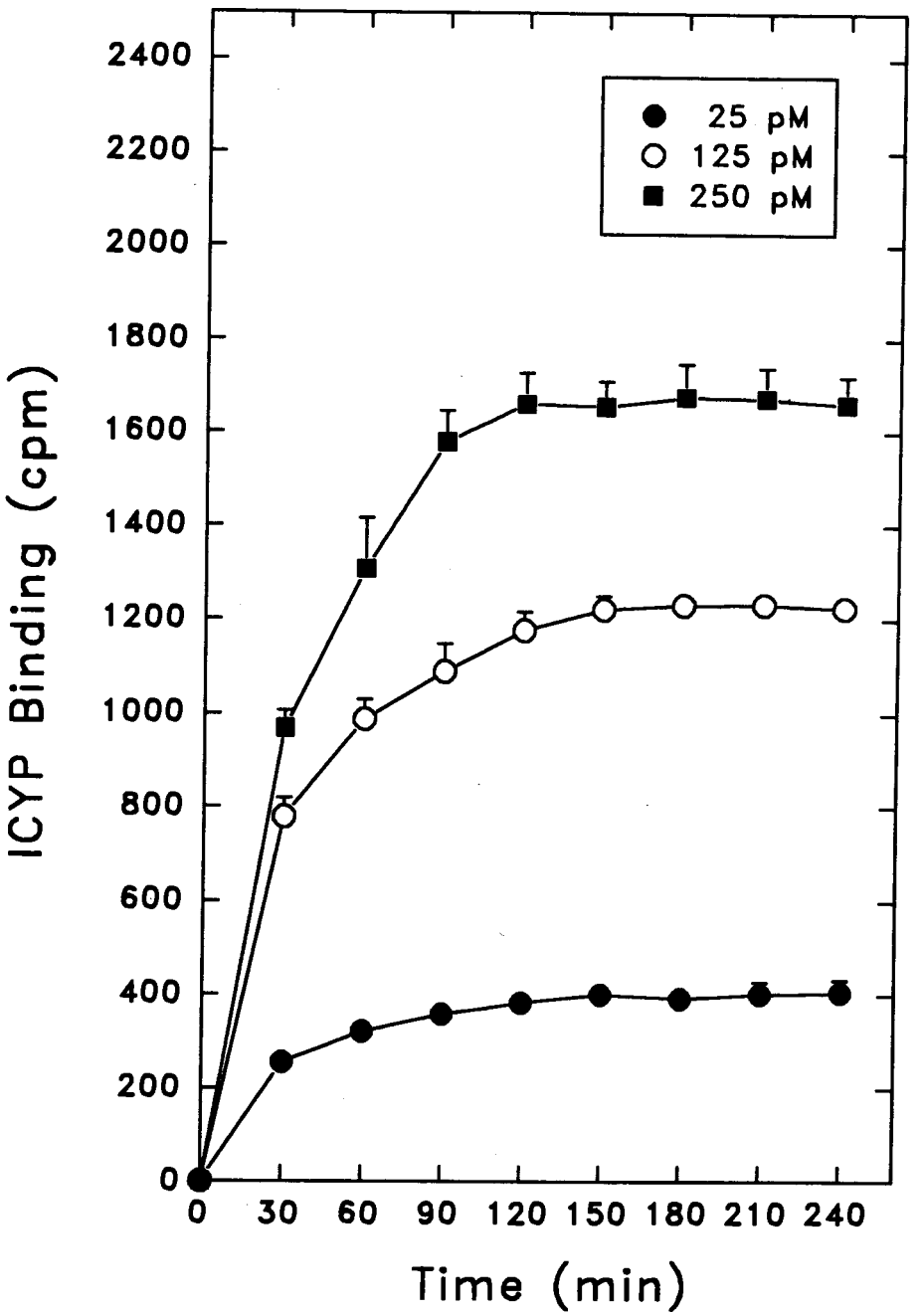
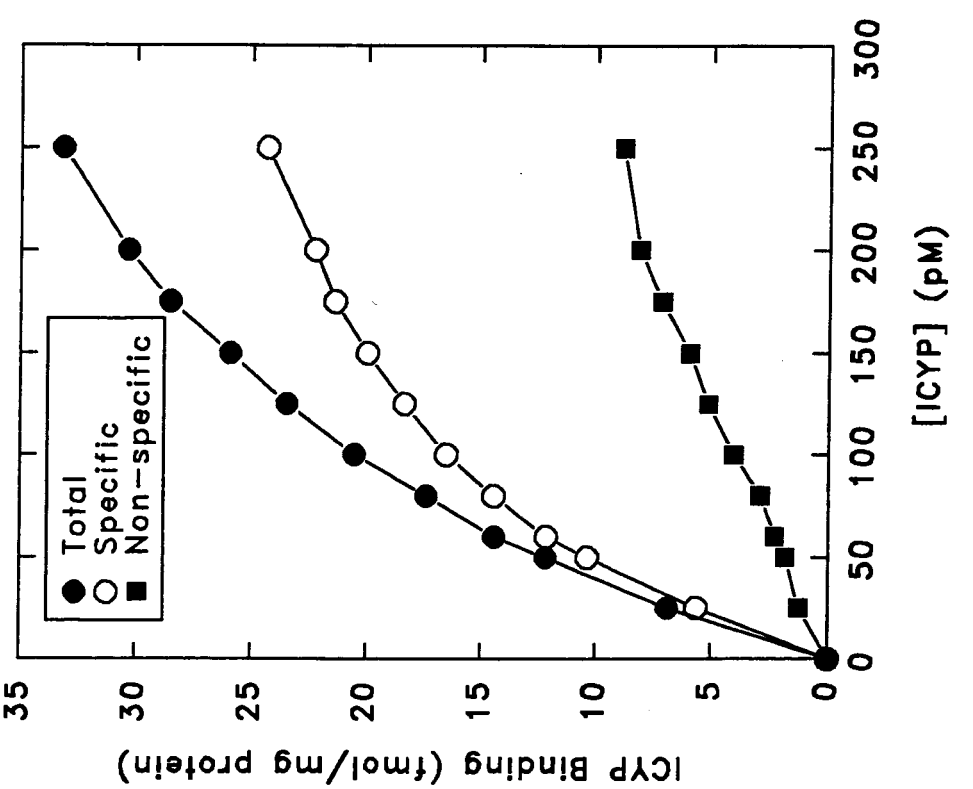
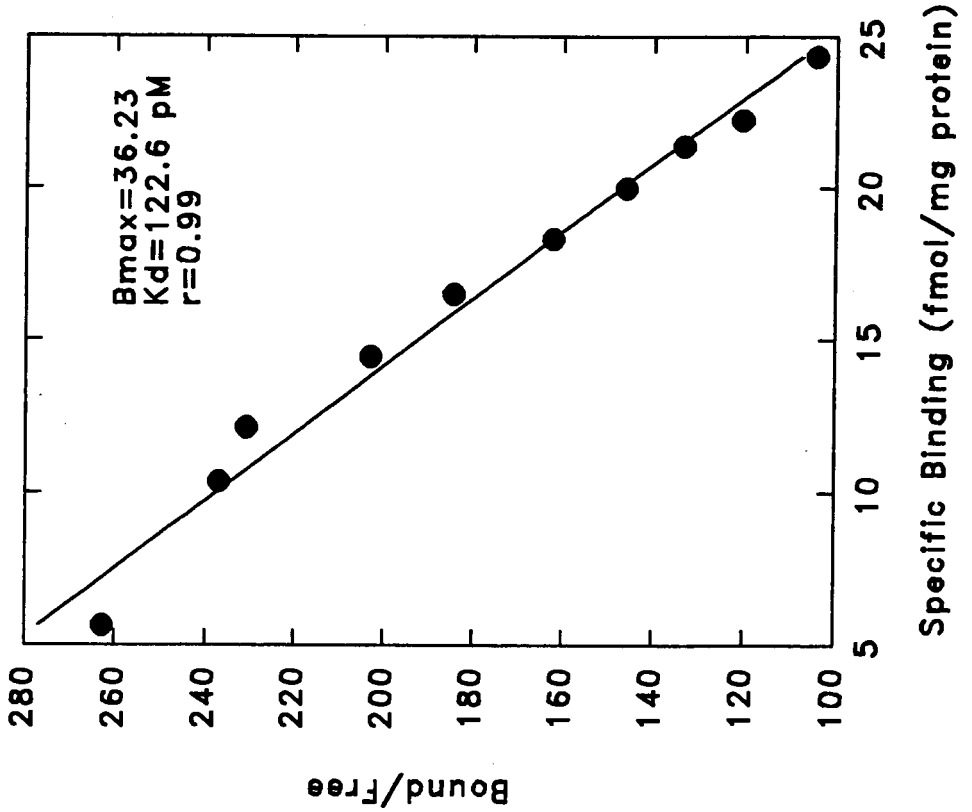

25 pM
125 pM
250 pM
ICYP Binding (cpm)
Time (min)
0
200
400
600
800
1000
1200
1400
1600
1800
2000
2200
2400
0
30
60
90
120
150
180
210
240

Figure 3.4. Typical saturation binding (left) and Scatchard (right) plots of ICYP binding to crude homogenate.


ICYP Binding (fmol/mg protein)
[ICYP] (pM)
Total
Specific
Non-specific
0
5
10
15
20
25
30
35
0
50
100
150
200
250
300

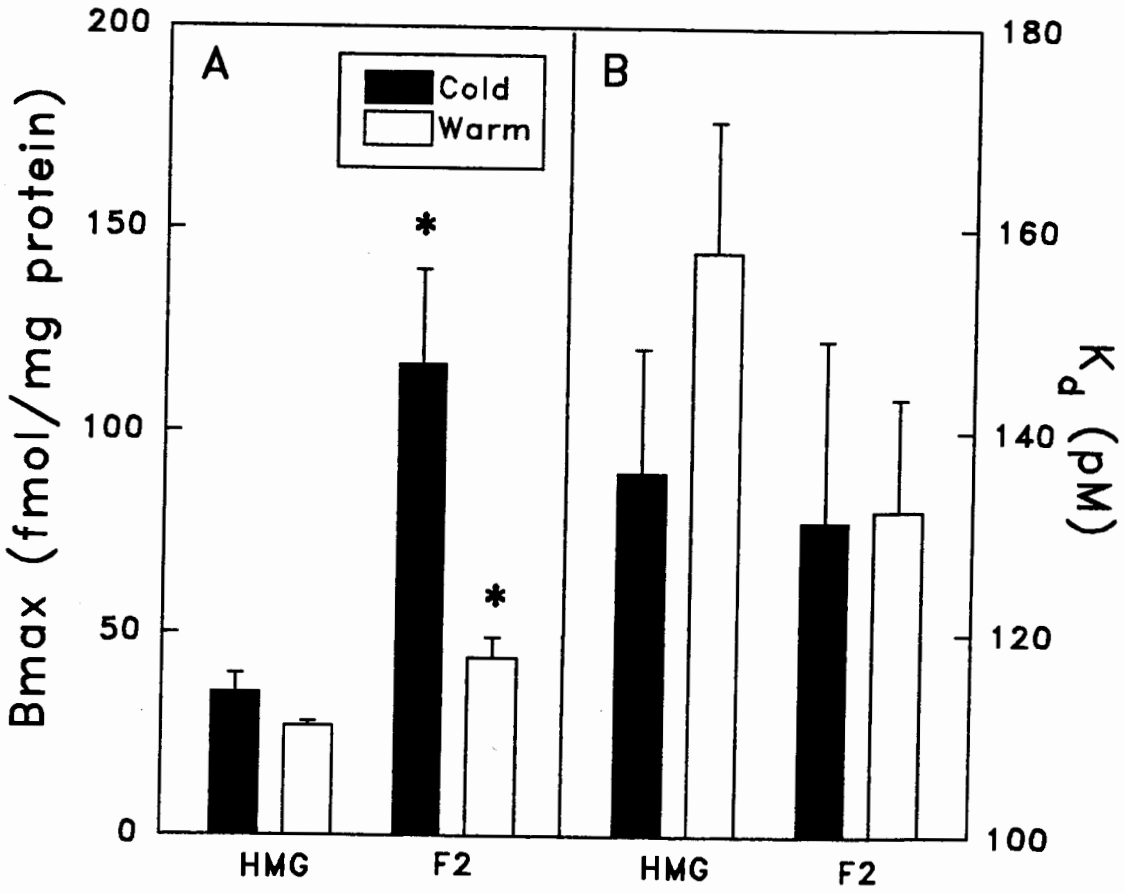

Bound/Free
Specific Binding (fmol/mg protein)
Bmax=36.23
Kd=122.6 pM
r=0.99
100
120
140
160
180
200
220
240
260
280
5
10
15
20
25

the difference between total and non-specific binding, represented >85% of total ligand binding at 125 pM while <10 % of the total available ligand was bound. Scatchard plot analysis (Figure 3.4) revealed no significant ($p>0.05$) differences in receptor density (B_{max}) or dissociation constant (K_d) between homogenate fractions from warm- and cold-acclimated trout hearts (Figure 3.5). ICYP binding was significantly greater ($p<0.05$) in F2 fractions derived from cold-acclimated hearts. B_{max} in these fractions was 125.6±26.3 fmoles·mg protein^{-1} while a B_{max} of 56.4±9.0 fmoles·mg protein^{-1} was found in F2 from warm-acclimated hearts (Figure 3.5). No significant differences in K_d were observed ($p<0.05$; Figure 3.5).

D. Discussion

The scope of adrenergically-mediated increases in cardiac output, stroke volume and power output were all significantly ($p<0.05$) greater in cold- than warm-acclimated WHPs. Cardiac output in cold-acclimated trout hearts was increased by 37% when maximally-stimulated by adrenaline compared to an increase of only 26% in warm-acclimated counterparts. These values agree qualitatively with those of Graham and Farrell (1989) but differ quantitatively. In their study, cardiac output was increased by 63% in trout hearts acclimated to 5°C when maximally stimulated by adrenaline but only by 38% in warm-acclimated (15°C) hearts. The disparity between the two studies may reflect a constraining influence of an intact pericardium on cardiac scope. However, the differences in cardiac output were primarily a function of a lower response of heart rate to adrenergic stimulation in the present study and not due to differences in stroke volume. Heart rate is highly temperature dependent (Wood *et al.*, 1979; Farrell, 1984) and the 3°C difference between acclimation temperatures in the two studies likely contribute to the differing results. The

Figure 3.5. B_{max} (panel A) and K_d (panel B) values for crude homogenate and isolated SL (F2) from cold- and warm-acclimated trout ventricular tissue. Histograms are the mean+SEM of 4 preparations. Significant ($p<0.05$) differences are indicated by an asterisk.


A
B
Cold
Warm
Bmax (fmol/mg protein)
K_d (pM)
200
150
100
50
0
180
160
140
120
100
*
*
HMG
F2
HMG
F2

change in heart rate with temperature is curvilinear (Randall and Brauner, 1991) and heart rate decreases more rapidly at temperatures below ~10°C and the stimulatory abilities of adrenaline may be influenced as a consequence. Alternatively, the differences between the studies may be a result of the different work regimes employed (maximal vs "working" levels). Work regimes are set through adjustments in filling pressure and thus differences in atrial and ventricular stretch (Starling effects) could affect relative increases in stroke volume and cardiac output. Stretch-related effects on heart rate could also be partially responsible for the lower heart rates found in the present study. If atrial expansion is limited by the pericardium, the stimulatory influences of atrial stretch on chronotropy could be attenuated as a result. Stretch (Starling) responses likely do not, however, account for differences in scope observed *in situ* between cold- and warm-acclimated hearts as the effect was not found in VSPs in which the degree of stretch is maximal in all instances.

The increased sensitivity to applied adrenaline in cold-acclimated trout hearts is in agreement with the results of Graham and Farrell (1989) using a similar preparation. Heart rate, stroke volume, cardiac output and power output were all significantly more responsive to adrenaline in cold-acclimated hearts. A more detailed dose-response curve was conducted in the present study yet the 10-fold shift in sensitivity is essentially equivalent to that found by Graham and Farrell (1989). In their study, threshold stimulatory actions of adrenaline were observed at concentrations of 10 and 100 nM for cold- and warm-acclimated hearts, respectively. These values are similar to the concentrations (5 nM [cold] and 50 nM [warm]) found in this study. Furthermore, EC_{50} values determined from dose-response curves indicated a similar 10-fold shift in sensitivity, demonstrating that the change in sensitivity was not a consequence of a "broadening" of the range of concentrations producing a stimulatory effects.

Adrenaline is considered to be ~10-fold more effective than noradrenaline in beta-adrenergic stimulation of the trout heart (Ask *et al.*, 1981; Farrell *et al.*, 1986). Circulating levels of adrenaline have been determined for winter and summer acclimated trout at both rest (5 nM [cold], 10 nM [warm]) and under conditions of extreme stress (190 *u*M [cold], 250 *u*M [warm]) (Milligan *et al.*, 1989). If the results from the *in situ* WHP experiments are applicable to *in vivo* conditions, resting catecholamine levels in cold-acclimated trout would generate a slight stimulation of the heart and underlies the importance of a tonic stimulation at cold temperatures previously reported by Graham and Farrell (1989). Resting catecholamine levels in warm-acclimated trout would be expected to have little influence on cardiac performance. In summary, the basic findings of Graham and Farrell (1989) using a WHP with a disrupted pericardium are largely corroborated by the present study utilizing a WHP with the pericardium intact. In both instances, adrenergic sensitivity was increased 10-fold with cold-acclimation and stimulatory responses were first evidenced in cold-acclimated hearts at low nanomolar concentrations of adrenaline. Similarly, relative increases in force at maximal adrenaline concentrations (scope) was higher in cold-acclimated trout hearts. Differences in acclimation temperature and work regimes, however, prevent assessment of the mechanisms responsible for quantitative differences between the two studies, especially in regard to differences in scope of stroke volume and heart rate response.

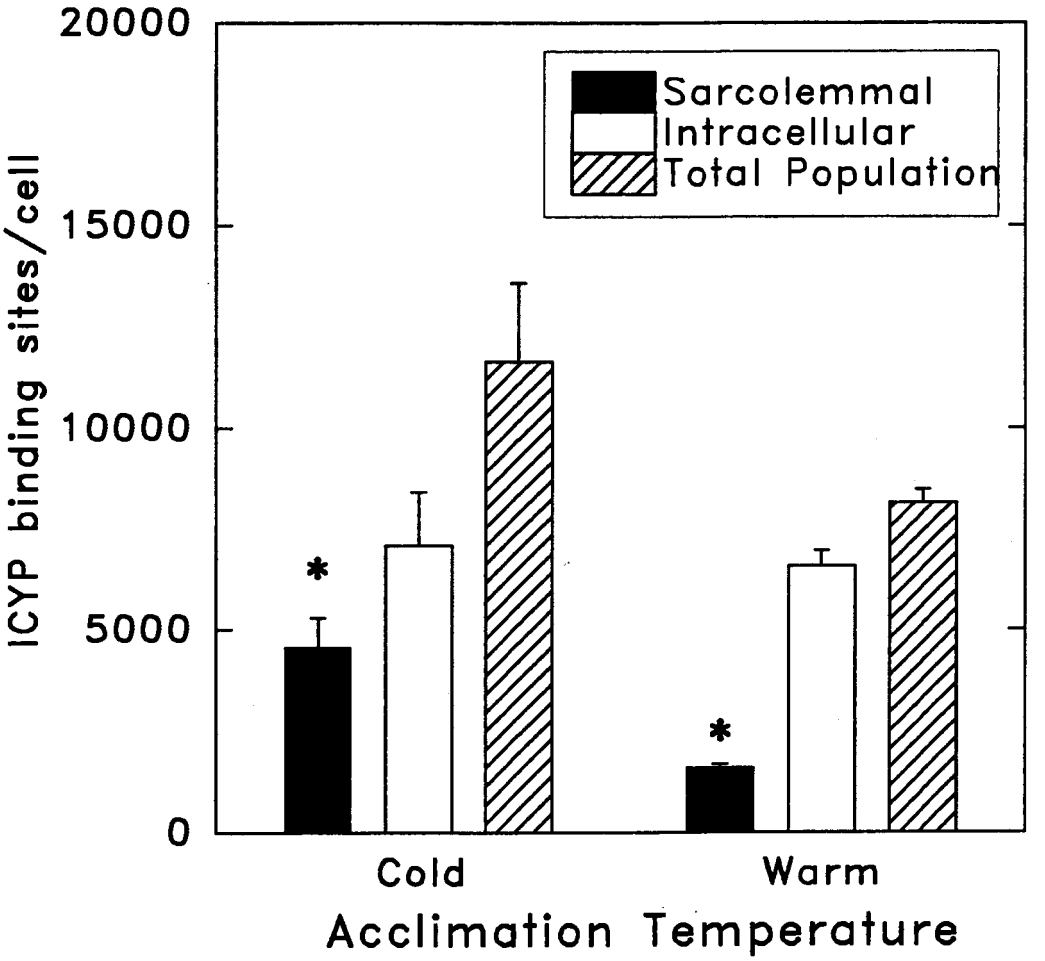
The shift in beta-adrenergic sensitivity was found to occur at both levels of organization examined in this study. Moreover, the demonstrated left-shift in sensitivity with cold-acclimation in WHPs and VSPs was correlated with the heightened sarcolemmal beta-receptor density determined in this study.

Using the methodology outlined in Tibbits *et al.* (1990), and assuming myocyte size to be unchanged by thermal acclimation, the number of sites per

cell and site density (per um^2 of SL) were calculated (see Appendix A) from Scatchard analysis of ICYP binding to the isolated SL fraction F2 (Figure 3.6). The calculated surface beta-adrenoceptor density of 1.39 ± 0.22 sites·um^{-2} for ventricular tissue from cold-acclimated trout was significantly higher ($p < 0.05$) than the value of 0.58 ± 0.10 sites·um^{-2} calculated for warm-acclimated heart tissue. Neither the total cell population (in sites·$cell^{-1}$) or the estimated intracellular population were found to be significantly different between the two acclimation groups. The mean total adrenoceptor population of 10,000 sites·$cell^{-1}$ is an order of magnitude less than that determined by Reid *et al.* (1991) for trout erythrocytes, and that estimated for rat cardiomyocytes (Moustafa *et al.*, 1978; Buxton and Brunton, 1985). Similarly, from the Reid *et al.* (1991) data, I estimate a surface density of ~20-30 adrenoreceptor sites·um^{-2} of red blood cell which is at least an order of magnitude greater than our estimates of ~1.0 sites·um^{-2} sarcolemma. My estimate is also lower than the surface density of 33 sites·um^{-2} estimated for rat myocytes. My estimate does compare favorably with densities of 3 and ~1 sites·um^{-2} determined for S49 lymphoma and pigeon red blood cells (Buxton and Brunton, 1985). These comparisons indicate that surface receptor densities vary as a function of tissue and species.

The greater than 2-fold increase in surface beta-adrenoceptor density in ventricular tissue from cold-acclimated trout is in agreement with the greater sensitivity observed in the working heart and ventricular strip preparations. As outlined in the General Introduction, receptor occupancy by adrenaline, via stimulation of a GTP-binding protein (G protein) intermediary, catalyses adenylate cyclase to convert ATP to cAMP (Lefkowitz *et al.*, 1983; Levitzki, 1986). L-type calcium channel current can be increased both directly, through interaction with the stimulated G protein, and indirectly, through phosphorylation by a cAMP-dependent kinase (PK A). A greater influx of

Figure 3.6. Calculated cellular ICYP receptor density of ventricles isolated from trout acclimated to cold (8°C) and warm (18°C) temperatures. Densities are divided into surface (sarcolemmal), intracellular and total populations and calculations were performed as described in Appendix A. Histograms are mean+SEM of 4 preparations. Significant ($p<0.05$) differences are indicated by an asterisk.


20000
15000
10000
5000
0
ICYP binding sites/cell
Sarcolemmal
Intracellular
Total Population
*
*
Cold
Warm
Acclimation Temperature

calcium across the sarcolemma should be manifest as a greater amount of force produced per beat. A greater surface density of beta-receptors in cold-acclimated hearts permits a greater likelihood of receptor occupancy at lower concentrations of adrenaline and consequently a greater sensitivity. This has been demonstrated previously in dog heart through comparisons of responsiveness of neonate and adult tissues to applied adrenaline in conjunction with determination of surface receptor number (Rockson *et al.*, 1981). Neonates possess a 50% greater number of surface beta-adrenoceptors than adults and this difference was correlated with a decrease in the EC_{50} of isoproterenol required to half-maximally activate adenylate cyclase.

No significant differences in total receptor population per cell were found between warm- and cold-acclimated ventricular tissue. Surface beta-receptor density in cold-acclimated tissues was significantly higher than found in warm-acclimated cardiac tissue. I was unable to determine whether the increase in surface receptors was a result of an increase in total receptor population or indicates a shift in receptor trafficking. Comparisons between total receptor populations were nearly significant ($p=0.11$) and thus I believe it more likely that the observed changes stem from an increase in the total available receptor pool. The mechanisms responsible for stimulation of adrenoceptor synthesis and routing of beta-adrenoceptors have not been examined in fishes. Heightened cortisol levels, however, have been correlated with an increase in beta-receptor number and with the routing of internalized receptors to the surface in trout red blood cells (Reid *et al.*, 1991) under a wide variety of stressors including hypoxia (Fievet *et al.*, 1987), hypercapnia (Perry *et al.*, 1989) and during strenuous exercise (Primmett *et al.*, 1986). The action of cortisol represents a short-term adaptation to an imposed stress but the mechanisms utilized may be related to the long term adaptational change in surface receptor density observed in this

study. In mammals, long-term upregulation of beta-adrenoceptor levels in a number of tissues has been demonstrated to occur in response to stimulation by glucocorticoids (Collins *et al.*, 1989; 1991) through increases in transcription of the beta-adrenoceptor gene. Whether such a mechanism exists in fishes has yet to be determined.

In conclusion, *in situ* heart preparations and *in vitro* ventricular strips from rainbow trout acclimated to cold-temperatures had a greater sensitivity to adrenaline than warm-acclimated counterparts. The effect was clearly demonstrated in ventricular strips to be an acclimatory response to temperature change and not a function of the trial temperature. Furthermore, binding studies conducted on isolated sarcolemmal fractions indicated a 2-fold increase in surface beta-receptor density in cold-acclimated fish hearts. It is suggested that the increase in surface beta-receptor density is at least partially responsible for the increased adrenergic sensitivity found in the cardiac tissue of cold-acclimated rainbow trout.

Increases in surface beta-adrenoceptor populations thus provide at least one method by which cold-acclimated trout hearts are made more sensitive to adrenaline. Occupation of the receptor by adrenaline stimulates a cascade of reactions which also possesses the potential for modification and could influence adrenergic sensitivity. The various components involved in the linking of hormone-receptor complexation with a modification of the intracellular milieu include the G_s protein and adenylate cyclase, and these are examined in the next Chapter.

CHAPTER IV

THERMAL MODIFICATION OF TRANSMEMBRANE SIGNALING

A. Introduction

In situ hearts from cold-acclimated trout have been shown to be approximately 10-times more sensitive to adrenaline than warm-acclimated counterparts (Graham and Farrell, 1989; Chapter III). Shifts in adrenergic response must be correlated with changes in the mechanisms involved in transduction of adrenergic receptor occupation into an intracellular response. *In vitro* radioligand binding analysis of ventricular tissue from warm- and cold-acclimated trout has revealed the surface beta-receptor density to be significantly increased at cold temperatures (Chapter III), explaining in part this heightened sensitivity of cold-acclimated trout hearts. Since acclimatory changes in the remaining components of the transduction system have yet to be detailed, this Chapter reports the experiments in which I examined adenylate cyclase activity *in vitro* in crude homogenates from hearts of warm- (18°C) and cold- (8°C) acclimated rainbow trout using agents which stimulate beta-adrenoceptor, G protein and adenylate cyclase.

B. Methods and Materials

Stock origin and maintenance was as described in Chapter II. Fish used in this particular study ranged from 372-623 g.

Tissue preparation. The ventricles from acclimated rainbow trout were homogenized as described in Chapter III except the pH of the homogenizing

medium was 7.1 at 23°C.

Adenylate cyclase activity. Adenylate cyclase activity was initiated and quantified as outlined in Chapter III with the following modifications. Tissues were incubated at acclimation temperature (8 or 18°C) unless otherwise specified. The pH at these temperatures was 7.4 (8°C) and 7.2 (18°C) and correspond to physiologically-realistic intracellular pH values in fish cardiac muscle (Nielsen and Gesser, 1984; Farrell and Milligan, 1986). Basal adenylate cyclase activity was determined using various concentrations of ATP (range 0.5-800 uM). The concentrations of agents used to stimulate specific components of the transmembrane signaling system are included in either the text or in Figure captions and assays were carried out with 500 uM ATP as a substrate, a level which produced a near maximal stimulation of basal adenylate cyclase activity.

C. Results

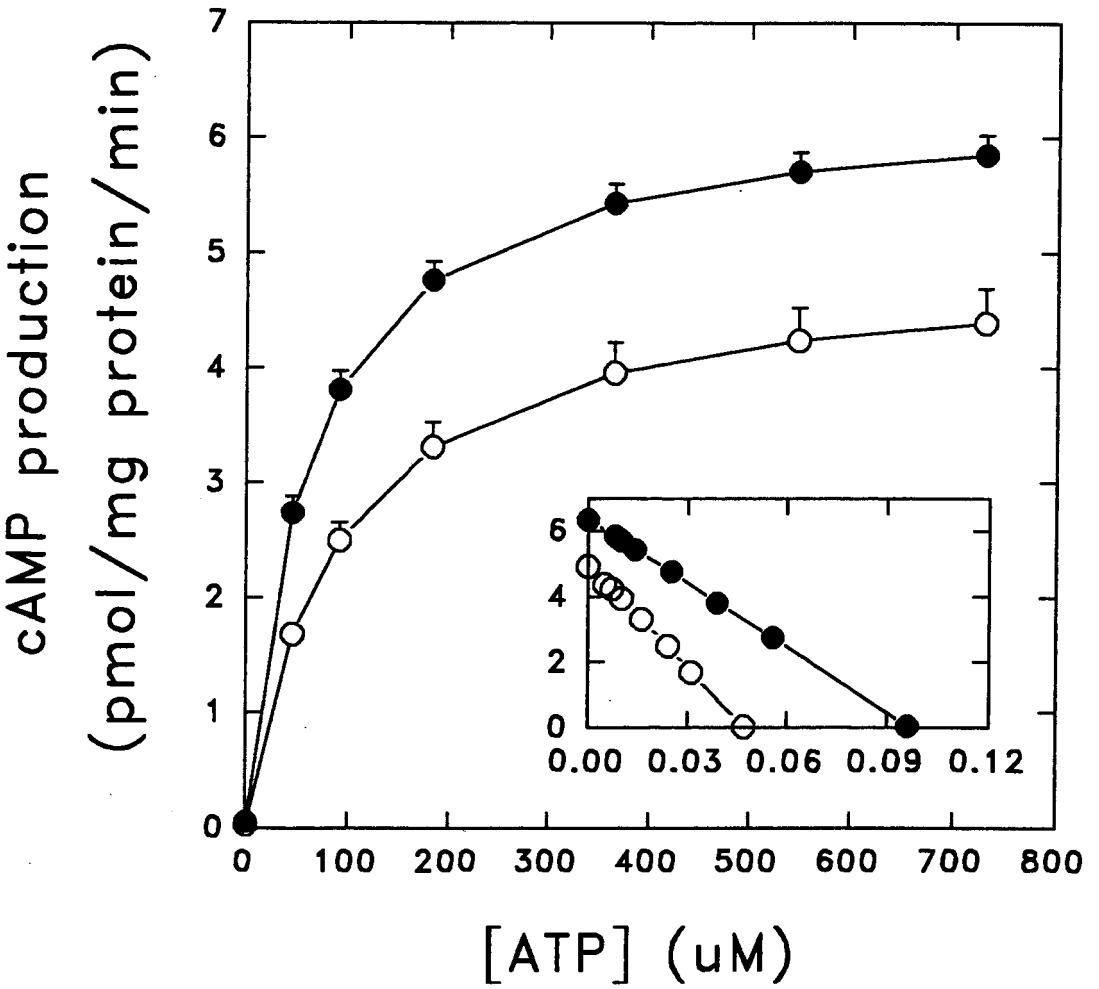
Morphometric data for cold- and warm-acclimated trout used in this study are presented in Table 4.1. Cold-acclimated trout were found to have significantly ($p<0.05$) higher heart-to-body mass ratio, consistent with previous reports of relative increases in ventricular mass with acclimation to cold temperatures (Farrell *et al.*, 1988a).

The basal activity level of adenylate cyclase, assayed in the absence of any stimulatory agents and at acclimation temperature, was examined as a function of substrate (ATP) concentration (Figure 4.1). Eadie-Hofstee analysis of activity profiles revealed the rate of adenylate cyclase activation to average 6.3 ± 0.2 pmoles·mg protein^{-1}·min^{-1} in ventricular homogenate from cold-acclimated fish and this level was found to be significantly ($p<0.05$) greater than the value of

Table 4.1. Morphometric data of the cold- and warm-acclimated rainbow trout used in the enzyme studies. Relative ventricular mass, as a function of body mass, (V/B) was calculated from (ventricular mass/body mass) x 100. Significant differences ($p < 0.05$) in V/B between acclimation groups are indicated by paired lower case letters. N = 15 in all instances.

Acclimation Group	Vent Mass (g)	Body Mass (g)	(V/B) x 100
Cold (8°C)	0.47±0.02	483±19	0.097±0.003[a]
Warm (18°C)	0.47±0.07	511±18	0.092±0.002[a]

Figure 4.1. Basal adenylate cyclase activity as a function of substrate (ATP) concentration. Curves are for ventricular homogenates from cold (solid) and warm (open) -acclimated trout. Assays were conducted at acclimation temperature (8 or 18°C). Eadie-Hofstee analysis (inset) indicated adenylate cyclase activities of 6.3±0.2 (cold) and 4.9±0.4 (warm) pmoles·mg^{-1}·min^{-1}. K_m values averaged 61±5 *u*M (cold) and 89±4 *u*M (warm). N=5 for each group and points are mean+SEM.

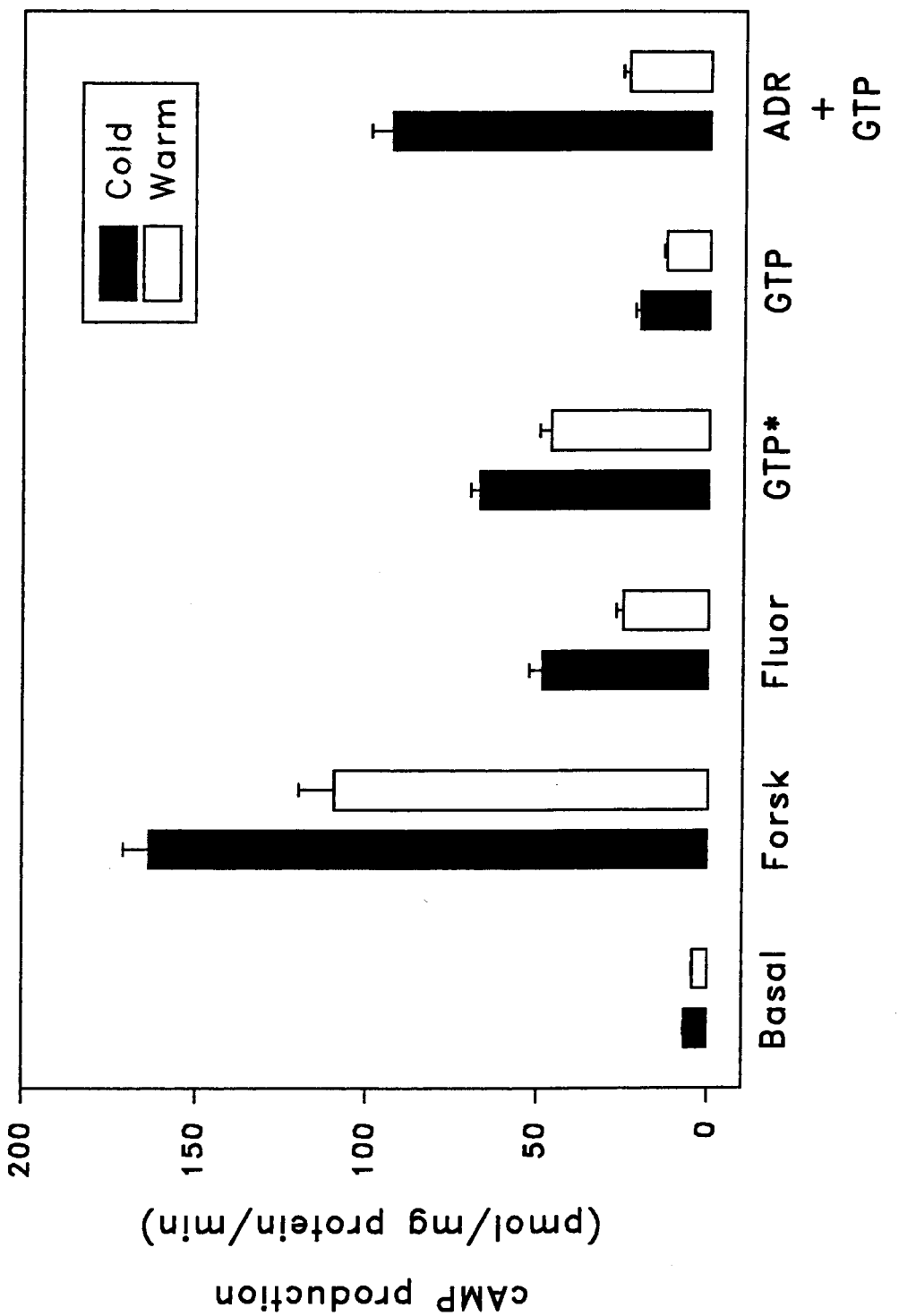

cAMP production
(pmol/mg protein/min)
[ATP] (uM)
0
1
2
3
4
5
6
7
0
100
200
300
400
500
600
700
800
0.00
0.03
0.06
0.09
0.12

4.9±0.4 pmoles·mg protein^{-1}·min^{-1} found in homogenates from warm-acclimated counterparts. The concentration of substrate producing half-maximal activation (K_m [ATP]) was also found to be significantly different ($p<0.05$). The apparent K_m(ATP) for cold-acclimated ventricular homogenate tested at 8°C was 61±5 uM and was significantly less than the estimate of 92±6 uM determined for warm-acclimated homogenate tested at 18°C. These differences were found to be related to the incubation temperature and not related to intrinsic differences in the enzymes. When tested at room temperature (23°C), the apparent K_m(ATP) values were not significantly different ($p>0.05$) and averaged 118±7 (cold) and 113±4 uM (warm), respectively. The rate of cAMP production in cold-acclimated tissue was increased to 10.3±0.8 pmoles·mg protein^{-1}·min^{-1} when tested at 23°C and was significantly greater ($p>0.05$) than the rate of 6.2±0.4 pmoles·mg protein^{-1}·min^{-1} found in warm-acclimated tissue.

The remaining assays were conducted at acclimation temperature in order to ensure physiological pH conditions. A substrate (ATP) concentration of 500 uM was used in all remaining assays, this concentration generating maximal basal adenylate cyclase activity in homogenates tested at acclimation temperatures (8 and 18°C).

Further direct assessment of adenylate cyclase activity was made using the diterpene forskolin and fluoride anion. Forskolin (1 mM) directly stimulates the catalytic subunit of adenylate cyclase and typically stimulates the enzyme to a greater degree than any other agent (Susanni *et al.*, 1992). This was also found to be true for trout heart (Figure 4.2). While a significantly higher activation of cAMP production was observed using homogenate from cold-acclimated trout hearts, the relative increase in activity over basal levels was not found to be significantly different. Forskolin generated a 24-fold increase in cAMP production in ventricular homogenate from cold-acclimated trout which was not

Figure 4.2. Effect of stimulation of components of the beta-adrenergic signaling pathway on adenylate cyclase activity. Substrate (ATP) concentration was 500 *u*M in all assays. Agonist concentrations are as follows: fluoride, 10 mM; forskolin, 1 mM; GTP, 100 *u*M; GTP-gamma-S, 100 *u*M. The adrenaline concentrations used were 10^{-6} M [cold] and 10^{-5} M [warm] and represent the concentration producing the maximal cAMP production. Adrenaline assays were conducted in the presence of 100 *u*M GTP. All assays were conducted at acclimation temperature (8 or 18°C). N=5 is all instances and histograms are mean+SEM.


cAMP production
(pmol/mg protein/min)
200
150
100
50
0
Cold
Warm
Basal
Forsk
Fluor
GTP*
GTP
ADR
+
GTP

statistically different from the 25-fold increase observed in homogenates from warm-acclimated trout hearts (Figure 4.3).

Fluoride anion stimulates the catalytic subunit of adenylate cyclase in a similar fashion as the GTP-occupied G_s protein by mimicking the gamma-phosphate of GTP in the GDP-bound G_s protein (Bigay *et al.*, 1985) and thus providing persistent stimulation of adenylate cyclase activity. Fluoride-stimulated (10 mM) adenylate cyclase activity was significantly greater ($p<0.05$) in cold- than in warm-acclimated heart homogenates (Figure 4.2). As with forskolin, however, the relative increase in activity above basal levels following fluoride addition was not significantly different ($p>0.05$; Figure 4.3)).

The stimulatory effects of G_s protein were examined using both GTP (100 uM) and a non-hydrolyzable analogue, GTP-gamma-S (100 uM). GTP addition resulted in a significantly greater ($p<0.05$) stimulation of adenylate cyclase in homogenate from cold-acclimated trout than was found in homogenate from warm-acclimated trout (Figure 4.2). Stimulation of adenylate cyclase activity using the non-hydrolyzable analogue was significantly greater ($p<0.05$) than found using GTP and, as with GTP, the rate of cAMP production was greater in cold-acclimated tissue (Figure 4.2). No significant difference ($p>0.05$) in the relative increase in adenylate cyclase activity above basal levels, either using GTP or GTP-gamma-S, was found between the two acclimation groups (Figure 4.3).

Adrenergic activation of adenylate cyclase was examined in dose-dependent fashion through application of adrenaline (10 pM to 10 uM) in the presence of 100 uM GTP. Maximal adrenergic activation of cAMP production was significantly greater in cold- than in warm-acclimated tissues (Figure 4.4). The relative increase in activation of cAMP production was also significantly ($p<0.05$) different between the two acclimation groups. Maximal adrenergic stimulation increased basal activity by 12.7 ± 1.0 times in cold-acclimated tissue but only by

Figure 4.3. The fold-increase in activation of adenylate cyclase by agonists stimulating various components of the transmembrane signaling pathway. The fold-increase was calculated from ($Activity_{agonist}/Activity_{basal}$) using the data presented in Figure 4.2. Significant differences are indicated by asterisks. Values are mean+SEM; N=5.

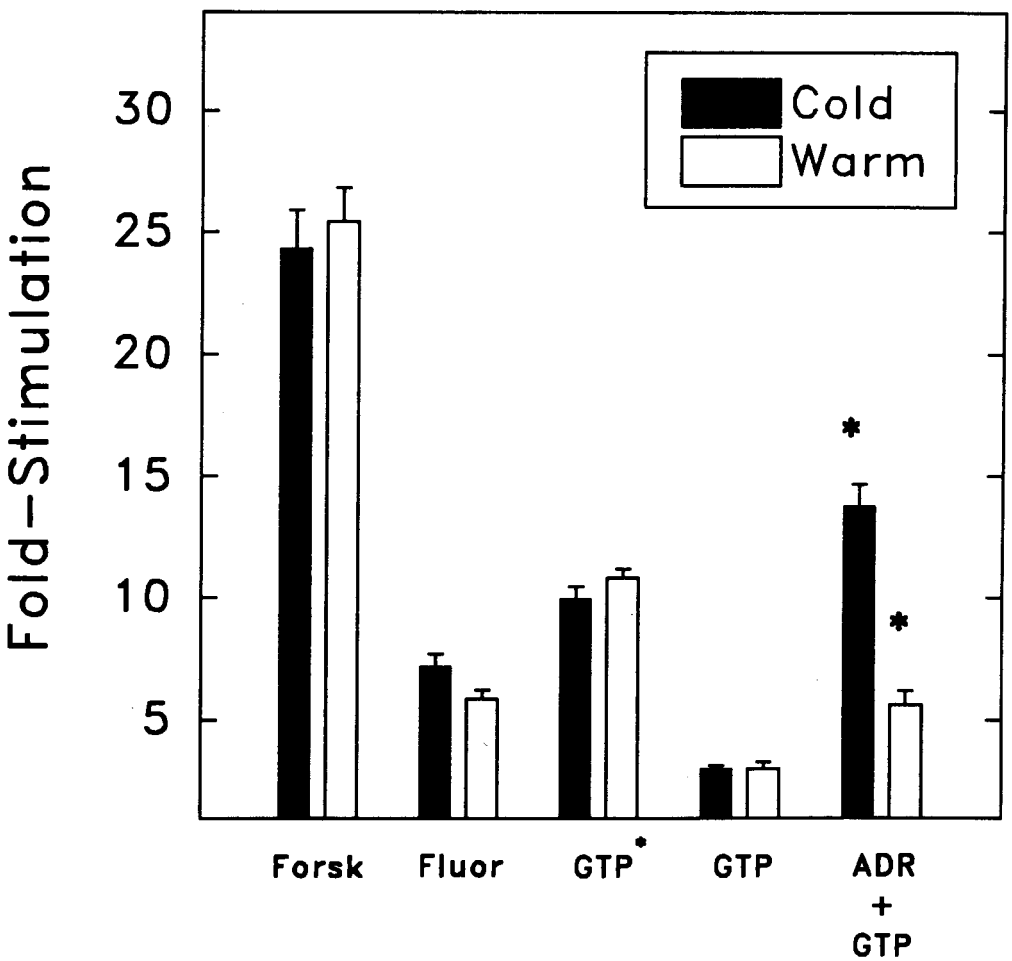
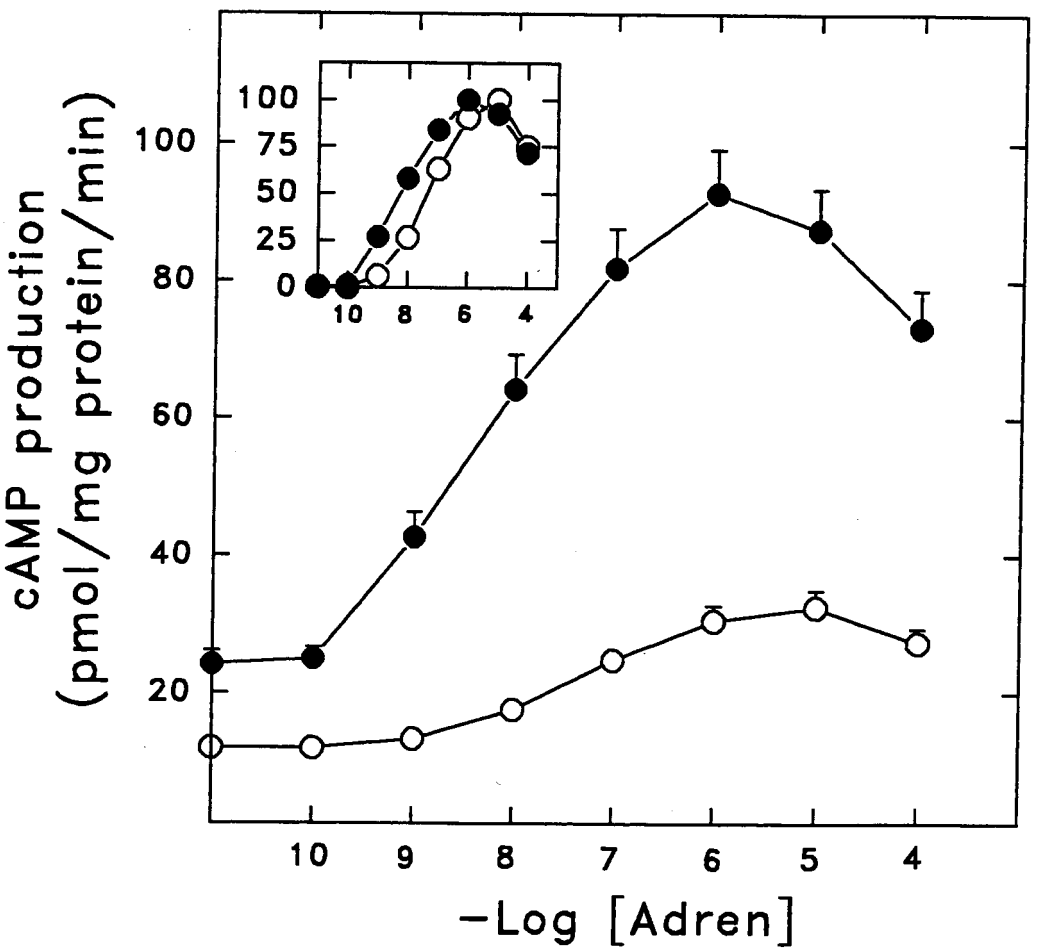

Fold-Stimulation
30
25
20
15
10
5
Cold
Warm
*
*
Forsk
Fluor
GTP*
GTP
ADR
+
GTP

Figure 4.4. Activation of adenyl cyclase activity as a function of applied adrenaline. Assays were conducted at acclimation temperature (8 [solid] or 18 [open] °C) in the presnece of 100 *u*M GTP and concentrations of adrenaline ranging from 10^{-11} to 10^{-4} M. Inset: EC_{50} values of $6.2x10^{-9}$ (cold-acclimated) and $4.7x10^{-8}$ (warm-acclimated) were calculated after normalization of the data as a function of the maximal rate of cAMP production. N=5 in all instances and points are mean$\pm$SEM.


cAMP production
(pmol/mg protein/min)
100
80
60
40
20
10
9
8
7
6
5
4
−Log [Adren]
100
75
50
25
0
10
8
6
4

5.7±0.2 times in warm-acclimated tissue (Figure 4.3).

The sensitivity of cardiac homogenates to adrenergic stimulation, estimated from the concentration producing half-maximal activation (EC_{50}) was found to be significantly greater in cold-acclimated tissues (6 nM) than in warm-acclimated (47 nM) tissues (Figure 4.4 [inset]).

D. Discussion

Thermal acclimation was found to significantly ($p<0.05$) alter the basal activity level of adenylate cyclase. A greater basal level of adenylate cyclase activity was found in cardiac homogenates from cold-acclimated trout than was found from warm-acclimated trout when incubated either at acclimation temperature or at room temperature (23°C). Whether the differences in reaction rate result from an increase in the number of enzyme units, an alteration in the ratio of enzyme types or through expression of different adenylate cyclase isoforms (Susanni *et al.*, 1992) has not been ascertained. The K_m(ATP) was also found to be significantly different when tissues were incubated at acclimation temperature but not at 23°C. Whether this stems from pH or temperature effects, or from differential isozyme expression has not been determined.

The involvement of G_s protein in activation of adenylate cyclase was investigated using GTP and a non-hydrolyzable analogue, GTP-gamma-S. No differences between acclimation groups were found in the stimulatory capacities of G_s protein, above those produced by the heightened basal activity levels. This suggests the G_s protein levels to be qualitatively similar in both preparations and not subject to thermal modification. This is not surprising given that G_s proteins have been found to be stimulated by at least 17 different types of surface receptors (Birnbaumer, 1990). If G_s protein levels were increased, then

occupation of any of the other receptors which stimulate G_s protein would produce large increases in cAMP levels, rather than beta-adrenergic agonists alone.

In Chapter III, *in situ* heart preparations and *in vitro* ventricular strip preparations demonstrated heart tissue from cold-acclimated trout to be more sensitive to either perfused or applied adrenaline than was found for cardiac tissue from warm-acclimated hearts. The shift in sensitivity was further demonstrated to be correlated with a heightened sarcolemmal density of beta-adrenoceptors in hearts from cold-acclimated trout. In the present study, a similar shift in sensitivity was observed in adrenergically-stimulated cAMP production with cold acclimation. This heightened adenylate cyclase activity in cold-acclimated tissues likely stems from the greater surface density in cold-acclimated tissue. An increase in adrenoceptor number should increase the likelihood of receptor occupancy at lower concentrations of agonist. G_s protein is stimulated by the hormone-occupied receptor and stimulates the catalytic subunit of adenylate cyclase. As a consequence, cAMP levels and the activity of cAMP-dependent processes are increased at lower concentrations of extracellular agonist. This is in good agreement with the *in situ* and *in vitro* findings in Chapter III.

At adrenaline concentrations producing maximal stimulation of adenylate cyclase activity, however, the rate of cAMP production was significantly higher in cold-acclimated tissues (Figure 4.4). Assuming cAMP-dependent processes are not substantially affected by thermal acclimation and inotropy is tightly correlated with cAMP levels, inotropic increases might be expected to be significantly greater in hearts from cold-acclimated trout. Such a condition was found in *in situ* heart preparations although it must be noted that they were not found in ventricular strip preparations (Chapter III). The reasons underlying this

disparity are not clear at present.

In conclusion, thermal acclimation produced modifications in the transmembrane-signalling components on two levels. Firstly, cold-acclimation produces an alteration in the adenylate cyclase enzyme such that cAMP production is enhanced under both stimulated and non-stimulated conditions. Secondly, the sensitivity of the system to applied adrenaline and subsequent adenylate cyclase stimulation are enhanced in cold-acclimated tissue. This likely results from the increased surface receptor density in cold-acclimated ventricular tissue demonstrated in Chapter III. No alteration in the ability of G_s protein to stimulate adenylate cyclase activity, however, were found.

These results thus provide some biochemical correlates for the thermally-induced shift in adrenergic sensitivity found using *in situ* WHPs (Chapter III). Adrenaline operates in part to increase sarcolemmal calcium influx which can either directly activate the myofilaments or stimulate the release of a quantity of calcium stored in the SR. Given the high thermal sensitivity of the ryanodine channel, adrenaline may operate as a more direct modulator of contractility in cold temperatures than may occur during summer months. Correspondingly, the last Chapter examines the effect of temperature on the role of the SR in contractility in the trout myocardium.

CHAPTER V

ALTERED CALCIUM SENSITIVITY AND CALCIUM HANDLING CAPACITIES OF CARDIAC TISSUE FOLLOWING TEMPERATURE ACCLIMATION

A. Introduction

Previous Chapters demonstrate cold-acclimation to increase the adrenergic sensitivity of the trout heart both *in situ* and *in vivo*. Biochemical studies suggest this phenomenon to be at least partially achieved through an increase in sarcolemmal adrenoceptor density and rate of intracellular cAMP production. An increase in cAMP formation, stimulated by hormone occupation of receptor, could result in a higher cytosolic cAMP concentration and an increased stimulation of cAMP-dependent protein kinases (PK As). In cardiac tissue, PK As act to phosphorylate a number of intracellular proteins (Tada *et al.*, 1975; Ray and England, 1976; Lindemann *et al.*, 1983; Solaro, 1986), including the L-type (DHP sensitive) calcium channel (Osterrieder *et al.*, 1982; Rinaldi *et al.*, 1982; Brum *et al.*, 1983; Kameyama *et al.*, 1985). Phosphorylation of this channel, in conjunction with the direct effects of adrenergic receptor-activated G_s protein (Yatani *et al.*, 1987; Mattera *et al.*, 1989), increases the magnitude of Ca^{2+} influx across the SL per membrane depolarization event (Reuter and Scholz, 1977; Osterrieder *et al.*, 1982; Reuter, 1983; Brum *et al.*, 1984).

In mammals, as outlined in the General Introduction, the influx of calcium across the SL serves primarily as a trigger for the release of a greater quantity of stored calcium from the SR which then activates the contractile element. Adrenergic amplification of SL Ca^{2+} influx increases the magnitude of SR calcium release and, in coordination with other cAMP- and Ca^{2+}/calmodulin-

stimulated processes (Warber and Potter, 1986; Shabb and Corbin, 1992), produces a positive inotropy.

Recent work (Vornanen, 1989; Tibbits *et al.*, 1990, 1991), in conjunction with ultrastructural evidence (Santer, 1974, 1985), has suggested that contractility in the teleost myocardium, like that of the frog (Hadju, 1969; Fabiato and Fabiato, 1978; Klitzner and Morad, 1983; Nabauer *et al.*, 1989), may have a greater dependence upon SL Ca^{2+} influx for direct activation of the myofilaments, with little or no contribution from SR release. Adrenergic magnification of calcium influx across the SL under these conditions would provide a direct method of improving force generation.

One of the more compelling arguments for the non-involvement of the SR in E-C coupling is the lack of an effect of ryanodine, a noted blocker of SR Ca^{2+} release in mammals (Sutko and Kenyon, 1983), on force development in fish hearts under physiologically-appropriate conditions (Driedzic and Gesser, 1988; El-Sayed and Gesser, 1989). However, the ryanodine-sensitive, Ca^{2+}-release channel of the SR has been shown to be highly temperature sensitive (Bers, 1987, 1989; Sitsapesan *et al.*, 1991) and essentially non-functional at cold temperatures. The colder incubation temperatures used in teleost studies may therefore, preclude an observable effect of ryanodine on cardiac contractility. This idea is further supported by a recent study using atrial strips from skipjack tuna (*Katsuwonus pelamis*) incubated at 25°C, in which 10 uM ryanodine depressed contractile force by 30% (Keen *et al.*, 1992).

The primary objective of this study was, therefore, to examine the effect of temperature change, both acute and chronic, on the role of the SR in the supply of calcium to the contractile machinery. The involvement of the SR in force development was estimated through the use of ryanodine at concentrations (10 nM, 10 uM) known to affect contractility in mammalian cardiac tissue (Sutko and

Kenyon, 1983). Two experimental preparations were used. Physiological significance was determined using an *in situ* working heart preparation (WHP) which closely approximates cardiac performance found *in vivo*. In order to look at the influence of acute temperature change on the ability of ryanodine to impair contractile function, without the confounding influences of changes in heart rate and fiber length, an *in vitro* isometric ventricular strip preparation (VSP) was also used.

A second series of experiments were conducted which examined the effect of extracellular calcium and temperature on twitch tension. This was conducted for two reasons. Firstly, a body of data exists which relates the sensitivity of ventricular tissue to changes in Ca^{2+}_{o} in a number of teleost and elasmobranch species (Driedzic and Gesser, 1988; Keen *et al.*, 1992) and it was therefore of interest to compare the response of trout ventricular tissue to these. Secondly, temperature has been demonstrated in mammals to affect the sensitivity of cardiac myofilaments to cytosolic calcium (Harrison and Bers, 1989). A decrease in temperature both reduces the maximal tension development and the calcium sensitivity of chemically skinned ventricular tissue (Harrison and Bers, 1989). While alterations in extracellular calcium concentration will affect cytosolic calcium levels in working heart and ventricular strip preparations, the magnitude of the change in cytosolic calcium cannot be easily quantified. Nevertheless, it does provide a gross means of adjusting intracellular calcium content and SL calcium influx. Correspondingly, given the thermal dependence of the response of the contractile element to changes in cytosolic calcium in mammals, it was of interest to examine the effect of temperature on the sensitivity of trout ventricular strips to extracellular calcium.

B. Methods and Materials

Stock origin and maintenance was as described in Chapter II. Fish used in this particular study averaged (±SEM) 573±15 g.

In situ working heart preparation (WHP). Surgical procedures, methods of data acquisition and salines used were as described in Chapter II. Upon stabilization of the preparations at "resting" levels of cardiac flow, one of two experimental series was undertaken.

WHP-Series A. Design limitations of systems are more readily observed under "working" rather than "resting" conditions. Correspondingly, after stabilization of the preparation (5-10 minutes), cardiac output was increased 1.5-2 fold to approximate that of a swimming fish but not one in a condition of *extremis*. This performance level of the heart represented 40-50% of maximum power output. In this series of experiments, the input line, receiving saline with a calcium concentration of 2.5 mM (control), was switched such that it received saline having a calcium concentration of 1.25 mM and the heart response monitored over a seven minute period. Following this, the heart was reperfused with 2.5 mM Ca^{2+} saline and recovery monitored for an additional seven minute period. After this, the heart was then exposed to either 1.25 mM Ca^{2+} saline or 1.25 mM Ca^{2+} saline + 10 *u*M ryanodine and depletion and recovery followed for additional seven minute periods.

WHP-Series B. As in the previous study, cardiac output was initially set at a level representative of a swimming fish. Following this, input saline was switched from control (2.5 mM Ca^{2+}_{o}) to a trial saline (1.25, 1.00 or 0.50 mM Ca^{2+}_{o}) and

depletion followed for seven minutes. The stopcock was then returned to its original position such that the heart received control saline and recovery monitored for seven minutes. The next depletion trial was then initiated and the procedure repeated.

Ventricular strip preparations (VSP). Strips (length 7.9±0.2 mm; width 0.7±0.1 mm) were set up as described in Chapter III. The stimulation voltage used in this experimental series averaged 11.7±0.3 V. After setup and stabilization of the preparation, bath saline was replenished and the strips were exposed to one of the following protocols.

VSP-Series A. The involvement of the SR in contractile function was assessed through sequential application of a low (10 nM) and high (10 *u*M) dose of saline-based ryanodine to ventricular strips. One hour incubations were used for each concentration. Trials for both acclimation groups were performed at stimulation rates of 0.2 and 0.6 Hz. An additional and more physiologically-relevant pacing frequency was also tested; 1.0 Hz for cold-acclimated and 1.5 Hz for warm-acclimated. Each strip received both ryanodine concentrations but no strip was tested at more than one stimulation rate or trial temperature.

VSP-Series B. The thermal dependency of ventricular strips to changes in extracellular calcium response was examined through the construction of concentration-response curves using hearts from warm- and cold-acclimated trout. The calcium concentration of the bathing (extracellular) solution was increased through addition of a saline-based $CaCl_2$ stock solution to produce a $Ca^{2+}{}_o$ range of 0.5 to 10.0 mM Ca^{2+}. A stimulation frequency of 0.2 Hz was used in all instances. After completion of a concentration-response trial, a new strip was used and the experiment repeated at the opposite temperature (either 8 or

18°C).

Salines. The following were common elements to all salines used (in mM): NaCl, 124.1; KCl, 3.1; $MgSO_4$, 0.9; dextrose, 5.0. Salines were buffered with 20 mM N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES, sodium salt and free acid combinations). Saline pH was 7.90 and 7.74 at 8 and 18°C, respectively, and approximate *in vivo* blood values at these temperatures (Howell *et al.*, 1970; Randall and Cameron, 1973; Railo *et al.*, 1985). Adrenaline at a concentration of 5 nM was added to all salines used in WHP experiments. This level approximates *in vivo* resting levels (Milligan *et al.*, 1989) and, *in situ*, both maintains a tonic cardiac stimulation and reduces deterioration of the preparation (Graham and Farrell, 1989). All chemicals used were reagent grade and purchased from Sigma Chemical (St. Louis, MO) with the exception of ryanodine which was purchased through Calbiochem (San Diego, CA).

Statistical Evaluation. Non-parametric statistical comparisons were made using Mann-Whitney U-tests and a significance level of 0.05.

C. Results

Examination of morphometric data from fish acclimated to cold and warm temperatures indicated ventricular mass, as a function of body mass, to be significantly ($p<0.05$) higher in cold-acclimated individuals (Table 5.1). An increase in relative heart mass associated with thermal acclimation has been previously described (Farrell *et al.*, 1988a) and provided an indication that the three week acclimation period (as a minimum) was sufficient to stimulate acclimatory responses.

Table 5.1. Morphometric data from warm- and cold-acclimated used in the WHP and VSP experiments. Data are mean±SEM. Significant differences ($p<0.05$) between groups in relative ventricular mass (V/B) are by lower case letters.

Temp	Series Type	Ventricle Mass (g)	Body Mass (g)	V/B (x100)
18°C	WHP (N=14)	0.49±0.03	567±36	0.087±0.002[a]
	VSP (N=12)	0.45±0.03	490±28	0.091±0.001[b]
	Pooled (N=26)	0.47±0.02	532±24	0.089±0.001[c]
8°C	WHP (N=14)	0.42±0.01	430±10	0.097±0.002[a]
	VSP (N=11)	0.38±0.02	388±16	0.098±0.002[b]
	Pooled (N=25)	0.40±0.01	412±9	0.097±0.001[c]

Preliminary studies of the form described for the *WHP-Series B* experiments (see Methods and Materials) suggested 1.25 mM Ca^{2+}_o to be, in a general sense, a threshold level for the maintenance of *in situ* cardiac performance. This level of Ca^{2+}_o was therefore chosen for *WHP-Series A* (ryanodine) trials with the expectation that any impairment of contractile performance due to an impairment of SR function would be exacerbated. Neither cold-acclimated hearts tested at 8°C or warm-acclimated hearts tested at 18°C demonstrated a significant difference in any of the cardiac variables in trials using 1.25 mM Ca^{2+}_o alone or with the addition of 10 *u*M ryanodine (Table 5.2a,b). In all cases, variables returned to within 95% of pre-depletion levels during the seven minute period of reperfusion with 2.5 mM Ca^{2+}_o (not shown).

Unlike the WHP series, a temperature dependency of ryanodine action was clearly demonstrated in studies using ventricular strips. At 0.2 Hz, strips from both warm- and cold-acclimated trout, when tested at 8°C, did not show any significant response to application of either 10 nM or 10 *u*M ryanodine (Figure 5.1, Panel A). In strips incubated at 18°C, however, active tension development was reduced by ryanodine. The degree of contractile impairment was significantly ($p<0.05$) different between the two acclimation groups after 1 hour exposure to high (10 *u*M) and low (10 nM) ryanodine doses. Low dose ryanodine application depressed twitch tension by 18.0±2.2% at 18°C in strips from cold-acclimated fish and by 13.1±2.2% at 18°C in strips from warm-acclimated fish. At high ryanodine levels, tension generation was depressed by 58.7±10.1% from control values in ventricular strips derived from cold-acclimated trout but only by 26.3±3.1% ($p<0.05$) in strips from warm-acclimated trout.

The effect of ryanodine also depended upon the rate of muscle stimulation (Figure 5.1, Panels B and C). In strips from cold-acclimated fish tested at 0.6 Hz and 18°C, low dose ryanodine application produced only a 9.9±0.8% decline in

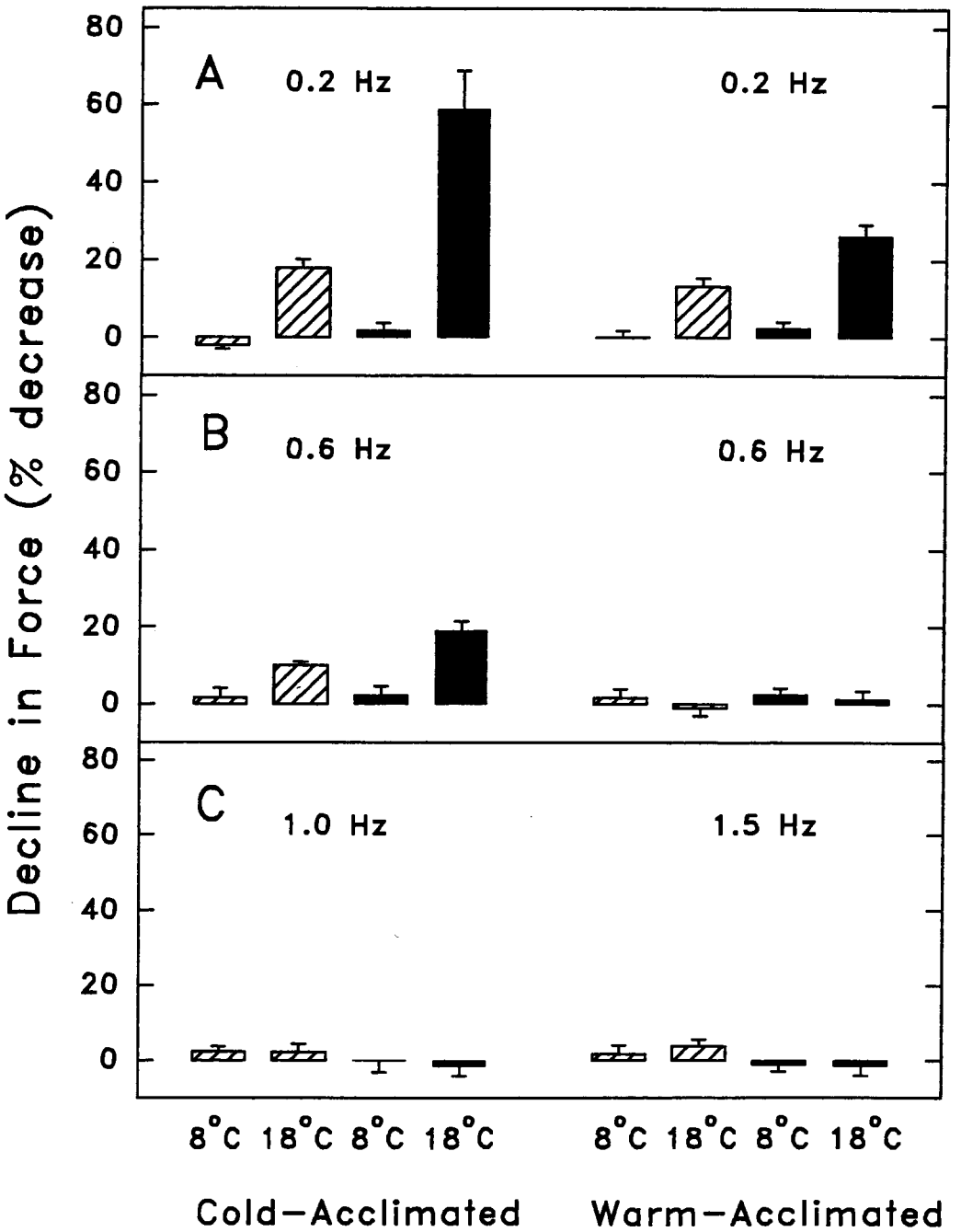
Table 5.2a. The effect of ryanodine on cardiac performance of hearts from cold- and warm-acclimated trout. A depletion trial using 1.25 mM Ca_o^{2+} was initially conducted, changes in cardiac variables paralleling those found in the *WHP-Series A* trials. After return to control saline, the depletion trial with 1.25 mM was repeated either with or without the addition of 10 *u*M ryanodine (RY). No significant ($p>0.05$) effect of ryanodine was observed within either acclimation group. N=4 for each group. Data are mean (±SEM) and are in the following units: stroke volume (SV), $mL \cdot beat^{-1}$; heart rate (f_H), $beats \cdot min^{-1}$; cardiac output ($\dot{Q}$), $mL \cdot min^{-1} \cdot kg^{-1}$ body weight; power, $mW \cdot g^{-1}$ ventricle.

Temp	$[Ca_o^{2+}]$	SV	f_H	$\dot{Q}$	Power
Cold Ryanodine	1.25 mM	0.17 (±0.2)	58.8 (±3.3)	24.51 (±0.60)	2.10 (±0.11)
	1.25 mM + 10 *u*M RY	0.17 (±0.02)	59.3 (±3.2)	23.93 (±0.80)	2.05 (±0.13)
Cold Control	1.25 mM	0.18 (±0.01)	56.2 (±2.4)	23.35 (±0.88)	2.21 (±0.07)
	1.25 mM	0.18 (±0.01)	56.0 (±2.3)	22.97 (±0.91)	2.18 (±0.07)
Warm Ryanodine	1.25 mM	0.24 (±0.01)	84.6 (±1.5)	40.64 (±1.44)	3.86 (±0.14)
	1.25 mM + 10 *u*M RY	0.23 (±0.01)	84.7 (±1.5)	40.33 (±1.36)	3.84 (±0.12)
Warm Control	1.25 mM	0.24 (±0.01)	86.1 (±2.2)	39.61 (±1.08)	3.81 (±0.15)
	1.25 mM	0.24 (±0.01)	86.0 (±2.1)	38.88 (±1.30)	3.73 (±0.17)

Table 5.2b. The effect of ryanodine on cardiac performance of hearts from cold- and warm-acclimated trout. A depletion trial using 1.25 mM Ca_o^{2+} was initially conducted, changes in cardiac variables paralleling those found in the *WHP-Series A* trials. After return to control saline, the depletion trial with 1.25 mM was repeated either with or without the addition of 10 *u*M ryanodine (RY). No significant ($p > 0.05$) effect of ryanodine was observed either within or between acclimation groups. Data are expressed as a percentage of control values and are mean (±SEM). N=4 for each group. Percentages were calculated from variables in the following units: stroke volume (SV), $mL \cdot beat^{-1}$; heart rate (f_H), $beats \cdot min^{-1}$; cardiac output (Q), $mL \cdot min^{-1} \cdot kg^{-1}$ body weight; power, $mW \cdot g^{-1}$ ventricle.

Temp	$[Ca_o^{2+}]$	SV	f_H	Q	Power
Cold Ryanodine	1.25 mM	84.4 (±2.2)	105.2 (±1.8)	88.8 (±0.9)	89.2 (±1.0)
	1.25 mM + 10 *u*M RY	83.7 (±1.7)	105.6 (±1.6)	88.3 (±1.1)	87.7 (±1.3)
Cold Control	1.25 mM	84.4 (±0.8)	105.9 (±0.7)	89.4 (±1.2)	89.4 (±1.3)
	1.25 mM	83.9 (±1.1)	106.0 (±0.5)	88.9 (±1.5)	88.6 (±1.5)
Warm Ryanodine	1.25 mM	86.2 (±1.1)	104.1 (±0.4)	89.9 (±1.2)	89.9 (±1.2)
	1.25 mM + 10 *u*M RY	86.1 (±0.8)	104.4 (±0.2)	89.9 (±0.7)	89.9 (±0.6)
Warm Control	1.25 mM	87.1 (±1.3)	104.8 (±0.5)	91.3 (±1.1)	91.3 (±1.0)
	1.25 mM	86.0 (±1.4)	104.6 (±0.6)	90.1 (±1.3)	89.8 (±1.3)

Figure 5.1. Effect of ryanodine application (10 nM [hatched] or 10 uM [solid]) on force development of ventricular strips from fish acclimated to either 8 or 18°C. Trial temperature (in °C) is specified on the abscissa. Acclimation temperature is indicated as either cold (left side) or warm (right side). Stimulation frequencies were 0.2 and 0.6 Hz in panels A and B, respectively. In panel C, physiologically-realistic stimulation rates of 1.0 and 1.5 Hz were imposed for ventricular strips from cold- (left) and warm- (right) acclimated fish. Histograms are mean$\pm$SEM and are the results from 5-6 strips.


Decline in Force (% decrease)
80
60
40
20
0
A
0.2 Hz
0.2 Hz
B
0.6 Hz
0.6 Hz
C
1.0 Hz
1.5 Hz
8°C 18°C 8°C 18°C
8°C 18°C 8°C 18°C
Cold-Acclimated
Warm-Acclimated

active tension production while high ryanodine concentration depressed force by 19.1±2.0%. No ryanodine effect on tension development was observed with strips from warm-acclimated fish tested at 18°C and 0.6 Hz (Figure 5.1, Panel B). Using physiologically-appropriate pacing regimes of 1.0 Hz (cold-acclimated) and 1.5 Hz (warm-acclimated), no effect of ryanodine was observed at 18°C in either group. In no instance was resting tension significantly affected by ryanodine treatment ($p > 0.05$).

Calcium concentration-response trials on hearts from warm- and cold-acclimated fish demonstrated performance to decline with decreasing Ca^{2+}_o. In both thermal groups, performance variables (power, cardiac output, stroke volume, heart rate) were only moderately compromised by a 50% reduction in Ca^{2+}_o. Power output, as an example, is presented in Figure 5.2. However, at Ca^{2+}_o levels below 1.25 mM, stroke volume, cardiac output and power were severely depressed and heart rate was significantly increased. Perfusion with 0.5 mM Ca^{2+}_o saline reduced stroke volume, cardiac output and power output to 50-60% of control (pre-exposure) levels while increases in heart rate were modest (< 10% on average) in comparison (Table 5.3).

Absolute values of stroke volume, cardiac output and power output were higher in hearts from warm-acclimated trout (Table 5.3) as a result of the greater work regime imposed. Acclimation temperature did not exert a significant ($p > 0.05$) effect, however, on the relative response of the WHP to reductions in Ca^{2+}_o (Table 5.3). No significant differences were found in relative changes (as a percentage of control values) after seven minutes exposure in any of the parameters examined (Table 5.3).

Hearts were not severely damaged by the Ca^{2+}_o-depletion trials as indicated by recovery profiles (see Figure 5.2 as an example). In all instances, variables returned to within 95% of pre-depletion levels within 7 minutes of re-exposure to

Figure 5.2. The effect of reductions in perfusate calcium on mass specific power output. Acclimation temperature was the same as trial temperature in these experiments. Temperature (warm [18°C]; cold [8°C]) and calcium concentration (1.25, 1.00 or 0.50 mM) are indicated in the legend. Depletion was initialed at time 0. At the arrowhead, the saline being received by the heart was switched from trial saline (1.25, 1.00 or 0.50 mM calcium) to control (2.50 mM calcium) saline. Points are mean values. Standard errors have not been included for reasons of clarity. N=6 in all instances.

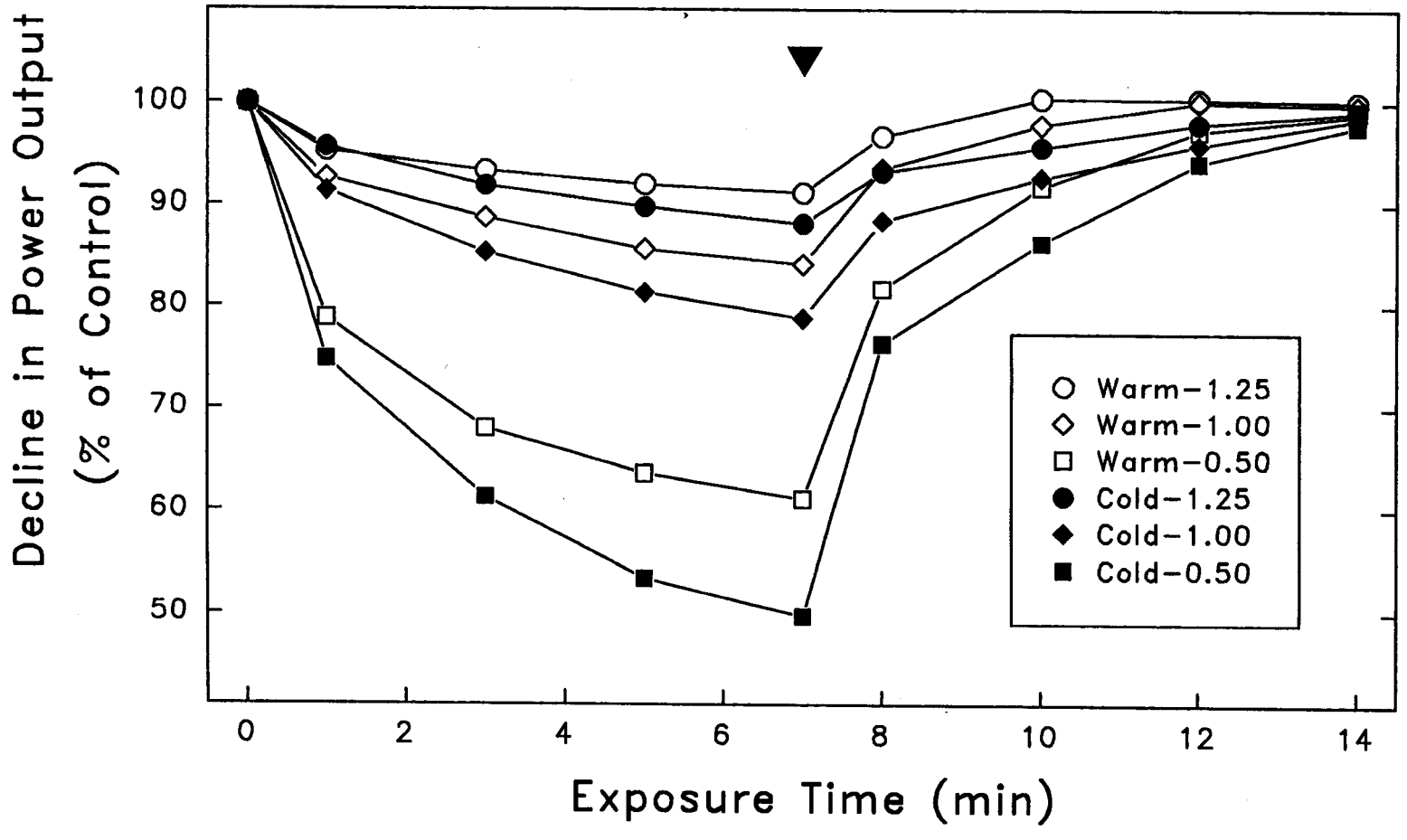

Decline in Power Output
(% of Control)
100
90
80
70
60
50
0
2
4
6
8
10
12
14
Exposure Time (min)
Warm–1.25
Warm–1.00
Warm–0.50
Cold–1.25
Cold–1.00
Cold–0.50

Table 5.3. Effect of seven minute calcium depletion on in situ **cardiac performance of hearts from cold- and warm-acclimated trout.** Data are expressed in absolute values (top panel) and as a percentage of control values. Data are mean (±SEM). N=6 in all groups. Percentages were calculated from absolute values in the following units: stroke volume (SV), $mL \cdot beat^{-1}$; heart rate (f_H), $beats \cdot min^{-1}$; cardiac output ($\dot{Q}$), $mL \cdot min^{-1} \cdot kg^{-1}$ body weight; power, $mW \cdot g^{-1}$ ventricle. No significant ($p > 0.05$) differences were found between groups under similar conditions of Ca_o^{2+} (in mM).

Temp	$[Ca_o^{2+}]$	SV	f_H	$\dot{Q}$	Power
Cold (8°C)	1.25	0.20 (±0.01)	53.9 (±1.0)	23.82 (±0.89)	2.09 (±0.05)
	1.00	0.17 (±0.01)	55.0 (±1.0)	21.22 (±0.84)	1.85 (±0.08)
	0.50	0.11 (±0.02)	55.3 (±1.5)	13.40 (±1.05)	1.15 (±0.13)
Warm (18°C)	1.25	0.33 (±0.03)	81.0 (±2.0)	41.23 (±0.70)	3.97 (±0.19)
	1.00	0.29 (±0.03)	82.8 (±2.1)	37.80 (±0.87)	3.67 (±0.23)
	0.50	0.20 (±0.02)	86.4 (±2.0)	27.00 (±1.88)	2.68 (±0.29)
Cold (8°C)	1.25	85.5 (±1.0)	103.9 (±1.3)	88.8 (±1.0)	88.1 (±0.9)
	1.00	73.9 (±3.3)	107.8 (±2.8)	79.5 (±2.6)	78.8 (±2.6)
	0.50	46.6 (±7.6)	109.6 (±2.2)	50.9 (±5.5)	49.7 (±6.4)
Warm (18°C)	1.25	88.4 (±1.7)	102.6 (±0.9)	90.6 (±1.7)	91.2 (±1.7)
	1.00	79.4 (±2.2)	104.8 (±2.4)	83.2 (±2.5)	84.1 (±2.4)
	0.50	55.0 (±6.4)	109.0 (±0.9)	59.8 (±6.5)	61.1 (±9.4)

control calcium (2.5 mM) saline. Hearts exposed to control saline for a period of time approximating the duration of these experiments (~60 minutes) displayed a slow decline in power generation of similar magnitude (not shown).

Ventricular strip preparations were exposed to a much broader range of extracellular calcium concentrations (0.5-10.0 mM). Force generation increased with increasing Ca^{2+}_o in both acclimation groups tested at both experimental temperatures. The scope of force development, estimated through comparison of developed force at 2.5 mM and 10.0 mM, was not significantly affected ($p > 0.05$) by temperature (Figure 5.3).

Unlike the scope of force development, the sensitivity of ventricular strips to Ca^{2+}_o, estimated through comparison of EC_{50} values (concentration producing one-half of maximal force), was affected by temperature and temperature acclimation (Figure 5.4). EC_{50} estimates of calcium sensitivity of cold- and warm-acclimated ventricular strips were not significantly different when incubated at warm (18°C) temperature. However, incubation at cold (8°C) temperature significantly ($p < 0.05$) decreased the sensitivity of both cold- and warm-acclimated strips to Ca^{2+}_o (increased the EC_{50}). Furthermore, the effect of cold-incubation on Ca^{2+}_o sensitivity was significantly ($p < 0.05$) greater in warm- than in cold-acclimated tissue (Figure 5.4 [inset]).

Resting tension increased as extracellular calcium was increased but represented a significantly ($p < 0.05$) smaller percentage of total tension at 10.0 mM than at 2.5 mM Ca^{2+}_o. No significant differences ($p > 0.05$) in the percent resting tension (as a function of total tension) were observed between acclimation groups as a function of trial temperature; resting tension averaged 17% and 10% of total tension at 2.5 and 10.0 mM Ca^{2+}_o, respectively. The increase in resting tension at 10 mM Ca^{2+}_o, however, relative to that at 2.5 mM Ca^{2+}_o was significantly greater ($p < 0.05$) in warm- than in cold-acclimated trout

Figure 5.3 Effect of trial and acclimation temperature on scope of force development with increasing Ca^{2+}_{o}. Values were calculated from (force @ 10.0 mM)/(force @ 2.5 mM). Strips were stimulated at 0.2 Hz. Histograms are mean+SEM. N=5 in all instances.

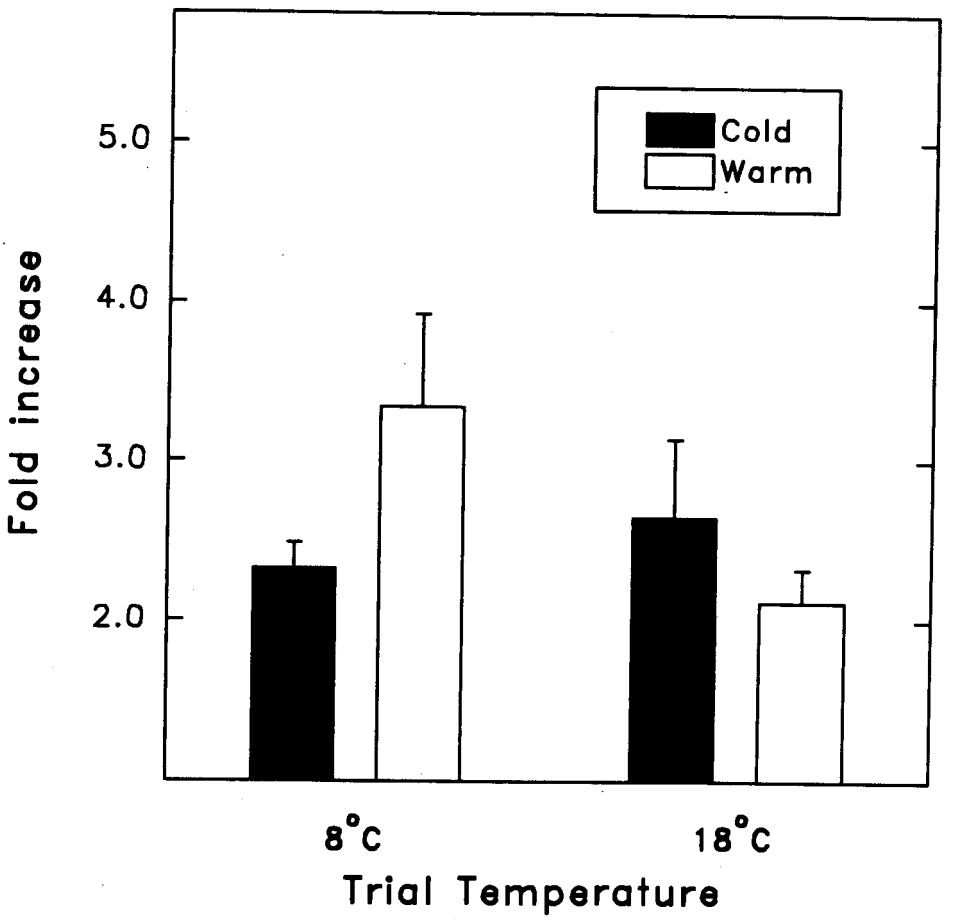
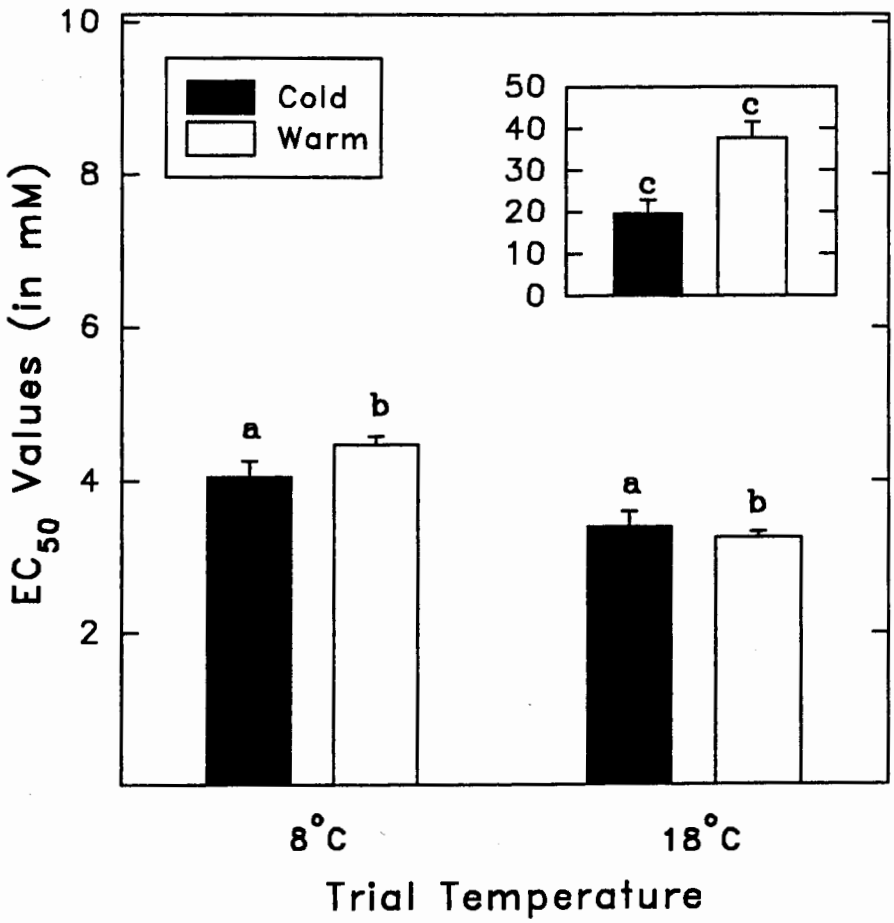

Cold
Warm
5.0
4.0
3.0
2.0
Fold increase
8°C
18°C
Trial Temperature

Figure 5.4. Effect of trial and acclimation temperature on the sensitivity of ventricular strips to Ca^{2+}_o as estimated by EC_{50} values. Histograms are mean+SEM. Significant differences are indicated by paired lower case letters. N=5 in all instances. Inset: The percent increase in EC_{50} at 8°C from that obtained at 18°C. Bar fill in both plots are for strips from cold- (solid) and warm- (open) acclimated trout. Stimulation frequency was 0.2 Hz.


10
8
6
4
2
EC_{50} Values (in mM)
Cold
Warm
50
40
30
20
10
0
c
c
a
b
a
b
8°C
18°C
Trial Temperature

ventricular strips (Figure 5.5) when tested at cold incubation temperature. No significant difference was observed between acclimation groups at warm incubation temperature ($p > 0.05$; Figure 5.5).

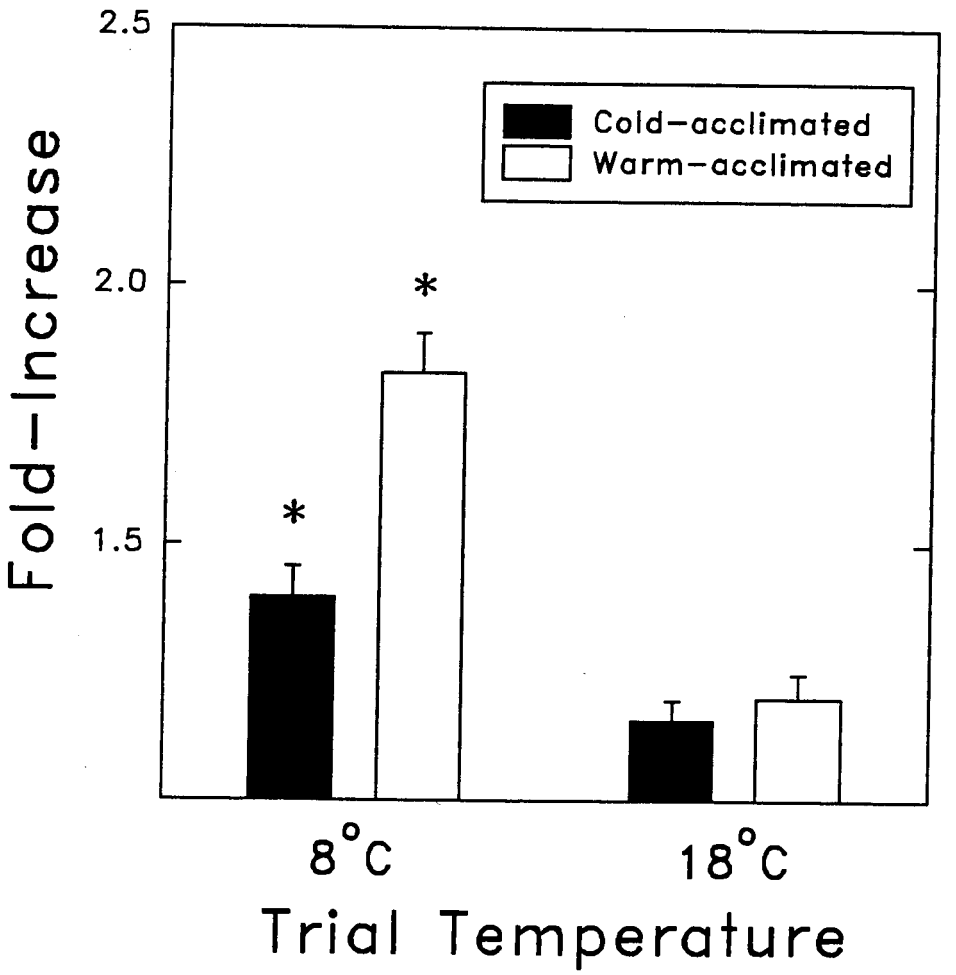
D. Discussion

Previous studies using cardiac tissue from temperate water fishes and elasmobranchs (Driedzic and Gesser, 1988; El-Sayed and Gesser, 1989) have been unable to demonstrate a significant effect of ryanodine on force generation. In agreement with this, ryanodine was not found to affect force generation at 8°C in VSPs at any stimulation frequency or in either acclimation group.

The lack of an effect of ryanodine at cold temperatures in VSPs could be a function of an insufficient incubation period. While incomplete equilibration cannot be ruled out, pilot trials using 10 *u*M ryanodine indicated reductions in twitch tension after 1 hr of incubation to be indistinguishable from reductions in control strips, and are in agreement with another study producing qualitatively similar results using a 45 min incubation period and a temperature of 5°C (L. Hove-Madsen, *pers. comm.*). It is conceivable, however, that the time required to reach equilibrium at 8°C may be substantially greater using lower concentrations (10 nM) of ryanodine.

It is also possible that differences in affinity of the SR calcium release channel for ryanodine exist between species and preclude substantial interaction at the concentrations used in this study. However, radioligand binding of [^{3}H]-ryanodine to enriched microsomal fractions and *in vitro* dose-response curves using ventricular strips (Tibbits *et al.*, 1991) have indicated both the K_d and $K_{1/2}$ of ryanodine in rainbow trout ventricle to be similar to those in mammalian cardiac tissues (Pessah *et al.*, 1985; Shattock and Bers, 1987) at the same

Figure 5.5. Effect of trial and acclimation temperature on the response of resting tension to changes in Ca^{2+}_{o}. Values are expressed as the fold-increase in resting tension at 10.0 mM in relation to that at 2.5 mM Ca^{2+}_{o}. N=5 in all instances and values are mean+SEM. Significant differences are indicated by asterisks.


2.5
2.0
1.5
Fold-Increase
Cold-acclimated
Warm-acclimated
*
*
8°C
18°C
Trial Temperature

temperature (25 °C). It is unlikely, therefore, that species differences in ligand affinity of the SR calcium release channel for ryanodine would have a major impact on the results.

The $K_{1/2}$ is highly temperature sensitive, however, and has been found to increase ~10-fold when temperature is dropped from 25 to 15°C (L. Hove-Madsen, *pers. comm.*). The effect of incubation at temperatures less than 15°C (as used in this study) has not been assessed and may be partially responsible for the lack of an observable response.

At 18°C ryanodine affected both warm- and cold-acclimated strips paced at 0.2 Hz. The thermal dependence of ryanodine action is consistent with that of the gating and conductance properties of the SR calcium-release channel described for mammalian heart tissue (Sitsapesan *et al.*, 1991). Electrophysiological experiments have demonstrated temperature reductions to increase open state probability of the channel and thus overall SR calcium current (Sitsapesan *et al.*, 1991). Cold temperatures, therefore, should promote the SR calcium channel to be in the "open" configuration a greater proportion of the time and, as a consequence, reduce the effectiveness of the SR Ca^{2+}-ATPase in sequestering calcium which might be utilized, through calcium-induced calcium release (Fabiato and Fabiato, 1975), in activation of the contractile apparatus. Ryanodine applied under these conditions would be expected to have no effect on contractility (Tibbits *et al.*, 1991). At warm temperatures, however, the open state probability of the channel is reduced and calcium-sequestration is much more effective. Calcium release from the SR under these conditions could occur and ryanodine, as demonstrated in this study, could exert an effect on contractility. Ventricular strips studies therefore suggest that at warm (18°C) temperatures the SR is able to accumulate and release calcium and to play a role in force development under nonphysiological conditions.

The greater effect of ryanodine on force development (in ventricular strips at 18°C at 0.2 and 0.6 Hz) in cold-acclimated cardiac tissue appears to indicate either a greater releasable pool of calcium to exist or the relative contributions of SL- and SR-derived calcium to force development to be different from that found in warm-acclimated tissue. The former may be accomplished through, among other possibilities, a greater uptake capacity of the SR Ca^{2+}-pump, a greater SR Ca^{2+}-ATPase pump density, a greater amount of organellar SR, or a greater cytoplasmic calcium level during systole (or combinations thereof).

The physiological relevance of a ryanodine-sensitive SR-derived calcium pool to E-C coupling was determined in *WHP-Series A* and *VSP-Series A* (1.0 and 1.5 Hz) trials. There was no effect of ryanodine at either warm or cold temperatures in either experimental series. The underlying reasons for this have yet to be determined but may be an indication that the SR calcium uptake rate during diastole (at physiological heart rates) is insufficient to provide a significant amount of releasable calcium during systole. In contrast to this observation, trials using tuna atrial strips at warm (25°C) temperatures demonstrate ryanodine to have an effect on contractility at both physiological and subphysiological stimulation rates (Keen *et al.*, 1992) and may indicate an important species difference between rainbow trout and tuna in terms of E-C coupling mechanisms.

Calcium depletion experiments in the *WHP-Series B* indicated 1.25 mM Ca^{2+}_o to be "threshold" level for cardiac performance. *In vivo* plasma calcium concentration in freshwater teleosts generally range from 2-4 mM (Holmes and Donaldson, 1969; Ruben and Bennett, 1981; Andreasen, 1985). Assuming 40% of plasma calcium to be bound (Driedzic and Gesser, 1985), it is likely that small variations in plasma calcium concentration could have profound effects on contractile function by increasing sarcolemmal influx per depolarization, particularly if extracellular calcium serves to directly activate the contractile

element as suggested in ryanodine experiments. This threshold level is also in general agreement with previous estimates using a similar preparation (Farrell *et al.*, 1986) in which 1.0 mM Ca^{2+}_o was found to generate cardiac failure within 5-10 minutes. Unlike the results of Farrell *et al.* (1986), however, our preparation did not fail at concentrations below 1.25 mM Ca^{2+}_o. Heart viability in the present study may be related to the higher work level imposed or, more likely, underlies the importance of an intact pericardium (see Chapter II). In the previous study (Farrell *et al.*, 1986), the pericardium was ruptured and the heart open to the bathing saline. The calcium dependency of cardiac function was examined through sequential trials in which maximum power output was determined under maximally volume-loaded conditions. In the absence of an intact pericardium, atrial overdistention could easily occur and affect viability of the preparation.

Thermal acclimation in the *WHP-Series B* did not demonstrate a reduction in extracellular calcium sensitivity with decreasing temperature as was apparent with the *VSP-Series B* experiments. The failure to detect a significant effect of temperature in the *WHP-Series B* trials, as was found in ventricular strip experiments, may be due to the abbreviated range of $[Ca^{2+}_o]$ used in *in situ* trials. Additionally, comparison of "working" power outputs with maximal power outputs (determined at the end of each trial) indicated trials using cold-acclimated fish hearts to represent 40% of maximum power output while warm-acclimated hearts were operating at 52% of maximum. The relationship between intracellular calcium concentration and force developments has been demonstrated to have an associated length-dependent component in mammals. If calcium influx across the SL and cytosolic calcium levels are altered by changes in Ca^{2+}_o in the trout heart, it is possible that in testing hearts from warm- and cold-acclimated fish at different points on the Starling curve (Lakatta, 1986; Babu *et*

al., 1988), differences in calcium sensitivity were equalized.

A decrease in calcium sensitivity with a decrease in temperature has been described for mammalian cardiac tissue, particularly in skinned fiber preparations in which bathing calcium concentrations are equivalent to cytosolic levels (Harrison and Bers, 1989; 1990a,b). In such studies, the intrinsic thermal effect on calcium sensitivity has been demonstrated to be a property of the myofilament apparatus in general, and may be explained in part by troponin C (TnC) interactions. Cooling of mammalian skeletal muscle, unlike cardiac tissue, produces an increase in calcium sensitivity. Replacement of cardiac TnC with skeletal TnC has been shown to reduce the effect of temperature in cardiac muscle (Harrison and Bers, 1990b). Not all of the thermally-induced changes can be accounted for by TnC, however, as a temperature-sensitive component remains even after TnC extraction (Harrison and Bers, 1990b). The relationship between cardiac TnC and cytosolic calcium levels, and the effect of temperature on this relationship, is not known in fishes.

The magnitude of the shift in calcium sensitivity with temperature change found in this study may not be representative of that solely due to calcium interactions alone. A change in temperature also produces a change in pH. The inhibitory effect of temperature decline has been demonstrated to be partially offset by a reduction in proton load (Fabiato and Fabiato, 1978b; Gulati and Babu, 1989) and the shift observed in this study may have been reduced as a consequence. Again, the occurrence of this phenomenon in fishes has not been investigated.

Chronic temperature exposure (Figure 5.4) also produces a shift in sensitivity, cardiac tissue from cold-acclimated fish being less sensitive to changes in extracellular calcium levels than is found in that from warm-acclimated fish. The underlying reasons for this have yet to be determined but it is not

inconceivable that they are related to changes in isoforms of various contractile proteins as has been described for skeletal muscle in a variety of other fishes (Gerlach *et al.*, 1990; Johnston *et al.*, 1990; Johnson and Johnston, 1991).

Peak twitch tension in VSPs increased 2-3 fold with a 4-fold increase in $Ca^{2+}{}_{o}$, independent of acclimation or trial condition. The sensitivity to changes in $Ca^{2+}{}_{o}$, in terms of twitch tension, was thus relatively greater than that found in ventricular strips of the spiny dogfish (*Squalus acanthias*), hagfish (*Myxine glutinosa*) and skipjack tuna (*Katsuwonus pelamis*) but less than that of black dogfish (*Etmopterus spinax*) and little skate (*Raja erinacea*) (Driedzic and Gesser, 1988; Keen *et al.*, 1992).

Resting tension increased as $Ca^{2+}{}_{o}$ increased in all instances. The relative increase, however, was significantly greater in warm- than in cold-acclimated tissue when tested at cold temperature. Increases in resting tension indicate an increase in resting cytosolic calcium level such that calcium remains available to the contractile proteins. Cytosolic calcium levels are regulated by sarcolemmal (Na/Ca exchanger, Ca-ATPase), sarcoplasmic reticular (Ca-ATPase) and mitochondrial (Na/Ca exchanger, Ca-ATPase) proteins. The greater relative increase in warm-acclimated tissue suggests a lesser ability to remove calcium from the cytosol. Whether acclimatory changes occur in the activity or quantity of these Ca pumps/exchangers is not known. However, a shift in calcium handling capacity was indicated in VSP-Series A experiments (0.2 and 0.6 Hz) in which cold-acclimated strips tested at warm temperature were observed to have a greater ryanodine sensitive component, and thus SR contribution, to contractility. The role this component could play in cytosolic calcium regulation during diastole is difficult to envision, particularly as this component is lost with incubation at cold temperature.

In summary, decreases in environmental temperature have a profound effect

on overall cardiac performance. At low temperature, the sensitivity of ventricular tissue to extracellular calcium is reduced and, *in vivo*, may have an effect on cardiac performance. Chronic exposure to cold-temperatures, however, provides some improvement in extracellular calcium sensitivity. The larger ryanodine sensitive component of contractility found at slow stimulation frequencies in cold-acclimated trout at warm temperatures suggests a relatively greater SR calcium handling capability than warm-acclimated ventricular tissue. *In situ* preparations and ventricular strips at high pacing frequencies, however, do not indicate the SR to contribute to force generation under physiologically-relevant conditions. The role of the SR in regulation of cytoplasmic calcium levels during diastole (as well as calcium transport systems of the SL and mitochondria) remains to be determined. Colder temperatures would be expected to increase the duration of the action potential and, coupled with a putative increase in DHP-sensitive L-type calcium channel density after acclimation to cold temperatures (G. Tibbits, *pers. comm.*), a greater influx of calcium across the SL might be expected in the hearts of cold-acclimated fishes. This is also consistent with adaptations expected to offset the reduced calcium sensitivity of heart tissue from cold-acclimated hearts.

CHAPTER VI

SUMMARY

This thesis examined the general effects of temperature on cardiac function in rainbow trout, and specifically those effects relating to beta-adrenergic responsiveness. The major findings, presented in relation to the initial stated objectives (Chapter I), were as follows:

Objective 1. To determine if the thermal dependence of maximum aerobic swimming capacity and cardiac performance are similar in trout (Chapter II).

Findings:

1. Maximum prolonged swimming speed increased with increasing ambient water temperature.

2. Maximum *in situ* cardiac power output increased with temperature.

3. Stroke volume decreased with increased temperature and, in part, offset increased heart rate.

4. The Q_{10} values for U_{crit} and maximum *in situ* cardiac performance were found to be similar and suggested that swimming speed is closely related to intrinsic heart performance.

Objective 2. To determine if beta-adrenergic sensitivity of the trout heart is altered by acclimation temperature (Chapter III).

Findings:

1. Acclimation to cold temperatures increased the sensitivity of the *in situ* and *in vitro* heart to beta-adrenergic stimulation.

2. After cold-acclimation, maximal beta-adrenergic stimulation produced a greater relative increase in *in situ* power output but not in *in vitro* force generation.

Objective 3. To determine if the change in beta-adrenergic sensitivity of the trout heart that occurs with thermal acclimation is correlated with alteration of components involved in the beta-adrenergic signal transduction pathway (Chapter III and IV).

Findings:

1. Cold-acclimated hearts had a greater surface beta-adrenoceptor population than warm-acclimated hearts.

2. Basal adenylate cyclase activity was greater in cold-acclimated heart tissue.

3. Adrenaline application to cold-acclimated heart tissue, in the presence of GTP, produced both a greater maximal stimulation of adenylate cyclase and initially stimulated adenylate cyclase activity at a lower adrenaline concentration than in warm-acclimated tissue.

4. Cold-acclimation did not change the stimulatory ability of either fluoride and forskolin on adenylate cyclase, or GTP and GTP-gamma-S on G_s protein.

Objective 4. To examine the role of the SR in myocardial E-C coupling in the trout and the effect of temperature on this role (Chapter V).

Findings:

1. No significant role was found for SR calcium release in E-C coupling in either cold- or warm-acclimated trout hearts. Ryanodine had no significant effect on either *in situ* cardiac performance or on *in vitro* force development (provided

pacing frequency was greater than or equal to 1.0 Hz).

2. Ryanodine produced a moderate decline in force production at 18°C provided the pacing frequency was less than or equal to 0.6 Hz. This effect was significantly greater in cold- than in warm-acclimated ventricular strips at a pacing frequency of 0.2 Hz but not at 0.6 Hz.

3. *In situ* calcium depletion below 1.25 mM severely reduced cardiac performance. No significant differences were observed, however, between warm- and cold-acclimated hearts.

4. In contrast, the sensitivity of ventricular strips to calcium (EC_{50}) was decreased at cold test temperature. This effect, however, was significantly lower in cold-acclimated tissue.

5. Resting tension increased to a greater extent when faced by a 10 mM $Ca^{2+}{}_{o}$ challenge in warm- than in cold-acclimated ventricular strips when tested at cold temperature.

Swimming in fishes is brought about through the interaction of numerous neural, mechanical and cardiovascular components, many of which are highly temperature dependent. An acute reduction in temperature reduces axonal conduction velocities, decreases the refractory period and decreases the amplitude of the action potential. Skeletal muscle (mechanical component) is affected by temperature such that a decrease in temperature reduces the maximum velocity of shortening and power generation.

Cardiac function is also affected by acute decreases in temperature. As demonstrated in this Thesis (Chapters II, III) and elsewhere, cardiac output is highly temperature dependent. Decreased temperature produces a large reduction in heart rate, and thus cardiac output, and in maximum force development, which in part reflects a decrease in the calcium sensitivity of the

ventricular tissue (Chapter V).

Intrinsic mechanisms exist, however, which partially offset the effects of temperature on performance. In cardiac tissue, the duration of the action potential is prolonged, as are the open times of membrane channels. Additional mechanisms also exist in cardiovascular design which partially compensate for thermal declines. The reduction in heart rate, and thus cardiac output, generated by a decrease in temperature, produces an increase in the filling time of the atrium and thus an increase in stroke volume which partially offsets the thermal dependency of beat frequency (Chapter II). At the myofilament level, the reduction in calcium sensitivity induced by a decrease in temperature may be partially offset by concomitant increases in pH which, in mammals, increases the calcium sensitivity of the myofilaments.

While these (and other) mechanisms ameliorate swimming and systemic performance after an acute temperature decrease, compensation is incomplete and performance remains reduced at low temperatures (Chapter II). Therefore, with the onset of cold temperatures, rainbow trout must either avoid cold waters (migrate) or, as demonstrated in this thesis and elsewhere, undergo adaptations which permit the maintenance of a high activity pattern despite thermal limitations and augment existing intrinsic compensatory mechanisms.

These mechanisms were found to occur both *in vivo* and *in vitro* and, in a general sense, appear to confer a degree of thermal independence at low temperatures. Acute temperature change was found to produce a greater effect upon the U_{crit} of warm-acclimated fish than of cold-acclimated (Chapter II). Acclimation to cold-temperatures thus altered swimming performance in such a way that thermal-dependency was reduced (Chapter II). Similar effects must occur in the cardiovascular system in order to maintain a matching between swimming and cardiac performance. Stroke volume has only a limited thermal-

dependency and thermal effects on cardiac output are largely the result of changes in heart rate (Chapter II). The acute Q_{10} of heart rate has been previously demonstrated to be about 2.0 and, despite the compensating influence of an increased stroke volume at cold-temperatures, cardiac output is substantially depressed. With acclimation however, heart rate increases such that the Q_{10} of warm- and cold-acclimated trout hearts is reduced to ~1.5 (Chapter II) and cardiac output is increased as a consequence. A similar reduction of thermal dependency was observed in force production in ventricular strips. While the sensitivity to extracellular calcium was increased upon incubation at warm (18°C) temperatures in both warm- and cold-acclimated strips, the magnitude of the sensitivity shift was significantly reduced in cold-acclimated tissues (Chapter V).

These elements operate to reduce the effects of temperature change and should result in maintenance of a tight relationship between maximal aerobic cardiac and swimming capacities at all acclimation temperatures. Analysis of *in situ* heart performance indicated a close relationship to exist between cardiac and swimming performance (Chapter II). It was therefore concluded that intrinsic *in situ* heart performance is nearly sufficient to fully meet the demands presumed to exist at U_{crit} but other factors which influence the heart *in vivo* likely play a role in augmenting cardiac function.

At U_{crit}, beta-adrenergic stimulation of the heart (and other tissues) likely contributes to improvement of performance and, based on studies using *in situ* heart preparations, should affect the Q_{10} of cardiac performance such that maximum cardiac and swimming capacity should be even more closely matched. Cold-acclimation was demonstrated to produce a shift in sensitivity such that at low temperatures, the heart responded to a lower adrenaline concentration (Chapter III). The relative increase in power output (scope) was also determined to be greater in cold-acclimated *in situ* heart preparations (Chapter III). The

acclimatory shift in adrenergic sensitivity is produced by at least two principle subcellular modifications. Radioligand binding studies revealed the population of surface (sarcolemmal) adrenoceptors to be more than 2 times greater in cold-acclimated cardiac tissue (Chapter III). Additionally, biochemical analysis of the transduction mechanisms responsible for converting the binding of beta-adrenergic hormone into a cytosolic response indicated basal adenylate cyclase activity to be significantly greater in cold-acclimated ventricular tissue (Chapter IV). Beta-adrenergic agonists, via interaction with G_s protein and indirect activation of adenylate cyclase activity, catalyze the phosphorylation of the L-type calcium channel and thus increase transsarcolemmal calcium influx. The insensitivity of the trout ventricular strips to ryanodine at physiologically-realistic stimulation frequencies, regardless of acclimation temperature, suggests the trout heart to have a greater dependency on transsarcolemmal calcium in the direct activation of force generation than is found in mammals. The greater density of L-type calcium channels found in cold-acclimated tissues (G.F. Tibbits, *pers. comm.*), in conjunction with an assumed cold-induced prolongation of the action potential and mean open state of membrane channels, further indicates that a shift in beta-adrenergic sensitivity, in conjunction with intrinsic mechanisms, represents an attractive mechanism to compensate for the effect of cold temperatures on cardiac, and thus swimming, performance.

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Appendix A

This is an example of the determination of the number of sites·cell^{-1} for SL. From the K^+-*p*NPPase activity derived yield and recovery for B8-1, we calculate a SL content of 9.62 mg protein·g^{-1} wet weight. From the B_{max} and an assumed stoichiometry of 1 ^{125}I-ICYP binding per beta-receptor, we determine 74.85 sites·pg^{-1} SL. Assuming cardiac myocytes to approximate a right cylinder of 86.6 *u*m (length) and 8.3 *u*m (diameter), and a specific gravity of 1.06, we estimate a cell wet weight of 5.3 ng and a SL content of 64.43 pg SL·cell^{-1}. This produces an estimated density of 4823 sites·cell^{-1}. Assuming caveolae to increase surface area by 50% we calculate a SL surface area of 3550 *u*m^2 and a beta-adrenoceptor surface density of 1.36 sites·*u*m^{-2}. Similarly, utilizing a homogenate yield of 101.7 mg protein·g^{-1} wet weight we calculate a total receptor population of 11821 sites·cell^{-1}. Intracellular receptor density is thus estimated as 6998 sites·cell^{-1} (59.2%) and surface receptors to represent 40.8% of the total receptor population.

APPENDIX B

Other published or accepted submissions resulting from work conducted during my Ph.D. candidacy

Keen, J.E., Farrell, A.P., Tibbits, G.F. and R.W. Brill. 1992. Cardiac dynamics in tunas. II. Effect of ryanodine, calcium and adrenaline on force-frequency relationships in atrial strips from skipjack tuna, *Katsuwonus pelamis*. Can. J. Zool. (In press).

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