THE RELATIVE IMPORTANCE OF THE SR IN CONTRIBUTING Ca^{2+} TO FORCE DEVELOPMENT IN ISOLATED VENTRICULAR TRABECULAE FROM RAINBOW TROUT (*Oncorhynchus mykiss*): THE EFFECTS OF TEMPERATURE, ADRENALINE AND RYANODINE.

by

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THE RELATIVE IMPORTANCE OF THE SR IN CONTRIBUTING Ca^{2+} TO FORCE DEVELOPMENT IN ISOLATED VENTRICULAR TRABECULAE FROM RAINBOW TROUT (*Oncorhynchus mykiss*): THE EFFECTS OF TEMPERATURE, ADRENALINE, AND RYANODINE.

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ABSTRACT

The sarcoplasmic reticulum (SR) is central in regulating intracellular Ca^{2+} during excitation-contraction (E-C) coupling in mammalian cardiac tissue. The role of the SR in E-C coupling in lower vertebrates is less certain. This uncertainty can be partially attributed to the temperature dependency of the Ca^{2+}-release channel and to the varying ryanodine sensitivity of ectotherm cardiac muscle. Additionally, adrenaline (AD) is known to influence sarcolemmal (SL) (extracellular) Ca^{2+} influx in lower vertebrates, further obscuring the relative significance of SR (intracellular) Ca^{2+} contribution. As such, the objective of this thesis was to assesses the relative importance of the SR versus the SL in contributing Ca^{2+} to force development in isolated ventricular trabeculae from rainbow trout performing both isometric and oscillatory contractions. Ryanodine, a noted blocker of SR Ca^{2+} release in mammals, was used to assess SR involvement. Additionally, adrenergic stimulation was used to assess the effect of modulating sarcolemmal Ca^{2+} flux, on the relative importance of SR Ca^{2+} contribution. The muscles were tested to two temperatures (12°C and 22°C) with and without acclimation to determine the modulating effect of temperature. Further, unlike previous studies, a tonic level (10 nM) of adrenaline was used as a control to better approximate the *in vivo* Ca^{2+} availability to the muscle.

The SR Ca^{2+}-release channel in rainbow trout was temperature dependent, being more operative at 22°C than 12°C and displaying a greater sensitivity to test temperature than to acclimation temperature. At 22°C, ryanodine caused significant reductions in peak tension over a range of pacing frequencies (40-50% at 0.2 Hz and 25-30% at 1.2-2.0 Hz), including physiologically realistic pacing frequencies. This suggests significant, but secondary, involvement of SR Ca^{2+} in force development under acute exposure to warm temperatures. At a colder test temperatures (12°C), ryanodine sensitivity is observed only at low, sub-physiological (0.2 Hz) pacing frequencies regardless of temperature

acclimation status. This finding suggests that at lower temperatures the involvement of the SR is not physiologically important and that the sarcolemma plays the primary role in supplying Ca^{2+} to the contractile elements of the fish heart. At both warm and cold test and acclimation temperatures the positive inotropy associated with 10 μM AD ameliorated the negative effects of ryanodine, demonstrating that the Ca^{2+} contribution from the SR can be overwhelmed by adrenergic stimulation of SL Ca^{2+} influx.

Additionally, results from the muscles performing oscillatory contractions suggest that at 12°C, the optimum frequency for power production is between 0.8 Hz and 1.2 Hz. This optimum frequency appears to be ryanodine-sensitive, a finding which may provide insight into a possible link between SR Ca^{2+} recruitment and cardiac performance at high temperatures and high cardiac frequencies.

Thus, unlike mammals, E-C coupling in trout predominantly relies on transsarcolemmal Ca^{2+} influx with SR-Ca^{2+} playing a significant secondary role at 22°C but not 12°C. Regardless of temperature, this secondary role can be easily overwhelmed by increased Ca^{2+} influx upon adrenergic stimulation of the sarcolemma. Certainly this relationship between the relative predominance of SL versus SR Ca^{2+} flux observed in trout, may vary in stenothermic warm-water fish, whose body temperatures are more compatible with those of mammals.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

General Introduction and Literature Review

The purpose of this thesis is to investigate the influence of temperature and adrenaline on the relative importance of the sarcoplasmic reticulum (SR) and the sarcolemma (SL) in mediating Ca^{2+} delivery to the myofilaments for force development in the ventricular muscle of rainbow trout. Over the last decade, an extensive body of research has developed concerning the mechanistic and physiological consequences of temperature change and adrenergic stimulation on excitation-contraction coupling and cardiac contractility in mammals, and, to a lesser extent in lower vertebrates. Therefore, the first chapter will be devoted to a literature review establishing the framework for this thesis.

Topics Discussed:

- Cardiac muscle excitation-contraction coupling and the involvement of the SR-Ca^{2+}-release channel (ryanodine receptor)
- The influence of temperature and temperature acclimation on cardiac contractility and the SR-Ca^{2+}-release channel
- The role of adrenaline in modulating cardiac contractility and transsarcolemmal Ca^{2+} influx
- Force-frequency relationships in cardiac muscle
- Oscillating muscle preparations

Cardiac Muscle Excitation-Contraction Coupling and the Involvement of the SR-Ca^{2+}-Release Channel (Ryanodine Receptor)

Contractility is determined primarily by the magnitude and rate of Ca^{2+} delivery to the myofilaments (Tibbits *et al.*, 1991). The process by which Ca^{2+} is delivered to the contractile elements and initiates contraction, is referred to as excitation-contraction (E-C) coupling. The Ca^{2+} which activates the contractile elements can be of both intracellular and extracellular origin depending upon the vertebrate group examined. Mammals primarily rely on intracellular Ca^{2+} stores (SR) for contraction whereas many lower vertebrates bring Ca^{2+} across the cell membrane (SL) with each pulse. As such, two general models for E-C coupling have been suggested for the cardiac muscle of mammals and lower vertebrates respectively. In mammals, the action potential (AP) is conducted deep within the cell via invaginations in the sarcolemmal membrane known as transverse tubules (T-tubules). The terminus of the T-tubules contain voltage gated L-type channels which are responsible for the inward flow of Ca^{2+} from the extracellular space. These T-tubules terminate at the terminal cisternae of the SR, where the SR-Ca^{2+}-release channels are located. The SR-Ca^{2+}-release channel, or ryanodine receptor, is responsible for controlling the release of Ca^{2+} stored within the SR. When the SL membrane is depolarized, a relatively small Ca^{2+} influx occurs through the L-type channel, triggering the release of a much greater pool of Ca^{2+} from the SR, through a process known as 'Ca^{2+}-induced Ca^{2+}-release' (Fabiato, 1983). Thus in mammals, transsarcolemmal Ca^{2+} influx is small, playing a minor role in the direct activation of the myofilaments, but is essential for triggering the release of activator Ca^{2+} from the SR.

In mammals, the Ca^{2+} released from the SR is essential for E-C coupling. However, despite this central role, there exists a graded dependence on SR-Ca^{2+} release among mammals. This gradation is apparent among different species (Sutko and Willerson, 1980), and at different stages of development (Fabiato and Fabiato, 1978; Nakanishi and

Jarmakani, 1984; Tanaka, and Shigenobu, 1989), as well as regionally within the heart (Bers, 1991). This is demonstrated by the varying response of rabbit and rat ventricular muscle to ryanodine, a powerful tool for assessing SR involvement. For example, ryanodine causes force to decline by ~20% in rabbit muscle, which is considerably less than the ~87% reduction in force observed in adult rat muscle (Bers, 1985). These varying responses to ryanodine suggest large differences in SR dependence, and are supported by ultrastructure correlates, where the adult rat ventricle demonstrates a much more highly developed SR than the rabbit (Bers, 1991). Within a species, SR dependence can be life stage specific. Ventricular muscle from neonatal rats show only a 55% reduction in force after ryanodine treatment compared with an ~87% reduction in force in the adult rat (Bers, 1991). This observation can be correlated with the ability to experimentally stimulate Ca^{2+}-induced Ca^{2+}-release. For example, Ca^{2+}-induced Ca^{2+}-release could not be observed in prenatal rat ventricle, where SR involvement is small, but was prominent in the adult rat ventricle which relies heavily on Ca^{2+} released from the SR. This developmental transition of relative SR dependence is also in accordance with ultrastructure studies which indicate the T-tubule/SR system is gradually developing from the prenatal stage through to the first weeks of life (Bers, 1991; Legato, 1979). Additionally, within a given species, atrial muscle seems to be more dependent on SR-Ca^{2+} release than ventricular muscle as demonstrated by varying ryanodine sensitivity. Force development in rabbit atrium declines by ~50% after ryanodine treatment compared with a 20% decline in rabbit ventricle (Bers, 1991). This difference may be partially attributed to the shorter action potential duration (APD) observed in atrial muscle compared with ventricular muscle, such that less Ca^{2+} enters via Ca^{2+} channels (or Na^{+}/Ca^{2+} exchange). Therefore, even within the mammalian world, the relative importance of the SR in contributing Ca^{2+} to force development can vary.

The identification of ryanodine as a specific and irreversible ligand for the SR-Ca^{2+}-release channel has lead to significant advancements in the study of E-C coupling in both skeletal and cardiac muscle. Other agents have also be used in assessing the role of the SR, but can cause side effects. For example, though caffeine is known to inhibit SR-Ca^{2+} uptake, it also increases myofilament Ca^{2+} sensitivity and Ca^{2+} influx (Bers, 1991). Ryanodine's actions are more specific. At low concentrations (nM), ryanodine locks the SR-Ca^{2+}-release channel in a open, subconducting state, leading to increased Ca^{2+} flux from the SR. In cardiac muscle, the Ca^{2+} lost from the SR can be extruded by the powerful Na^{+}/Ca^{2+} exchange system in the sarcolemma, thereby "unloading" Ca^{2+} from the SR and out of the cell. At higher concentrations (μM), ryanodine has been reported to cause irreversible channel closure. This may be a result of ryanodine binding to low affinity sites on the receptor (Tinker *et al.*, 1996). In either case, the SR of cardiac muscle treated with ryanodine, is ineffective in sequestering and releasing Ca^{2+}, which explains the observed reduction in force (Bers, 1991). Thus ryanodine, has become the single most useful tool for assessing the relative importance of the SR in E-C coupling.

The model of E-C coupling adopted for teleosts and other lower vertebrates, suggests a reduced dependence on intracellular (SR) stores for delivery of Ca^{2+} to the contractile elements. Indeed, it is suggested that the majority of activator Ca^{2+} crosses the SL membrane with each pulse (Tibbits *et al.*, 1990). This model is supported by direct and indirect observations. Relative to mammals, electron micrograph studies of frogs and many fish indicate sparse development of SR, however some fish, such as trout and mackerel (*Scomber scombrus*), display a much more abundant complement of SR (Santer, 1985). Additionally, electron microscopy indicates the lack of a T-tubular system in the hearts of fish, amphibians and reptiles (Bossen and Sommer, 1984; Santer, 1985). These findings provide strong morphological evidence to support a reduced role for SR Ca^{2+} in lower vertebrates compared with mammals. A less direct line of evidence can be drawn by

comparing the diameter of mammalian and lower vertebrate myocytes. The smaller diameter of the trout myocyte (~8 μM; Farrell *et al*, 1988) provides a higher sarcolemmal surface to cystolic volume than the mammalian myocyte (15-20 μM; Sommer and Jennings, 1986), which results in a higher capacity to deliver Ca^{2+} to the myofilaments via transsarcolemmal influx alone (Tibbits *et al.*, 1990). There is strong evidence from amphibian myocytes (~4 μM), which are smaller than those of both teleosts and mammals, to support this contention (Klitzner and Morad, 1983; Bers, 1985). Nevertheless, biochemical studies assessing SR activity report considerably higher Ca^{2+} uptake rates in trout cardiac muscle (10°C) than in frog (10°C) or rat (37°C) at their respective physiological temperatures (McArdle and Johnston, 1981). Dybro and Gesser (1986) also found the SR of trout (15°C) to be functionally well developed with crude ventricular homogenates showing greater Ca^{2+} uptake and greater Ca^{2+} uptake rates than turtle (25°C), flounder (15°C), or frog (15°C). In any case, the most compelling evidence for the lack of SR involvement in E-C coupling in lower vertebrates is the absence of a response to ryanodine in the small number of species studied to date (Driedzic and Gesser, 1988; Hove-Madsen and Gesser, 1989; Vornanen, 1996). These studies are reviewed below.

Whereas the treatment of adult mammalian hearts with ryanodine typically results in decreased twitch tension, this is not the case for most teleosts. In fish, the responsiveness to ryanodine treatment varies with temperature and pacing frequency. Ventricular strips from sea raven (*Hemitripterus americanus*) and cod (*Gadus morhua*) showed no change in force after ryanodine treatment at 10°C, irrespective of pacing frequency (Driedzic and Gesser, 1988). At 15°C, force development in ventricular strips from rainbow trout and plaice (*Pleuronectes flesus*) were also unresponsive to ryanodine even at the lowest pacing frequencies (El-Sayed and Gesser, 1989), though ryanodine abolished post-rest potentiations in trout (El-Sayed and Gesser, 1989; Hove-Madsen and Gesser, 1989) (post-rest potentiations are discussed under the heading 'Force-Frequency Response'). Møller-

Nielsen and Gesser (1992) concluded that at physiological pacing frequencies, ryanodine did not significantly effect force development in ventricular strips from rainbow trout at 10°C or 20°C. Keen *et al.* (1994) reached the same conclusion using ventricular strips and *in situ* heart preparations acclimated and tested at 8°C and 18°C. Further, Vornanen (1994, 1996) came to the same conclusion for crucian carp (*Carassius carassius*) at 12°C. The culmination of these studies is that there is no substantial reduction in contractile force at physiologically relevant pacing frequencies when ryanodine is applied to teleost ventricular strips or *in situ* perfused hearts at temperatures between 5° and 20°C.

At high experimental temperatures ryanodine can elicit a response in teleosts at sub-physiological frequencies (0.2 Hz). In the aforementioned study with ventricular muscle from rainbow trout by Møller-Nielsen and Gesser (1992), no reduction in force was observed at 10°C regardless of pacing frequency, however, at 20°C, ryanodine caused a 32% reduction in force at 0.2 Hz. Likewise, at 0.2 Hz, Keen *et al.* (1994), found a 60% reduction in force in rainbow trout ventricular strips after ryanodine treatment at 18°C but not 8°C. Additionally, Hove-Madsen (1992) noticed that ryanodine abolished post-rest potentiations at 0.2 Hz and at 25°C in trout myocardium but not at higher pacing frequencies or lower temperatures.

This temperature dependency of the ryanodine response in teleosts is in accordance with studies on the temperature dependency of the SR-Ca^{2+}-release-channel in mammals (Bers, 1987, 1989, Sitsapesan *et al.*, 1991). In mammals, as temperature decreases, the open-state probability of the SR-Ca^{2+}-release channel increases, making the SR less able to effectively sequester and release Ca^{2+} during contraction. If the SR-Ca^{2+}-release channel is also temperature dependent in lower vertebrates, then the failure of most teleosts to demonstrate a ryanodine response may merely be a result of the temperatures at which they are tested (Tibbits *et al.*, 1991; Tibbits *et al.*, 1992a; Keen *et al.*, 1992, 1994). In support

of this idea, a study with isolated atrial strips from skipjack tuna at 25°C, demonstrated a 30% decrease in contractile force after ryanodine treatment over the entire range of physiological pacing frequencies, suggesting that in this species, the SR is active in contributing a significant proportion of the activator Ca^{2+} to E-C coupling (Keen *et al.*, 1992). This was the first example of a teleost exhibiting a ryanodine response at physiological pacing frequencies.

The more mammalian-like role of the SR in the skipjack tuna separates it from other teleosts studied to date. Indeed, the tuna is already separate from other teleosts because (1) they demonstrate swimming heart rates of 154 bpm (Farrell *et al.*, 1992) exceeding the 120 bpm maximum observed for other fish (Farrell, 1991), and (2) they routinely occupy warm waters of approximately 25°C while most other work has been conducted on more temperate-water species (5°-15°C). Even rainbow trout has an upper incipient lethal temperature (UILT) of ~25°C and rarely survives temperatures greater than 22°C for a prolonged period of time. The temperature at which the animal lives is of considerable importance with respect to the thermal sensitivity of the SR-Ca^{2+}-release channel in mammals. At 25°C, the SR-Ca^{2+}-release channel may be functional in the skipjack tuna, allowing for significant SR-Ca^{2+} contribution during contraction. The fact that skipjack tuna have the highest maximal heart rates observed in lower vertebrates may suggest that by recruiting activator Ca^{2+} from a source (SR) closer to the myofilaments, thereby allowing faster Ca^{2+} delivery to and from the myofilaments, this ectotherm to is able to increase the rate of its cardiac cycle. This idea is supported by the observation that in addition to reducing maximum force, ryanodine also depressed the maximum attainable stimulation frequency by ~0.4 Hz in the skipjack tuna (Keen *et al.*, 1992).

Therefore, there is a broad range of responsiveness to ryanodine in fish, indicating that like mammals, the utilization of SR-Ca^{2+} varies between species. It remains unclear whether

these differences are primarily species dependent, as is the case with mammals, or whether the varied SR involvement is related to the temperature at which the animal lives. The lack of a significant ryanodine response at physiological pacing frequencies in studies with rainbow trout at 8°C and 18°C (Keen *et al.*, 1994), and 10°C and 20°C (Møller-Nielsen and Gesser, 1992) suggest that either these temperatures were not warm enough to induce adequate activation of the SR-Ca^{2+}-release channel, or that there are fundamental differences in the mechanisms behind E-C coupling in trout and tuna, which allow tuna to utilize SR-Ca^{2+} but not trout. This thesis examines the effect of acute and chronic exposure to 12°C and 22°C on the involvement of the SR in E-C coupling in rainbow trout. 22°C is approaching the UILT for this species, as salmonids are generally considered stenothermal with UILT values in the range of 20-26°C with a preferred temperature of around 15°C (Black, 1953; Brett, 1971). Therefore, if temperature is the variable upon which SR utilization depends, then 22°C is probably the highest temperature for trout, (keeping within a physiologically realistic range) at which one might expect the recruitment of SR-Ca^{2+} to routinely occur. Additionally, SR involvement in fish appears to be frequency dependent with *in vitro* studies indicating increased SR involvement at low frequencies and high temperatures. However, *in vivo*, heart rate increases with increased temperature, therefore if the SR is to be of physiological importance, the animal's ability to utilize SR-Ca^{2+} must extend to high contraction frequencies. Indeed, perhaps it is the ability to recruit SR-Ca^{2+} at the high contraction frequencies associated with high temperatures in tuna, and the inability in trout, that accounts for the disparity between the maximal heart rates in these teleosts. Whether the inability to utilize SR-Ca^{2+} at high frequencies limits the maximum attainable heart rates in lower vertebrates is unknown. This thesis probes some of these fascinating questions.

The Influence of Temperature and Temperature Acclimation on Cardiac Contractility and the SR-Ca^{2+}-Release Channel

Temperature is an important and all-pervasive factor that has profound effects on the physiological and biochemical processes of animals. This is especially true of ectothermic vertebrates whose thermal balance is predominated by external sources of heat. At its most elementary level, temperature dictates the thermal kinetic energy of molecules, and as such, is the prime determinant of physiological, chemical and biochemical reaction rates (Withers, 1992). It is not surprising then that temperature strongly influences the contractile performance of skeletal and cardiac muscle in both fish and mammals (Bennett, 1984; Farrell, 1984; Rall and Woledge, 1990) in part through a profound impact on the dynamics of E-C coupling (Blinks and Koch-Weser, 1963; Tibbits *et al.*, 1992a; Vornanen, 1989, 1994, 1996; Hove-Madsen, 1992, Keen *et al.*, 1994). However, physiological processes of animals are not entirely at the mercy of their thermal environment because the reaction rates of these processes can be adjusted to compensate for variations in temperature. Such compensation is called *acclimatization* if it occurs in nature, and *acclimation* if it is induced in the laboratory.

The ability to acclimatize is of particular importance for fish of temperate regions who are susceptible to large changes in environmental temperature. In addition to seasonal changes, many fish encounter acute temperature gradients. Indeed, observations of salmon (Brett, 1971), blue marlin (Block *et al.*, 1992) and tuna (Holland, *et al*, 1990; Block *et al*, 1996) show that fish transcend thermoclines in both lakes and oceans while foraging or escaping predation. In such circumstances, proper functioning of both skeletal and cardiac muscle over a range of temperatures, independent of acclimatized temperature, is essential for survival. Consequently, many fish alleviate the adverse effects of temperature by changing the metabolic, contractile or morphological properties of cardiac muscle (Matikainen and

Vornanen, 1992). These temperature-induced changes are complex and apparent at all levels of organization, from varying heart rate (Graham and Farrell, 1989), and isometric force development (Bailey and Driedzic, 1990) to altering myofibrillar protein structure (Vornanen, 1994).

Influence of Temperature on Heart Rate and Contraction Kinetics

Temperature is the single most important determinant of heart rate in fish. Indeed, intrinsic pacemaker rate varies directly with temperature (Farrell and Jones, 1992) as it affects the membrane permeability of pacemaker fibres (Randall, 1970). Moreover, many studies have suggested the Q_{10} for heart rate is 2.0 or greater. For example, in *in vitro* perfused working sea raven hearts, the Q_{10} is 2.0 (Graham and Farrell, 1985) and is 2.0-2.3 in non-working isolated goldfish hearts (*Carassius auratus*) (Tsukuda *et al*, 1985). Compensatory changes in heart rate have been demonstrated in several fish after cold-acclimation, such that cold-acclimated fish have higher pacemaker rates than warm-acclimated fish (goldfish, Tsukuda *et al*, 1985; trout, Graham and Farrell, 1989; sole (*Solea vulgaris*), Sureau *et al.*, 1989; and European eel (*Anguilla anguilla*), Seibert, 1979). In perch (*Perca fluviatilis*), Bowler and Tirri, (1990) found low temperature acclimation resulted in an increase in the volume density of SR, as well as an increased ability to maintain higher rates of contraction at lower temperatures. The increase in SR may be related to the shortening of the activation and relaxation phases of cardiac contraction, and the subsequent higher maximal contraction frequencies (Driedzic and Gesser, 1994), however such a conclusion presumes SR involvement which has yet to be demonstrated at these temperatures.

Warm temperature acclimation can increase the upper limit of the pacemaker rate (Tsukuda *et al*, 1985) which could result in heart rate increasing with temperature up to the UILT. However, even in warm-acclimated fish, maximum heart rate peaks around the 120 bpm maximum suggested by Farrell (1991). Though heart rate can increase with temperature up

to UILT, cardiac performance cannot. In fact, in a number of fish, the positive chronotropy associated with elevated temperature results in negative inotropic effects on force and power production. For example, at the organ level, in *in situ* rainbow trout hearts, the increases in heart rate associated with increased acclimation temperature (from 70 bpm at 15°C to 85 bpm at 22°C), resulted in reductions in maximum stroke volume and power output (Farrell *et al.*, 1996). This suggests increased inotropic failure at warm temperatures and an upper temperature limit (~18°C for rainbow trout, Farrell *et al.*, 1996) above which, temperature-induced positive chronotropy may have deleterious effects on cardiac performance. Consistent with this, at the tissue level, *in vitro* experiments with spontaneously beating trout and flounder atrial strips show decreases in maximum isometric tension with increased temperature (Ask, 1983). In contrast, Matikainen and Vornanen (1992) report that in carp, warm-acclimation confers greater isometric force development and pumping capacity compared with cold-acclimation. However, they determined this to be a particular adaptation to meet the extreme seasonal variations in water temperature and oxygen content experienced by the carp. Thus, the effects of temperature acclimation on chronotropy and the concomitant effect on inotropy and can be species specific. Additional species specific differences are observed between crucian carp and yellow perch. Cold-acclimation decreases heart rate and the ability of ventricular muscle to follow fast pacing in crucian carp (Vornanen, 1994) while in yellow perch, cold-acclimation improves the ability to maintain contractility at high frequencies (Bailey and Driedzic, 1990). Species differences in acclimatory changes are probably associated with activity patterns. Yellow perch remain active at low temperatures and compensatory changes in contractile properties are necessary for adequate cardiac function, while crucian carp remain torpid during winter months (Vornanen, 1996), thus decreasing the activity of the heart is energetically favourable. Generally though, increases in temperature (both acute and chronic) are regarded as having positive chronotropic effects on heart rate and negative inotropic effects on force development in the isolated teleost myocardium (Matikainen and Vornanen, 1992).

As implied by the temperature-induced changes in heart rate, the kinetics of cardiac contraction are also susceptible to thermal modulation. Examination of the kinetic parameters of contraction provide additional insight into the mechanisms resulting in the negative inotropy associated with temperature-induced elevation in heart rate. It is well known that decreasing the interval between contractions has a large effect on contractile force as noted by the prominence of a negative force-frequency relationship in fish (Driedzic and Gesser, 1985) (force-frequency relationships are discussed in detail in a later section). Acute increases in temperature cause a reduction in time to peak tension (TPT), an increased maximal rate of force development (trout; Hove-Madsen, 1992), and a shorter duration of activation and relaxation at high temperatures (carp; Matikainen and Vornanen, 1992). In addition to these acute effects, warm-acclimation induced faster isometric contraction, with increased rates of time dependent variables in both spontaneously beating hearts and paced ventricles (Vornanen, 1994). Thus, increases in the spontaneous heart rate associated with increases in temperature (both acute and chronic) may reduce the active state of the muscle, ultimately resulting in a decrease in inotropic force. The reduction in active state may be associated with a decrease in APD at warm temperatures, which would reduce Ca^{2+} availability to the contractile elements (Driedzic and Gesser, 1994). Consistent with this idea, Ca^{2+} influx in flounder heart tissue was reduced as temperature increased above 10°C, presumably due to reduced APD (Lennard and Huddart, 1991). In isolated *in vitro* preparations, where contraction frequency is controlled, it is possible to resolve the simultaneous negative inotropic and positive chronotropic effects of increased temperature. Indeed, when ventricular strips from carp were paced at a constant frequency (thereby maintaining active state, and APD constant), isometric force increased with increased temperature (Matikainen and Vornanen, 1992).

Therefore, though there are many well established adaptations to cold which protect cardiac contractility in fish at low temperatures (see below), at warm temperatures, when the heart is susceptible to a reduction in contractility and power generating ability, such adaptations appear limited (Farrell *et al.*, 1996). Increases in relative ventricle size with cold acclimation has been demonstrated in several teleosts (rainbow trout, Graham and Farrell, 1989, Farrell *et al.*, 1996; channel catfish and green sunfish, Kent *et al.*, 1988; goldfish Tsukuda, *et al*, 1985, and carp, Matikainen and Vornanen, 1992; Pelouch and Vornanen, 1996) and is considered an adaptive mechanism to compensate for the effects of low temperature on cardiac contractility and rates of enzyme catalysis (Driedzic and Gesser, 1994). In addition, an increased number of adrenoreceptors, which increase the sensitivity of the myocardium to adrenaline (Keen *et al.*, 1993), and an increase in stroke volume to compensate for decreased heart rate, have been demonstrated in cold-acclimated animals, both which aid in maintaining inotropic force at cold temperatures. However, at temperatures above preferred, and especially at temperatures approaching UILT, adaptive mechanisms to protect contractility appear limited. Therefore, force development is compromised by increased contraction frequency and the power production of the heart decreases. This decrease in performance at the organ and tissue level, suggests that some aspect of E-C coupling is limited at high temperatures and heart rates. The following section examines the effect of temperature on the cellular mechanisms of E-C coupling.

Influence of Temperature on Cellular Mechanisms

From the above, it is clear, that temperature influences force and time course of teleost contractions. Force of contraction reflects the myofilament sensitivity to Ca^{2+} and the number of active crossbridges, which is determined by cytosolic Ca^{2+} concentration. The time-course of contraction is dependent on the duration of the intracellular Ca^{2+} transient ($[Ca^{2+}]_i$) and crossbridge cycling rate, the latter being determined mainly by the properties of myosin (Katz, 1992). Therefore, the effect of temperature on the physiological aspects

of contractility must reflect the temperature sensitivity of the cellular processes of E-C coupling themselves. Indeed, seasonal and temperature-induced changes in myosin heavy chain composition, which would manifest changes in the duration of contraction, are reported for crucian carp (Vornanen, 1994). It was found that winter (or cold-acclimated) fish expressed only one form of myosin, but that in summer (or warm-acclimated) fish, part of the 'winter' heavy chain myosin was replaced by a heavier 'summer' myosin. These alterations in myosin heavy chain composition were associated with temperature-induced changes in myosin ATPase activity, heart rate and the kinetics of isometric contraction. Indeed, warm-acclimated fish had higher *in vitro* heart rates and shorter contraction durations than cold-acclimated fish. The rate and duration of the cardiac cycle are primarily controlled by the attachment and detachment rates of the crossbridges which can be quantified by myosin ATPase activity. Therefore, because the myosin ATPase activity of cardiac myofibrils containing summer myosin was higher than that of winter myosin, it was concluded that the changes in myosin heavy chain composition form the basis for the functional changes found in the duration of isometric contraction and heart rate between warm and cold-acclimated carp. To date, these changes have not been investigated in other fish species.

The time course of contraction is not solely dependent on cross-bridge cycling rate, it is also controlled by the duration of the Ca^{2+} transient which is regulated by the Ca^{2+} removal mechanisms of the cell. In mammalian cardiac muscle, the lowering of cytosolic Ca^{2+} is primarily achieved through the combined actions of the SR-Ca^{2+} pump (SR-Ca^{2+} ATPase) and the SL Na^{+}/Ca^{2+} exchanger (Bridge *et al.*, 1988). These extrusion mechanisms compete for Ca^{2+}, with the SR consuming 60-80% and the remainder leaving the cell via the Na^{+}/Ca^{2+} exchanger (Tibbits *et al.*, 1992a). Because of the reduced role of the SR in most teleosts, the Na^{+}/Ca^{2+} exchanger is considered the primary mechanism for Ca^{2+} extrusion, although little is known about the role played by the SL Ca^{2+}-pump or the

mitochondria (Tibbits *et al.*, 1992). Indeed, rainbow trout Na^+/Ca^{2+} exchange activity at 21°C is comparable to the highest activity observed in mammals (Tibbits *et al.*, 1992b). Also, indirect studies have demonstrated the importance of Na^+/Ca^{2+} exchanger in reducing $[Ca^{2+}]_i$ in teleost ventricular strips (Hove-Madsen and Gesser, 1989). Consistent with Na^+/Ca^{2+} exchange as the primary Ca^{2+} extrusion mechanism, the smaller diameter of teleost myocytes increases the surface are to volume ratio which increases the efficacy of SL transport mechanisms (Tibbits *et al.*, 1992b).

Temperature can effect the time course of relaxation by modulating these cellular extrusion mechanisms. In mammals, both the SL Na^+/Ca^{2+} exchanger and the SR-Ca^{2+}-pump are sensitive to temperature as demonstrated by rapid cooling contractures (RCC). Rapid cooling from 30°C to 1°C induces a rapid release of the available SR-Ca^{2+} to the cytoplasm due to the increased opening of the SR-Ca^{2+}-release channel at low temperatures. This Ca^{2+} then activates a contracture whose amplitude is indicative of the amount of Ca^{2+} available for release at the time of cooling (Bers, 1991). The contraction associated with rapid cooling is prolonged due to the cold-induced inhibition of the Ca^{2+} removal mechanisms, the SR-Ca^{2+}-pump and Na^+/Ca^{2+} exchanger (Bers, 1989). To prevent similar inhibition of relaxation in teleosts living at cold ambient temperatures, a decreased temperature sensitivity of these extrusion mechanisms would be expected. Bersohn *et al.* (1991) demonstrated that the Na^+/Ca^{2+} exchange in the frog heart was less temperature dependent that mammals, and Tibbits *et al.* (1992b) found the teleost cardiac Na^+/Ca^{2+} exchanger to be less temperature dependent than that of the frog. These changes in the temperature sensitivity of the Na^+/Ca^{2+} exchanger could represent important physiological adaptations which allow fish to maintain normal relaxation, and therefore activity, at cold temperatures (Tibbits *et al.*, 1992a).

If the SR-Ca^{2+}-pump were to contribute to cardiac relaxation in fish, then it too, would have to demonstrate a thermostability different from that of mammals. Indeed, this is the case for fish skeletal muscle. Studies on the SR-Ca^{2+}-pump from trout and rabbit skeletal muscle indicate different temperature dependencies, with SR vesicles from trout loading Ca^{2+} faster than SR vesicles from rabbit at temperatures below 20°C (Chini *et al.*, 1993; Toledo *et al.*, 1995). Structural differences in the SR-Ca^{2+}-pump proteins may be responsible for this varying thermostability as differences in proteins were observed in the Ca^{2+}-pump from rabbit and carp skeletal muscle (Dux *et al.*, 1989). However, these types of comparisons have yet to be conducted with fish cardiac muscle.

Thus far, the influence of temperature on variables influencing time course of contraction have been examined. In addition to these effects, temperature has been demonstrated to influence two components which determine maximal force development, the first of which is the Ca^{2+} sensitivity of the myofibrils. In mammalian cardiac muscle, decreasing temperature below physiological levels with rapid cooling contractures (RCC) dramatically desensitized the myofibrils with respect to Ca^{2+} (rat, Harrison and Bers, 1990; rabbit, Harrison and Bers, 1989). This reduced Ca^{2+} sensitivity has also been demonstrated in frog ventricular muscle, with a 45.7% decrease in maximum Ca^{2+} activated force from 22°C to 1°C (Harrison and Bers, 1990). However, this dramatic desensitization may be overcome in cold water fish by a greater inherent Ca^{2+} sensitivity of the myofibrils over the entire physiological temperature range. This was demonstrated by both increased ATPase activity and increased force development in skinned ventricular fibres from trout (~10°C) when compared with rat (37°C) (Churcott *et al.*, 1994). Interestingly, in carp, the Ca^{2+} sensitivity of myofibrillar ATPase was unaffected by thermal acclimation (Vornanen, 1996) supporting the contention that Ca^{2+} sensitivity is consistent over the physiological temperature range in fish. However, there may be drawbacks to high Ca^{2+} sensitivity, as the rate of unloading Ca^{2+} from the troponin C (TnC) may be very slow and maximum

attainable heart rate may be compromised (Churcott *et al.*, 1994). This possibility remains to be explored in teleosts.

The second way temperature can influence the force of contraction is by effecting an increase in $[Ca^{2+}]_i$. The rise in intracellular Ca^{2+} during contraction in mammals is supported by Ca^{2+} from both the extracellular space and the SR. The main route for transsarcolemmal Ca^{2+} influx is the L-type channel (though the reverse mode of the Na^+/Ca^{2+} exchanger may potentially contribute a small amount of Ca^{2+} to $[Ca^{2+}]_i$). In mammals, the L-type channel shows a strong thermal dependence with a Q_{10} of 3 (Cavalie *et al.*, 1985). However, little is known about the temperature dependence of this channel in teleost hearts, though, if similar to mammals, it would have a profound impact on contractility in cold-water species. If the activity of the teleost L-type channel is reduced at cold temperatures, the concomitant increase in adrenergic sensitivity may provide compensation, as it would result in a greater likelihood of an adrenergically mediated increase in SL Ca^{2+} influx (an effect that will be discussed in detail in the following section).

In addition to influencing Ca^{2+} influx via SL Ca^{2+}-channels, in mammals, temperature effects $[Ca^{2+}]_i$ through modulation of the SR-Ca^{2+}-release channel. Lowering of temperature in the interval of 37°-22°C diminished the relative importance of the SR to E-C coupling in rat and rabbit ventricle (Shattock and Bers, 1987). The effect of temperature on the SR from mammalian heart was briefly discussed in the first section. Lowering ambient temperature causes the Ca^{2+}-release channel of the SR to increase it's open probability, reducing the effectiveness of the SR in sequestering and releasing Ca^{2+} during E-C coupling. Cooling the SR-Ca^{2+}-release channel isolated from sheep cardiac muscle, resulted in an increased open probability from 0.13 at 23°C to 0.69 at 5-10°C (Sitsapesan *et al.*, 1991) This dramatic increase in open-state probability would severely impair the

channel's ability to effectively sequester and release Ca^{2+} for contraction. The temperature dependency of the channel in mammals has also been demonstrated by more indirect means through rapid cooling contractures (RCC), where the magnitude of the contraction after cooling is considered to reflect the Ca^{2+} content if the SR (Bers, 1987). Only very small RCC are observed in frog ventricular muscle, consistent with the reduced role of the SR in lower vertebrates (Harrison and Bers, 1990). However, a small RCC does not necessarily mean that the SR-Ca^{2+}-release channel in frog is less temperature dependent. The assessment of the importance of the SR by RCC's in lower vertebrates is complicated by the fact that other cellular Ca^{2+}-regulating mechanisms, such as the Na^{+}/Ca^{2+} exchanger, are still active in the cold (Tibbits *et al.*, 1992a). Thus, due to the temperature sensitivity of the SR-Ca^{2+}-release channel in mammals, the importance of the SR in contributing Ca^{2+} to force development can not be excluded for teleosts, frogs, and other lower vertebrates by the lack of involvement at low ambient temperatures. The experimental aspect of this thesis will involve indirectly assessing the temperature dependency of the SR-Ca^{2+}-release channel in trout at temperatures approaching their UILT, to better define the role of the SR in E-C coupling in a lower vertebrate.

Though temperature may modify the ability of the SR to sequester and release Ca^{2+}, Ca^{2+} flux across the SL will ultimately determine the importance of this intracellular source. The factors that determine SL Ca^{2+} flux include, (1) the number of Ca^{2+} channels in the membrane, (2) the electrochemical gradient driving Ca^{2+} through the channels and (3) the open probability of the channels. Using sarcolemmal vesicles isolated from the ventricles of cold-acclimated (8°C) rainbow trout, the number of L-type Ca^{2+} channels (DHP ligand binding sites) has been estimated as 3.06 ± 0.49 pmol/mg protein (Tibbits *et al.*, 1990). Consistent with the idea of a increased role for transsarcolemmal Ca^{2+} flux during E-C coupling in trout, this density of L-type Ca^{2+} channel is 3-4 fold higher than that estimated for mammals using a similar preparation. The extracellular Ca^{2+} concentration is usually

fairly constant, though Ruben and Bennett (1981) report an increase in the total plasma Ca^{2+} concentration from about 2.5 to 3.5 mM associated with strenuous activity in a number of teleosts. In any case, all cells maintain the Ca^{2+} concentration of their cytoplasm (~0.05-0.5 μM) orders of magnitude below that of the extracellular fluids (1-10 mM) (Taylor, 1985; Reuter, 1983). This results in a large driving force behind Ca^{2+} entry via the voltage dependent L-type channels during excitation. Transsarcolemmal Ca^{2+} flux can also depend on the probability of the L-type Ca^{2+} channel being open at any given time. Adrenaline is a potent modulator of the open probability of this channel in both fish and mammals, as will be discussed in the following section.

The Role of Adrenaline in Modulating Cardiac Contractility and Transsarcolemmal Ca^{2+} Influx

Catecholamines are potent regulators of cardiac function. In teleosts, adrenaline is the predominant catecholamine involved in modulating contractility and can arrive at the heart from both neural and humoral sources (Ask *et al.*, 1981). The effect of adrenaline on cardiac function is usually two fold; (1) adrenaline increases cardiac output by providing inotropic and chronotropic stimulation, and (2) adrenaline protects the integrity of the myocardium under stressful and adverse conditions (Farrell, 1985). The positive chronotropic effects of adrenaline on heart rate are well documented (Falck *et al*, 1966; Gannon and Burnstock, 1969; Cobb and Santer, 1973; Farrell *et al.*, 1982; Laurent *et al.*, 1983; Farrell, 1984; Farrell, *et al*, 1986) and are partially achieved through direct stimulation of the pace-maker cells (Cobb and Santer, 1973). Indeed, removal of adrenergic stimulation in cholinergically blocked hearts reduces *in vivo* heart rate by more than 20% in most teleosts (Axelsson *et al.*, 1987; Keen *et al.*, 1995). Likewise, adrenaline potentiated *in vitro* contraction frequency in spontaneously beating atria from frog, trout and flounder (Ask, 1983). Similarly, heart rates measured *in situ* from rainbow trout at 30 nM adrenaline were increased by increasing the adrenaline concentration to 200 nM (Farrell

et al., 1996). In mammals, adrenaline-induced positive chronotropy has also been partially attributed to an increased rate of relaxation. Adrenergic stimulation removes the inhibitory effect of phospholambam (an inhibitory protein of the SR-Ca^{2+}-pump) thereby stimulating the SR to take up Ca^{2+}, reducing $[Ca^{2+}]_i$, and ultimately increasing the rate of relaxation (Bers, 1991). Moreover, adrenergic stimulation has been demonstrated to decrease the affinity of troponin I (TnI) for TnC in mammals (Bers, 1991). The adrenaline-mediated phosphorylation of TnI weakens the TnI-TnC interaction, thereby increasing interaction between TnI and actin, and ultimately decreasing the systolic interval. These processes may contribute to the positive chronotropic effects of adrenaline; however, they have not been investigated in fish.

The positive inotropic effects of adrenaline are also well documented (Falck *et al*, 1966; Gannon and Burnstock, 1969; Cobb and Santer, 1973; Ask, 1983; Farrell, *et al*, 1986; Vornanen, 1989; Keen, *et al.*, 1992) and can be separated from the chronotropic effects by isolating the ventricle from the atrium and stimulating contractions electrically. Under such circumstances, a 2-fold increase in force is reported for trout after stimulation with 10 μM adrenaline (Gesser *et al.*, 1982; Hove-Madsen and Gesser, 1989) and a 3-fold increase in force was observed after adrenergic stimulation in the 10 nM to 10 μM range in skipjack tuna (Keen *et al.*, 1992). Additionally, adrenergic stimulation increased peak tension in ventricular strips from carp (Vornanen, 1989). The positive inotropy associated with adrenergic stimulation is attributed to a ß-adrenoreceptor-mediated increase in Ca^{2+} flux across the SL. There is an α-adrenoreceptor-mediated pathway which can also increase $[Ca^{2+}]_i$ via an inositol 1,4,5-triphosphate (IP_3)-dependent pathway in mammalian smooth muscle cells. However, the physiological importance of this pathway in releasing Ca^{2+} from SR stores in mammalian cardiac cells is usually dismissed as the rate and degree of IP_3-induced Ca^{2+}-release is significantly less than Ca^{2+}-induce Ca^{2+}-release. The role of the α-adrenoreceptor in mediating IP_3-induced Ca^{2+}-release has yet to be investigated in

fish myocytes, however, because of the relatively low heart rates in fish, it is conceivable that this method of Ca^{2+} release may contribute to the rise in $[Ca^{2+}]_i$ after adrenergic stimulation.

The prominent ß-adrenoreceptor-mediated increased Ca^{2+} current (I_{Ca}) was thought to be a result of an increase in the number of functional Ca^{2+} channels, (Reuter and Scholtz, 1977) however, it is now accepted that ß-adrenoreceptor agonists enhance I_{Ca} by increasing the open probability of Ca^{2+} channels during depolarization (Reuter, 1983). The SL surface ß-adrenoreceptor density in trout ventricular myocytes is 3.38 sites μm^{-2} or 12 000 sites per cell (Gamperl *et al.*, 1994). A similar receptor density of 1.29 sites μm^{-2} was estimated by Keen *et al.* (1993), using a different technique. When adrenaline binds to the ß-adrenoreceptor it activates a GTP-binding protein (G_s protein) which catalyses adenylate cyclase to convert ATP to cyclic AMP (cAMP) (Lefkowitz *et al.*, 1983). cAMP interacts with a cAMP-dependent protein kinase (PKA) in the cytosol, which in turn phosphorylates the L-type channel of the SL. Additionally, the G-protein can interact directly with the L-type channel (Yatani *et al.*, 1987). This phosphorylation results in the increased open probability of the channel (Reuter, 1983). Since force development is directly related to the amount of Ca^{2+} available for the myofilaments (Yue, 1987), an adrenergically stimulated increased Ca^{2+} influx would manifest in the positive inotropy observed in response to adrenaline in fish and other vertebrates.

Thus, adrenaline can be an important modulator of cardiac function increasing both the rate and the strength of contraction. In addition, adrenergic stimulation may protect the myocardium under adverse conditions. Milligan and Farrell (1986) suggested that the 6-15 fold increase in circulating levels of adrenaline after intense exercise protects the inotropic capabilities of the heart from exercise-induced acidosis and hypoxia. The inhibition of the contractile process in acidotic fish hearts is thought to be through a functional deficit of

intracellular Ca^{2+} (Gesser and Poupa, 1983) which could be ameliorated through elevated levels of circulating Ca^{2+} and adrenaline (Farrell, 1984). In a study by Farrell *et al.* (1986), elevated extracellular Ca^{2+} improved the inotropic state of the acidotic perfused trout heart but also induced negative chronotropy, whereas, physiological levels of adrenaline restored overall cardiac performance. Further, Farrell and Milligan (1986) found that the addition of 0.5 μM of adrenaline during acidosis, prevented the loss of contractility without changing intracellular pH, which led to the suggestion that adrenaline restored contractility through modulation of SL Ca^{2+} flux. Thus, adrenaline undoubtedly serves a protective role during acidosis which is related to SL Ca^{2+} flux, though the exact mechanisms are still not fully understood.

Likewise, adrenaline may confer a protective role in maintaining contractility at cold temperatures, when cellular processes are slowed. However, the influence of adrenaline in modulating contractility during temperature change is complicated by the fact that the adrenergic sensitivity of the myocardium, is itself, temperature-dependent. For example, Graham and Farrell (1989) observed a 10-fold increase in the sensitivity of rainbow trout *in situ* heart preparations to adrenaline in cold-acclimated animals. Additionally, Ask *et al.* (1981) observed that the effects of adrenaline on isolated trout atrium (and frog and flounder; Ask, 1983) were more pronounced at 2°C than 17°C, and suggested that sympathetic activation was especially important in allowing maximal performance at low environmental temperatures. Conversely, these studies could also be interpreted to indicate a reduced importance of adrenaline in maintaining cardiac performance at warm temperatures.

The cold-induced enhancement of adrenergic sensitivity has been explained, at least in part, by an elevation in the number of SL ß-adrenoreceptors (Keen *et al.*, 1993). An increase in number of receptors permits a greater likelihood of receptor occupancy upon adrenergic

stimulation, which would increase inotropic force through stimulation of the aforementioned signal transduction pathway. A decrease in the EC_{50} (concentration required for half maximal stimulation) for adrenaline in both *in situ* working heart preparations and isolated ventricular strips from cold-acclimated rainbow trout, was also correlated to the increased surface density of ß-adrenoreceptors (Keen *et al.*, 1993). Conversely, as temperatures increase, the number of SL ß-adrenoreceptors decrease. In a recent study, a 1°C increase in temperature was suggested to be associated with a ~30% decrease in receptor number (Gamperl *et al.*, 1996). This down regulation of the adrenergic response upon increased temperature has enormous implications for the ability of adrenaline to effectively modulate contractility at warm temperatures. This is especially true of temperatures approaching the UILT.

Given the proposed temperature-dependency of the SR-Ca^{2+}-release channel in fish, it is intriguing to speculate that the decreased ability of adrenaline to modulate SL Ca^{2+} influx at warm temperatures, corresponds with an increased ability to utilize SR-Ca^{2+}. Such interplay between the two potential sources of activator Ca^{2+} may help to maintain adequate Ca^{2+} availability to the myofibrils as temperatures approach the UILT. Thus, the experimental aspect of this thesis will examine for the first time, the interaction between Ca^{2+} released from the SR, and Ca^{2+} flux across the SL under varying levels of adrenergic stimulation in fish at warm temperatures. The outcome of this investigation may provide important clues into the biological relevance of SR-Ca^{2+} in maintaining contractility as temperatures increase.

Thus far, the effects of temperature and adrenaline on cardiac function have been discussed and speculated on in detail. Frequency, is another potent modulator of E-C coupling and cardiac performance whose effects have been eluded to throughout this discussion. Thus,

the next topic to be addressed in this literature review is the relationship between contraction frequency and force.

Force-Frequency Relationships

In 1972, Rumberger and Reichel noticed that the amplitude of contraction increased with increased frequency in 'warm-blooded animals' (guinea pig) but decreased with increased frequency in 'cold-blooded animals' (turtles and frogs). They attributed the changes in force observed within each species to changes in the action potential (AP), and attributed the inter-species (warm vs. cold-blooded) variation to differences in the relative Ca^{2+} storage capabilities of the SR. Today, 25 years later, researchers are still examining both the effect of stimulation frequency on APD and the amplitude of contraction, and on the importance of the SR in explaining the difference in force-frequency response between most mammals and most lower vertebrates.

Force-frequency relationships in mammals

In most mammalian ventricles, typified by the rabbit, an elevation in pacing frequency leads to an increase in contractile force. This positive relationship between increased frequency and increased force has been attributed to a net influx of Ca^{2+} during contraction. The negative force-frequency response, or decrease in force with increased frequency, is observed in a limited number of mammals (i.e. rat) and most teleosts, and has been attributed to net Ca^{2+} efflux during contraction. Since Na^{+}/Ca^{2+} exchange is the main mechanism by which Ca^{2+} is extruded from cardiac cells, it has been suggested that the net Ca^{2+} influx observed in rabbit ventricle and net Ca^{2+} efflux observed in rat ventricle is due to the varying activity of the Na^{+}/Ca^{2+} exchanger in each animal (Bers, 1989). Indeed, studies with rat ventricular muscle suggest that during contraction, Ca^{2+} released from the SR is removed from the cytoplasm by the Na^{+}/Ca^{2+} exchanger thereby depleting the cell of

Ca^{2+}. This net Ca^{2+} efflux occurs because during the pulse, when $[Ca^{2+}]_i$ is high, and aNa^+_i (intracellular Na^+ activity) is low, the reverse potential for Na^+/Ca^{2+} exchange ($E_{Na/Ca}$) is positive to the membrane potential (E_m) so there is a large driving force favouring Ca^{2+} extrusion via Na^+/Ca^{2+} exchange. Additionally, the APD in rat ventricle is very short (~50 msec, Shattock and Bers, 1987), limiting transsarcolemmal Ca^{2+} influx, but providing the necessary Ca^{2+} pulse for Ca^{2+}-induced Ca^{2+}-release from the SR. Recall that rat ventricles rely almost entirely on SR-Ca^{2+} for activation of the myofilaments, as indicated by the elaborate network of SR and the 87% reduction in force after ryanodine treatment (Bers, 1985). Therefore, as contraction frequency is increased in the rat, the Ca^{2+} that is released from the SR to initiate contraction, is removed from the cytoplasm because the Na^+/Ca^{2+} exchanger out competes the SR-Ca^{2+}-pump (Bers, 1991). This action gradually depletes the SR of its Ca^{2+} stores without replenishing them through transsarcolemmal influx. This process culminates in the overall decrease in force observed with increased frequency in the ventricle of the rat.

The rabbit ventricle (the mammalian tissue most representative of the positive force-frequency response) is less dependent on the SR than the rat, having a longer APD (372 ± 14 ms, Tibbits *et al.*, (1992b), compared with ~ 50 ms in rat), which allows for greater Ca^{2+} influx across the SL with each beat. This transsarcolemmal Ca^{2+} influx can contribute Ca^{2+} to contraction as well as stimulating Ca^{2+}-induced Ca^{2+}-release from the SR; a contention supported by the ability of the rabbit to maintain 80% of its contractile force in the absence of SR-Ca^{2+} contribution (Bers, 1985). During the contraction, E_m exceeds $E_{Na/Ca}$ in the ventricular cells of rabbit, such that there is a modest driving force favouring Ca^{2+} entry via Na^+/Ca^{2+} exchange. This process loads Ca^{2+} into the cell where it can be stored in the SR allowing for a greater Ca^{2+} release on the subsequent contraction. This results in the positive force-frequency relationship.

Therefore, in mammals, SR-Ca^{2+} content is critically linked to transsarcolemmal Ca^{2+} movements via Na^{+}/Ca^{2+} exchange (Bers, 1991). The activity of the Na^{+}/Ca^{2+} exchanger determines whether net Ca^{2+} efflux (resulting in a negative staircase) or influx (resulting in a positive staircase) occurs during a pulse. Because the negative force-frequency response in mammals is found in tissues and species where activation of the contractile elements relies heavily upon SR-Ca^{2+} it is surprising to observed a negative force-frequency response in fish heart, where, according to the proposed model for E-C coupling, force generation is largely SR independent.

Force-frequency relationships in lower vertebrates

Driedzic and Gesser (1985) examined the relationship between force and frequency in a diverse group of teleosts and found that the inherent response of ventricular muscle was a decrease in force with an increase in frequency. They found this negative-staircase was independent of the activity level of the fish, as both the active mackerel and more sluggish sea raven both demonstrated the same response. Subsequent studies with additional species including hagfish (*Myxine glutinosa*) and white sturgeon (*Acipenser transmontanus*) confirmed the earlier results with all species displaying a negative force-frequency response. In three elasmobranchs, the little skate (*Raja erinacea*), spiny dogfish (*Squalus acanthias*) and black dogfish (*Etmopterus spinax*) a positive force-frequency response was observed at low pacing frequencies, but became negative in the physiological range (>0.4 Hz) (Driedzic and Gesser, 1988). A similar result was observed for turtle and frog ventricle which revealed a positive force-frequency response over the 0.1 - 0.5 Hz range, and a negative response as frequency increased above 0.5 Hz (Driedzic and Gesser, 1985).

In light of the discussion concerning the force-frequency response in rat and rabbit, it is evident that there is complex interplay between APD, aNa^{+}_{i}, and the Na^{+}/Ca^{2+} exchange

which relates to a relative dependence on the SR. Though the information gained from mammalian studies may have limited application in the fish and amphibian world, the observed direction of the force-frequency response in lower vertebrates may still reflect the relative dependence on the SR for Ca^{2+}. Indeed, the frog, with virtually no SR demonstrates a positive force-frequency response (and post-rest decay), and the trout, with greater SR content (Santer, 1985) exhibits a negative response (and post-rest potentiation). In the absence of electrophysiological information on the fish myocyte, it may be useful to discuss the potential role played by the APD in determining the direction of the force-frequency response and the relative dependence on the SR in supplying Ca^{2+} for contraction.

The APD of most teleosts (300 ms for rainbow trout at 15°C; Hove-Madsen and Gesser, 1989) is shorter than that of the frog (732±106 ms, for atrium at 20°C, Hume and Giles, 1982). This suggests that the time for transsarcolemmal Ca^{2+} influx in fish is less than that in the frog which may account for the increased dependence on SR-Ca^{2+}observed in fish heart. In contrast, the prolonged APD in frog may allow sufficient Ca^{2+} influx across the sarcolemma to negate the need for intracellular contribution from the SR. These differences in APD and the resulting involvement of the SR, may influence the activity of the Na^+/Ca^{2+} exchanger and influence net Ca^{2+} loss or gain by the cell during contraction.

The observation that increased $[Ca^{2+}]_o$ reduced or eliminated the negative effect of increase frequency in teleost ventricular strips (Driedzic and Gesser, 1985) suggests that either Ca^{2+} efflux via Na^+/Ca^{2+} exchange is limited under high levels of $[Ca^{2+}]_o$, or that $[Ca^{2+}]_o$ increased the driving force behind transsarcolemmal Ca^{2+} influx such that the cell gained Ca^{2+} during contraction despite the activity of Na^+/Ca^{2+} exchange. Either being the case, one might expect adrenergic stimulation to eliminate the negative-force frequency response, as it too increases SL Ca^{2+} influx. However, such an effect has not been observed

experimentally. Hove-Madsen and Gesser (1989) suggested that adrenergic stimulation of the Na^+/K^+-ATPase would decrease $[Na^+]_i$ causing the gradual loss of $[Ca^{2+}]_i$ through Na^+/Ca^{2+} exchange. In this way, they explain the shift from a positive to a negative staircase after adrenergic stimulation they observed in trout at 15°C. This study is unusual as a positive force-frequency response was observed from 0.1 Hz to 1.0 Hz, however, they used high extracellular K^+ (~5 mM) which would affect $[Na^+]_i$. Interestingly, addition of ryanodine blocked the increased Ca^{2+} uptake observed in response to K^+ (El-Sayed and Gesser, 1989) suggesting SR involvement in this positive force-frequency display. These results have not been substantiated with subsequent experiments. Beyond this, not many studies have observed the effect of adrenaline on the force-frequency relationship *per se*, though Keen *et al.*, (1992) report that in skipjack tuna, adrenaline concentration-response curves and EC_{50} values are not affected by stimulation frequency.

Though APD may provide insight into the force-frequency response in vertebrates hearts, its use as a predictor of the direction of the curve is limited by its temperature sensitivity. For example, APD of the rabbit ventricle at 23°C is reported to be ~372 ms, compared with ~222 ms at 37°C (Shattock and Bers, 1987). Additionally, prolongation of the AP has been observed upon lowering of temperature in amphibians (Blinks and Koch-Weser, 1963), flounder (Lennard and Huddart, 1991), and trout (Møller-Nielsen and Gesser, 1992). In bullfrog atrial tissue, increased APD by low temperatures resulted in a potentiated Ca^{2+} current, indicating an increased Ca^{2+} influx (Goto, *et al.*, 1978). Furthermore, a prolonged depolarization may favour an inward shift of Ca^{2+} by Na^+/Ca^{2+} exchange. This tendency is further emphasized in that decreases in temperature may depress the Na^+/K^+-ATPase activity and increase intracellular Na^+ as shown for cat heart muscle (Page and Storm, 1965). Consistent with this, cellular Ca^{2+} uptake in flounder heart tissue was reduced as temperature increased above 10°C (Lennard and Huddart, 1991).

From this discussion it becomes obvious that the relationship between frequency and force is not straight forward. Many factors contribute to the shape and direction of the curve, yet a great deal of information regarding the activity of cellular mechanisms can still be derived by conducting force-frequency experiments. This has provoked numerous investigations into force-frequency relationships in fish, with countless variations of parameters known to influence contractility. Of particular interest is the effect of ryanodine on this relationship as it is central in discerning the role of the SR. Indeed, as discussed in previous sections, the SR may contribute Ca^{2+} to force production under certain circumstances in fish myocytes. Often SR involvement in fish is observed through the induction of post-rest potentiations.

A post-rest potentiation is elicited by an interruption of the regular stimulation rate by an unphysiologically long rest interval. During this rest period, aNa_i activity is high, such that $E_{Na/Ca}$ is negative to E_m, causing the Na^+/Ca^{2+} exchanger to operate in the reverse mode, causing net Ca^{2+} uptake, thereby enhancing the subsequently evoked contraction (El-Sayed and Gesser, 1989; Bers, 1991). The post-rest potentiation of trout myocardium is noteworthy because it compares well with that seen in rat myocardium where the potentiation is attributed to an exceptional dependence on the SR (Tibbits *et al.*, 1992a). However, Møller-Nielsen and Gesser (1992) point out that post-rest potentiation is not solely dependent on the SR, but also possibly on rest-induced enhancement of sarcolemmal Ca^{2+} influx. If this is the case, it could complicate assessment of SR involvement based on this parameter.

Ryanodine is a more reliable tool for assessing the relative importance of the SR. As such, ryanodine's effects on the force-frequency relationship in fish have been studied in detail. No effect of ryanodine was observed on the negative staircase in cod and sea raven (Driedzic and Gesser, 1988). Hove-Madsen (1992) noticed that the biphasic response to increased frequency, with a minimum around 0.2 Hz at 5°C and 15°C, became a

monophasic negative decline at 25°C in trout. This negative force-frequency response at 25°C, was interpreted as indicating increased SR-Ca^{2+} contribution to force development. Tests with ryanodine supported this contention as it reduced both isometric force and diminished post-rest potentiations at 25°C and low frequencies, but not at colder temperatures (5° and 15°C) and never at higher frequencies; suggesting that under physiological conditions the SR was not contributing Ca^{2+} to contraction. Likewise, Keen *et al.* (1994) concluded that the SR was not involved in routine contractions in rainbow trout as a ryanodine response did not exist at low temperatures (8°C) and only occurred at frequencies $\leq$ 0.2 Hz at warm temperatures (18°C). In skipjack tuna atrium, the shape of the biphasic response to increased frequency (positive below and negative above 1.5 Hz) was not affected by ryanodine, however a downward shift in the force curve (~30%) was observed at all pacing frequencies. This varying force-frequency response and ryanodine response from trout and tuna at 25°C points to either species-dependent differences in SR-Ca^{2+}-release channel function or complex temperature effects on its functional operation. However, which of these possibilities is responsible for the varying response at 25°C is unknown.

Experimental temperatures of 25°C are greater than the UILT for trout, but not tuna, therefore testing trout at 25°C has little biological relevance. At 22°C the temperature dependency of the SR can be tested, still keeping below the UILT for trout (Black, 1953; Farrell *et al.*, 1996). Incorporation of warm temperature acclimation to 22°C, in addition to acute temperature change may help to resolve the differences in SR involvement between trout and tuna. Additionally, because SL Ca^{2+} flux is the predominant source of Ca^{2+} in teleosts, realistic levels of adrenergic stimulation may help to identify the physiological importance (if any) of SR contribution. Indeed, the interplay between SR and SL Ca^{2+} flux becomes particularly important as temperatures approach the UILT, as the adrenergic sensitivity of the myocardium is reduced (Keen *et al.*, 1993), and the intrinsic properties of

the heart are operating a maximal levels or declining (Farrell *et al.*, 1996). Lastly, given that the tuna exhibits both the most significant dependence on SR-Ca^{2+}, and the highest heart rates recorded for lower vertebrates to date, the potential relationship between the ability to recruit intracellular Ca^{2+} and elevate contraction frequency is of vast biological importance.

Thus, in this thesis, the modulatory effects of temperature on the SR-Ca^{2+}-release channel and adrenaline on the SL will be examined over a range of applied frequencies in an isolated ventricular muscle preparation. For the purpose of these experiments, it is important to be able to control frequency so that the inotropic effects of temperature and adrenaline are separated from their potential chronotropic effects. The applied frequencies range from 0.2 Hz, (sub-physiological under routine conditions, but necessary for comparison with other studies), to 2.0 Hz, (the upper frequency limit for lower vertebrates suggested by Farrell (1991)) in an effort to determine what effect (if any) recruitment of SR-Ca^{2+} stores has on the ability of the fish heart to beat at high frequencies. Traditionally, experiments of this type have been performed on muscle undergoing isometric contractions. However, *in vivo*, muscle length changes over the cardiac cycle. Therefore the last topic to be discussed in this literature review is the oscillating or working muscle preparation which I had the opportunity to explore for the first time with ventricular muscle from rainbow trout.

Oscillating Muscle Preparation

When working with ventricular strips in an isometric muscle preparation, observations concerning the inotropic capabilities of the heart centre around the measurement of peak tension. Peak tension is a useful indicator of the beat-to-beat contractile properties of the heart. However, *in vivo*, cardiac muscle does not contract isometrically, the length of the muscle changes with lumen volume as blood enters and exits the heart. Indeed, length has

a profound influence on cardiac muscle performance, as noted by the Frank-Starling principle which demonstrates that cardiac output is a function of filling pressure; and by the length-tension relationship, which is determined by the amount of myofilament overlap and cross-bridge formation at varying lengths. The oscillatory muscle preparation or work loop technique forces the muscle to contract during a length change cycle and assesses both the contractile work done by the muscle and the work required to re-extend it (Syme, 1993). Using this technique, it is possible to precisely control muscle length, contraction frequency and strain for ventricular trabeculae, which correspond to chamber volume, heart rate and stroke volume respectively in the intact heart (Syme, 1993). Additionally, net work and power output (product of net work and cycle frequency) can be calculated. An examination of these parameters under varying conditions (i.e., temperature change, adrenergic stimulation and frequency) provides a more integrative assessment of cardiac performance than the isometric parameter "peak tension". This is especially true for assessing the frequency response as muscle length is known to influence activation processes such as the action potential, Ca^{2+} flux and the binding of Ca^{2+} to troponin (Allen and Kentish, 1985). These effects are largely attributed to increased Ca^{2+} release and increased Ca^{2+} sensitivity of the myofilaments with increasing length (Layland *et al.*, 1995b). As such, evaluating performance with the work loop technique may allow for better extrapolation to the physiological condition. Indeed, studies on skeletal muscle (Altringham and Young, 1991; James *et al.*, 1995) and cardiac muscle (Layland *et al.*, 1995a) using the work loop technique have demonstrated that muscles *in vitro* produce maximum power output at cycle frequencies corresponding to their normal operating frequencies *in vivo*. Therefore the last part of this thesis will re-examine the influences of temperature, adrenaline, ryanodine and frequency on trout ventricular trabeculae performing oscillatory contractions to provide an additional, and more physiological perspective to the results obtained isometrically.

OBJECTIVES

The overall objective of this thesis is to investigate the influence temperature change and adrenergic stimulation exert on the relative importance of SR-Ca^{2+}-release and SL Ca^{2+} flux in contributing Ca^{2+} to force development in the ventricle of rainbow trout.

The specific objectives of this thesis are as follows:

(1) To determine the effect of temperature and temperature acclimation (of 12°C and 22°C) on the relative importance of the SR-Ca^{2+}-release channel in contributing Ca^{2+} to force development at physiologically realistic pacing frequencies under tonic adrenergic stimulation in isolated ventricular trabeculae from rainbow trout. (Chapter 2)

(2) To examine the muscle's ability to maintain tension after blocking SR-Ca^{2+}-release with ryanodine, over a range of pacing frequencies, under temperature and adrenergic modulation. (Chapter 2)

(3) To examine the effect of modulating SL Ca^{2+} influx with maximal adrenergic stimulation on the relative importance of SR-Ca^{2+} contribution to E-C coupling at physiologically realistic pacing frequencies at 12°C and 22°C. (Chapter 2)

(4) To re-examine objective (1), (2) and (3) using an oscillatory muscle preparation, to substantiate the isometric results, and to assess modulatory effects of temperature and adrenaline on power production. (Chapter 3)

The approach used in objective (1), (2) and (3), is similar to that used by Keen *et al.* (1994) at 8°C and 18°C. By testing the same species of fish, from the same origin, held under the same conditions, at 12°C and 22°C, this thesis allows for comparison between studies, and adds information on the importance of the SR to force development in fish hearts at temperatures approaching the UILT.

Unlike previous studies, this study utilized a tonic level of adrenergic stimulation in all preparations to ensure Ca^{2+} availability *in vitro* best approximated that observed *in vivo.* Further, these experiments were carried out using isolated ventricular trabeculae as opposed to ventricular strips (Keen *et al.*, 1994) which minimized the number of severed (branched) fibers. The use of tonic adrenaline and isolated trabeculae in this study would allow greater force development at all pacing frequencies than was possible in earlier studies. Because many of my conclusions are based on observations concerning changes in force, the increase in attainable force development at control levels in my study may facilitate observations that were undetectable in previous work.

The oscillatory study was conducted at Dr. E. D. Stevens' lab at the University of Guelph, Guelph, Ontario.

Chapter 2 of this thesis appears in the form submitted to the Journal of Experimental Biology, August 1996.

ASSUMPTIONS

In this thesis, as with most scientific research, a certain number of assumptions concerning the experimental methodology have been made. Those of specific importance to the interpretation of the results are given below.

(1) After incubating ventricular muscle for 45 min at 12°C and 30 min at 22°C in a solution containing 10 μM of ryanodine, all the potential ryanodine binding sites on the SR-Ca^{2+}-release channel are occupied by ryanodine resulting in complete channel closure.

(2) It is also assumed that ryanodine binding is not affected by previous temperature acclimation.

(3) Though adrenergic stimulation can effect Ca^{2+} dynamics at both the SL and at the SR, by blocking Ca^{2+} release from the SR with ryanodine, it is assumed that adrenergically mediated increases in force are due primarily to changes in sarcolemmal Ca^{2+} flux.

CHAPTER 2

The effect of temperature and adrenaline on the relative importance of the SR in contributing Ca^{2+} to force development in isolated ventricular trabeculae performing isometric contractions

INTRODUCTION

Examination of excitation-contraction (E-C) coupling in the teleost heart has lead to the suggestion that, unlike mammals, the release of intracellular Ca^{2+} from the SR is not necessary to activate the tropomyosin complex, rather contraction is initiated by a large transsarcolemmal Ca^{2+} influx (Tibbits *et al.*, 1990). This assertion is supported by ultrastructure studies of fish myocytes which have indicated a lack of well developed SR (Santer, 1985). However, the most compelling evidence for the lack of SR involvement in teleost E-C coupling, is the absence of a ryanodine effect. Ryanodine is a neutral plant alkaloid which, when applied in high concentrations (10 μM), binds specifically and irreversibly to the SR-Ca^{2+}-release channel, locking it closed and thus rendering the SR ineffective in contributing Ca^{2+} to force production. As a result, contractile force is typically reduced in adult mammalian cardiac tissues after ryanodine application. In contrast, no substantial reduction in contractile force is observed at physiologically relevant pacing frequencies when ryanodine is applied to teleost ventricular strips (Driedzic and Gesser, 1988; Hove-Madsen and Gesser, 1989; Vornanen, 1996) or *in situ* perfused hearts (Keen *et al.*, 1994) at temperatures between 5° and 20°C. Mammalian cardiac studies have shown the SR-Ca^{2+}-release channel to be highly temperature sensitive (Bers, 1987, 1989, Sitsapesan *et al.*, 1991), spending an increasingly greater proportion of time in the "open-state" as temperature decreases. If the SR-Ca^{2+}-release channel is also temperature dependent in lower vertebrates, as has been suggested (Keen *et al.*, 1992, 1994; Tibbits *et al.*, 1992a; Møller-Nielsen and Gesser 1992), then the failure of most teleosts to

demonstrate a ryanodine response may merely be a result of the temperatures at which they are tested.

Another confounding factor in the assessment of the dynamics of teleost E-C coupling is the role of catecholamines, which are known to influence cardiac contractility (Farrell and Jones, 1992). Adrenaline is the predominant catecholamine in the circulation of most fish. Studies have demonstrated that a tonic level of adrenaline (AD) (5 nM) is present in resting fish (Milligan *et al.*, 1989), with concentrations increasing up to 1000 nM under conditions of extreme stress (McDonald and Milligan, 1992). Furthermore, the importance of this tonic (nM) level of AD for maintaining regular contractions in both teleost and elasmobranch hearts has been demonstrated with *in situ* perfused heart preparations (Graham and Farrell, 1989; Davie and Farrell, 1991). In the myocyte, AD is known to stimulate ß-adrenergic receptors, causing the phosphorylation of the SL L-type Ca^{2+}-channel via cAMP and PKA pathways (Tibbits *et al.*, 1992a). This phosphorylation increases the open probability of the channel (Bers, 1991) allowing for greater transsarcolemmal Ca^{2+} influx with each depolarization and producing in part, the positive inotropic effect of AD. Since the SL Ca^{2+} influx is so central to E-C coupling in teleosts any assessment of the physiological importance of the SR-Ca^{2+}-release channel must consider physiologically relevant states of adrenergic stimulation (tonic and maximal). This has not been done in previous studies. In addition, the adrenergic sensitivity of the myocardium has been demonstrated to decrease with increased temperatures (Graham and Farrell, 1989) due to a reduction in the number of ß-adrenoreceptors on the SL membrane (Keen *et al*, 1993; Gamperl *et al.*, 1996). This could have an effect on the ability of the heart to increase SL Ca^{2+} flux at warm temperatures.

These experiments reassess the significance of the SR in contributing Ca^{2+} to ventricular force development in trout under tonic and maximal adrenergic stimulation at "cold" (12°C)

and "warm" (22°C) temperatures. To ensure physiologically relevant pacing frequencies were studied, the temperature dependency of the ryanodine response was examined over a range of stimulation frequencies.

MATERIALS AND METHODS

Fish origin and maintenance.

Fish (mean weight 1224 ± 102 g, both sexes) were obtained from West Creek Trout Farms, B.C. and held outdoors in a 2000 L fiberglass tank receiving aerated, dechlorinated tap water. The water was maintained at 12°C (± 2°C) throughout the 12°C acclimation and experimental period. On completion of the 12°C acclimation experiments the remaining fish were moved indoors to a similar tank to begin the 22°C acclimation period. Experiments began after a two week acclimation. A water temperature of 22°C was achieved via a countercurrent heat exchanger of local construction. Fish were exposed to a neutral photoperiod (12L:12D) and were offered food three times a week.

Tissue Preparation.

Trout were killed by a sharp blow to the head and the heart was excised and placed in ice-cooled physiological saline (pH 7.8 at 15°C) of the following composition (in mM): NaCl, 124.1; KCl, 3.1; $CaCl_2$, 2.5; $MgSO_4$, 0.9; D-glucose, 5.0. Solutions were buffered with 20 mM TES (Na salt and free acid combinations). The ventricle was isolated and cut lengthwise to expose the lumen. Three trabeculae (mean length of 0.17 cm, and $\leq$ 1 mm in diameter; mean weight of 0.002 g) were dissected out and tied at either end with single strands of 5-0 surgical silk. Trabeculae were then attached by one end to a fixed post and to an isometric force transducer (Kulite Semiconductor Products, Leonia, N.J.) via a fine gold chain on the other. The muscle was then lowered into a water-jacketed organ bath containing 20 mL of oxygenated physiological saline. The organ bath was maintained at either 12°C or 22°C. Trabeculae were paced using a Harvard Student Stimulator (Harvard

Apparatus Ltd., Edenbridge, Kent) delivering charge via two flattened platinum electrodes positioned on either side of the muscle (0.2 Hz-2.0 Hz, 10 V, 10 msec duration). Signals from the force transducer were displayed on a Gould RS3400 chart recorder (Gould, Cleveland, Ohio). Prior to experiments L_{max} (the length at which active tension is maximized) was established. The muscle was allowed to equilibrate at this length for 30 min under basal stimulation (0.2 Hz) before being subjected to one of the following protocols.

Experimental Protocols

The protocols were intended to test the effects of ryanodine in the presence of high (10 μM) and low (10 nM) adrenaline concentrations on cardiac muscle properties at the two acclimation temperatures (12°C and 22°C) and with an acute temperature change (to 12°C from 22°C or to 22°C from 12°C). Because ryanodine binds irreversibly, two protocols were performed on different fish to separate the effects of ryanodine at the two adrenaline concentrations. The sample sizes for each group were as follows: for 12°C acclimated fish, n=7 for protocol 1, n=4 for protocol 2; for 22°C acclimated fish, n=8 for protocol 1, and n=7 for protocol 2. Some preparations became irregular at the highest pacing frequency under each protocol, as such the sample size at the high frequencies may be less than the aforementioned values, however, no mean values represent an n<4. Performance measured with low AD was intended to represent the tonic level of AD found *in vivo*. As such, this measurement acted as control for the subsequent tests on the effect of ryanodine and the effect of the high AD concentration (10 μM) which would result in maximal stimulation of the ventricular tissue (Keen *et al.*, 1993). The adrenaline doses used in our study were the minimum (10 nM) and maximum (10 μM) values of the dose-response curve generated by Keen *et al.* (1993).

Protocol 1

Low AD. Graham and Farrell (1989) established that a tonic level of AD is needed to maintain tone in *in situ* perfused hearts, therefore after the 30 min equilibration period, fresh saline containing 10 nM AD was added to the bath. Following a 5 min equilibration period, the muscle was then subjected to the force-frequency trial; pacing frequency was increased in 0.2 Hz increments, from 0.2 Hz to 2.0 Hz or until muscle failed to show regular contractions. The muscle remained at each frequency until peak force stabilized. At the end of the force-frequency trial, pacing frequency was returned to basal (0.2 Hz).

Ryanodine & Low AD. To examine the relative contribution of the SR in force development, bath saline was replaced with saline containing 10 μM of ryanodine. The preparation was then left to equilibrate at 0.2 Hz (30 min at a bath temperature of 22°C and for 45 min at 12°C). It was assumed that after these equilibration periods, ryanodine was irreversibly bound to the SR-Ca^{2+}-release channel locking it closed and rendering the SR effectively useless in contributing Ca^{2+} to force development. Five minutes prior to the force frequency trial, fresh saline was added containing 10 nM AD.

Ryanodine & High AD The final test in protocol 1 determined the effects of high AD on the ryanodine-treated muscle in an attempt to ascertain how an adrenergically stimulated increase in Ca^{2+} influx via SL channels effects force development during inhibition of the SR-Ca^{2+} release. Fresh saline containing 10 μM of AD was added 5 min before the force-frequency trial was repeated.

Protocol 2

Protocol 2 had a similar format to protocol 1, but the purpose of protocol 2 was to observe whether ryanodine effects were discernible at high levels of AD thereby indicating the relative importance of SR-Ca^{2+} in the presence of increased transsarcolemmal Ca^{2+} influx.

The first force-frequency trial with low AD established control, against which two further trials, one with high AD and the other with high AD after ryanodine, were evaluated.

Standardization

Force is expressed as $mg \cdot mm^{-2}$. Mean cross-sectional area was calculated using muscle mass, trabecular length and an assumed muscle density of 1.06 g cm^{-3}. The length of the trabeculae was measured using a pair of vernier calipers (to 0.1 mm) prior to removing from the apparatus. After removal, the tissue wet weight was determined (to the nearest 0.01 mg).

Drugs

All chemicals and drugs were purchased from either Sigma (St. Louis, MO.) or BDH (Toronto, ON), with the exception of ryanodine which was purchased from Calbiochem (San Diego).

Data Analysis and Statistics

Peak tension, time to peak tension (TPT) and time to half relaxation (THR) were measured from an expanded ($10\ mm \cdot s^{-1}$) chart recorder trace. Approximations of rates of contraction and relaxation were made by dividing peak tension by TPT or THR and are expressed as $mg \cdot s^{-1}$. The effects of pacing frequency, test temperature and acclimation temperature on measured parameters at low AD (basal) conditions are displayed on Table 2.1. Student's t-tests and factorial ANOVA's were used to establish significant differences ($P>0.05$). Differences between percentage (normalized) data were tested non-parametrically by either Krusal-Wallis tests or Mann-Whitney U-tests ($P>0.05$). Pumping capacity (an estimate of power output) was calculated as the product of frequency and peak tension as per Matikainen and Vornanen (1992).

RESULTS

Acclimation to 12°C: Tests at 12°C.

With 10 nM AD, the effect of increasing stimulation frequency from 0.2 Hz to 1.2 Hz was a 70% decrease in peak tension from 163.3 $mg \cdot mm^{-2}$ to 58.6 $mg \cdot mm^{-2}$ (Table 2.1, Fig. 2.1A). TPT also decreased with increased pacing frequency from 0.54 s at 0.2 Hz to 0.37 s at 1.2 Hz. Similarly, THR decreased from 0.36 s at 0.2 Hz to 0.2 s at 1.2 Hz. Rates of contraction and relaxation increased significantly with increases in pacing frequency (Table 2.1). Few preparations maintained regular contractions at pacing frequencies higher than 1.2 Hz at 12°C with 10 nM AD.

The addition of ryanodine, in the presence of 10 nM AD significantly decreased peak tension at low (0.2 Hz) frequencies but not at high (1.2 Hz) frequencies (Fig. 2.1A). Other measured variables were unchanged. Increasing the AD concentration to 10 μM induced a significant positive inotropic response at 0.2 Hz, but not at 1.2 Hz. As a result, the negative inotropic effect of ryanodine was reversed at low frequencies and contractility improved beyond that observed under tonic AD stimulation (Fig. 2.1A).

Protocol 2 specifically examined whether or not ryanodine effects could be detected in the presence of high AD. As with protocol 1, the force generated with 10 μM AD after ryanodine treatment was above that observed with 10 nM AD in all preparations. Addition of ryanodine reduced force by a small percentage in all 4 preparations, but the reduction was not statistically resolvable (Fig. 2.1C). Preparations could not be consistently paced at 1.2 Hz, therefore the high pacing frequency comparisons were made at 1.0 Hz.

Acclimation to 12°C: Tests at 22°C

Increasing the test temperature by 10°C significantly altered ventricular contractility. It also allowed most preparations to maintain regular contractions at frequencies above 1.2 Hz.

Under tonic AD stimulation at 0.2 Hz, peak tension was 35% lower at 22°C when compared with 12°C (156.7 versus 106.1 $mg \cdot mm^{-2}$). However, peak tension at 1.2 Hz was not significantly different between the two test temperatures (Table 2.1). TPT and THR were significantly shorter at 22°C when compared with the 12°C tests. TPT and THR were reduced by 50% and 35%, respectively at 0.2 Hz, and by 40% and 30%, respectively at 1.2 Hz. Rates of contraction and relaxation were not significantly different compared with the 12°C test temperature (Table 2.1).

The effects of increasing pacing frequency from 0.2 Hz to 1.2 Hz at 22°C were similar to those observed at 12°C. Peak tension decreased by 60% (Table 2.1, Fig. 2.1B), likewise, TPT and THR decreased from 0.27 s to 0.22 s and 0.24 s to 0.14 s, respectively. Additionally, the rate of contraction increased significantly with increased pacing frequency (Table 2.1).

In protocol 1, the application of ryanodine under tonic AD stimulation significantly reduced peak tension at 0.2 Hz and 1.2 Hz (Fig. 2.1B). This is in contrast to the 12°C test temperature where ryanodine effects were significant only at low (0.2 Hz) pacing frequencies. Also in contrast to the 12°C group, ryanodine effects were observed on THR (Fig. 2.2A), where values were significantly reduced at 0.2 Hz but not 1.2 Hz. As with the 12°C tests, increasing the AD concentration to 10 μM reversed the negative inotropic effect of ryanodine on peak tension, and improved contractility beyond that observed with tonic AD stimulation alone (Fig. 2.1B). However, unlike the 12°C tests, the AD-mediated restoration of tension was evident at both high and low pacing frequencies at 22°C (Fig. 2.1B). These results suggest that the negative inotropic effects of ryanodine and the ameliorative positive inotropic effects of high AD are inducible at more physiological (1.2 Hz) pacing frequencies when cold-acclimated muscles are tested at warmer (22°C) temperatures.

Table 2.1 The effect of acclimation temperature, test temperature and pacing frequency on measured variables under control conditions (Low AD) in ventricular trabeculae from rainbow trout. Values are group means. Below each value is (n; S.E.M.) Significance ($P<0.05$) is denoted by paired letters which represent the following: for differences between pacing frequencies, within a test temperature, (a) 1.2 Hz is different from 0.2 Hz, (b) 2.0 Hz is different from 0.2 Hz, (c) 1.2 Hz is different from 2.0 Hz; (d) denotes test temperature differences at the same frequency and same acclimation temperature and (e) denotes acclimation differences at the same frequency and same test temperature.

	12°C Acclimation				22°C Acclimation					
	12°C Test		22°C Test		12°C Test			22°C Test		
Variables	0.2 Hz	1.2 HZ	0.2 Hz	1.2 Hz	0.2 Hz	1.2 Hz	2.0 Hz	0.2 Hz	1.2 Hz	2.0 Hz
Peak Tension ($mg \cdot mm^{-2}$)	163.3 [ae]	58.6 [ae]	106.1 [ae]	33.3 [ae]	255.3 [aedb]	130.3 [ace]	75.5 [bc]	182.3 [aed]	106.6 [ac]	78.6 [bc]
	(11; 33.8)	(5; 16.6)	(11; 20.8)	(8; 8.3)	(15; 31.2)	(14; 15.3)	(4; 23.4)	(15; 16.9)	(14; 11.2)	(12; 8.02)
Normalized Peak Tension (%)	100 [a]	39.2 [ae]	100 [a]	35.5 [ae]	100 [abd]	52.3 [ace]	31.3 [bc]	100 [abd]	59 [ace]	42 [bc]
	(11; 0)	(5; 4.0)	(11; 0)	(8; 2.06)	(15; 0)	(14; 3.0)	(4; 10.7)	(15; 0)	(14; 1.82)	(12; 2.51)
Time to Peak Tension (s)	0.54 [ad]	0.37 [aed]	0.27 [ad]	0.22 [aed]	0.51 [abd]	0.33 [acde]	0.23 [cbd]	0.28 [abd]	0.21 [aed]	0.19 [bd]
	(11; 0.01)	(5; 0 .01)	(11; 0 .01)	(8; 0)	(15; 0.02)	(14; 0.01)	(4; 0.01)	(15; 0.006)	(14; 0.004)	(12; 0.002)
Time to Half Relaxation (s)	0.36 [aed]	0.2 [aed]	0.24 [aed]	0.14 [ad]	0.26 [abde]	0.16 [aed]	0.13 [bd]	0.17 [abde]	0.11 [ad]	0.1 [bd]
	(11; 0.03)	(5; 0)	(11; 0.01)	(8; 0.02)	(15; 0.01)	(14; 0.01)	(4; 0.01)	(15; 0.01)	(14; 0.004)	(12; 0.002)
rate of TPT ($mg \cdot s^{-1}$)	299 [e]	161 [e]	387.3 [ae]	154.3 [ae]	496.5 [e]	395.7 [e]	353.3	642.4 [eb]	513.3 [e]	409.5 [b]
	(11; 58.1)	(5; 45.9)	(11; 71.7)	(8; 40.7)	(15; 60.6)	(14; 49.8)	(4; 114.7)	(15; 55.6)	(14; 54.7)	(12; 41.8)
rate of THR ($mg \cdot s^{-1}$)	410 [e]	288 [e]	379.7 [e]	228.3 [e]	960.9 [e]	821.4 [e]	680.5	1112.7 [e]	984.3 [e]	804.1
	(11; 93.5)	(5; 73.6)	(11; 61.8)	(8; 49.6)	(15; 107.6)	(14; 101.0)	(4; 222.5)	(15; 126.3)	(14; 111.7)	(12; 90.3)

Increasing the AD concentration from 10 nM to 10 μM in the second protocol caused significant increases in peak tension at both 0.2 and 1.2 Hz (Fig. 2.1D), again demonstrating the positive inotropic effects of AD over the whole range of pacing frequencies. Other variables remained unchanged. Addition of ryanodine at this high AD concentration caused significant decreases peak tension at 0.2 Hz but not at higher pacing frequencies. Other variables were not significantly effected.

Acclimation to 22°C: Tests at 12°C.

Acclimation to 22°C resulted in significantly higher attainable pacing frequencies (2.0 Hz at 22°C versus 1.2 Hz at 12°C) and thus statistical comparisons are made at three pacing frequencies for the 22°C acclimated fish. Similar to the 12°C acclimation, increasing pacing frequency caused significant decreases in peak tension, TPT and THR. Under tonic AD stimulation, peak tension fell by 50% from 255 $mg \cdot mm^{-2}$ at 0.2 Hz to 130 $mg \cdot mm^{-2}$ at 1.2 Hz and by another 20% to 75 $mg \cdot mm^{-2}$ at 2.0 Hz (Table 2.1, Fig. 2.3A). TPT was 0.51 s at 0.2 Hz, and decreased significantly to 0.33 s at 1.2 Hz, and further to 0.23 s at 2.0 Hz. THR also decreased significantly from 0.26 s at 0.2 Hz to 0.16 s at 1.2 Hz, and then to 0.13 s at 2.0 Hz (Table 2.1). Additionally, rates of contraction and relaxation were significantly faster with increased pacing frequency (Table 2.1).

The effect of ryanodine application on peak tension development was similar to the 12°C tests after 12°C acclimation, showing significant reductions at 0.2 Hz (Fig. 2.3A) but not at 1.2 Hz. However, unlike 12°C acclimated fish, ryanodine affected other variables in 22°C acclimated fish when tested at 12°C test. THR was significantly reduced by ryanodine incubation (Fig. 2.2B) and the rate of relaxation was decreased at 0.2 Hz, but not at 1.2 Hz or 2.0 Hz (not shown). There were no significant effects on the other variables measured. Increasing the AD concentration to 10 μM caused a similar positive inotropic response in peak tension to that observed with the 12°C tests at 12°C acclimation. As such, at 0.2 Hz,

Figure 2.1 Force-frequency relationships from ventricular trabeculae from rainbow trout normalized to the Low AD and 0.2 Hz treatment for fish acclimated to 12°C, at each test temperature (upper right corner) and each protocol (protocol 1 A&B, top panel; protocol 2 C&D, lower panel). Results are displayed as line graphs to show progressive changes with increased frequency and as bar graphs to highlight drug effects at high and low frequencies. Values are group means. Vertical bars represent ±1 S.E.M. * denotes significant differences ($P<0.05$) between all drug treatments within each frequency. † denotes difference between Low AD and both High AD and High AD & ryanodine but no differences between the latter two. Within each drug treatment, there is a significant change in peak tension when pacing frequency is increased from 0.2 Hz to 1.2 Hz (or 1.0 Hz) with the exception of the High AD treatment in (C).

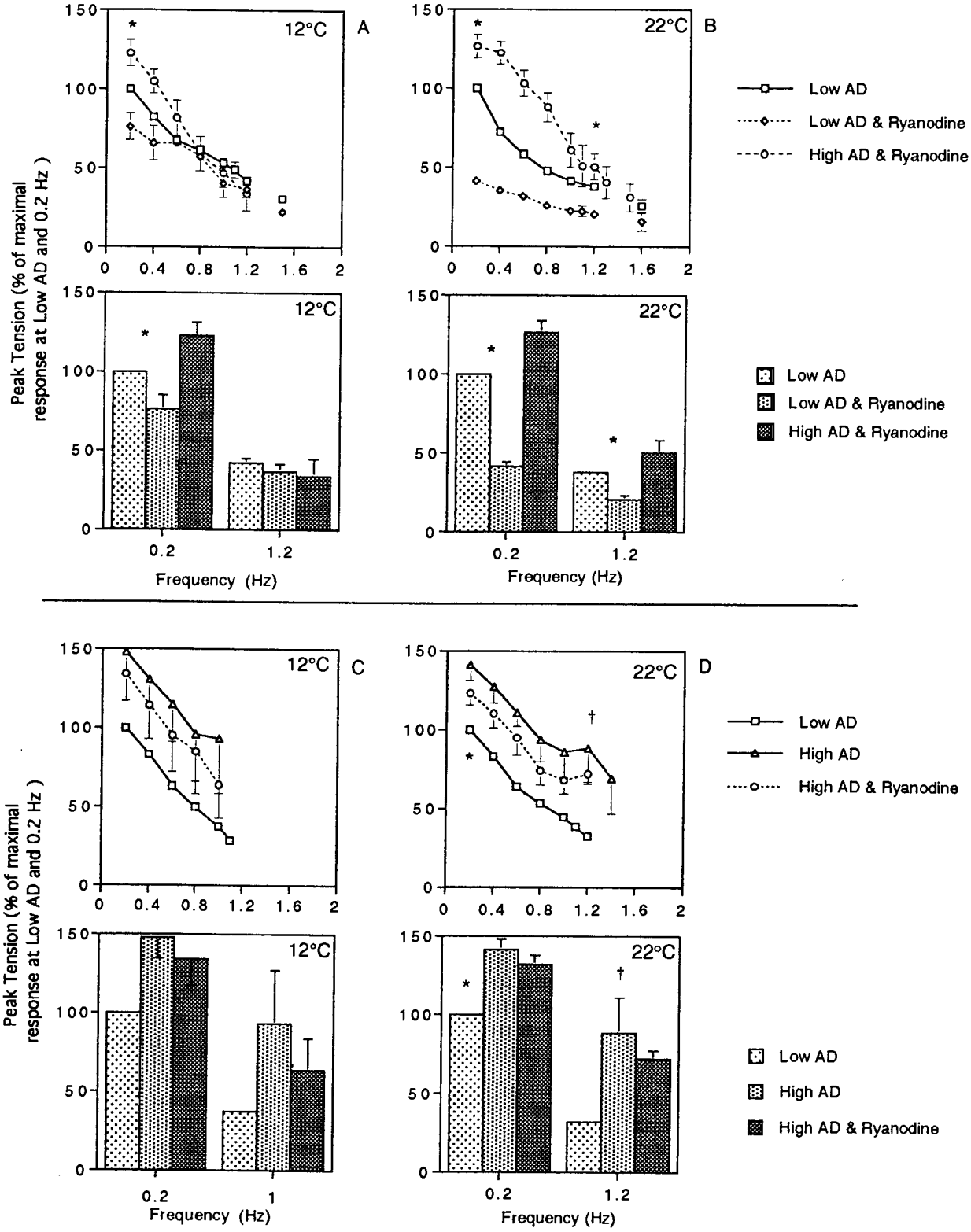
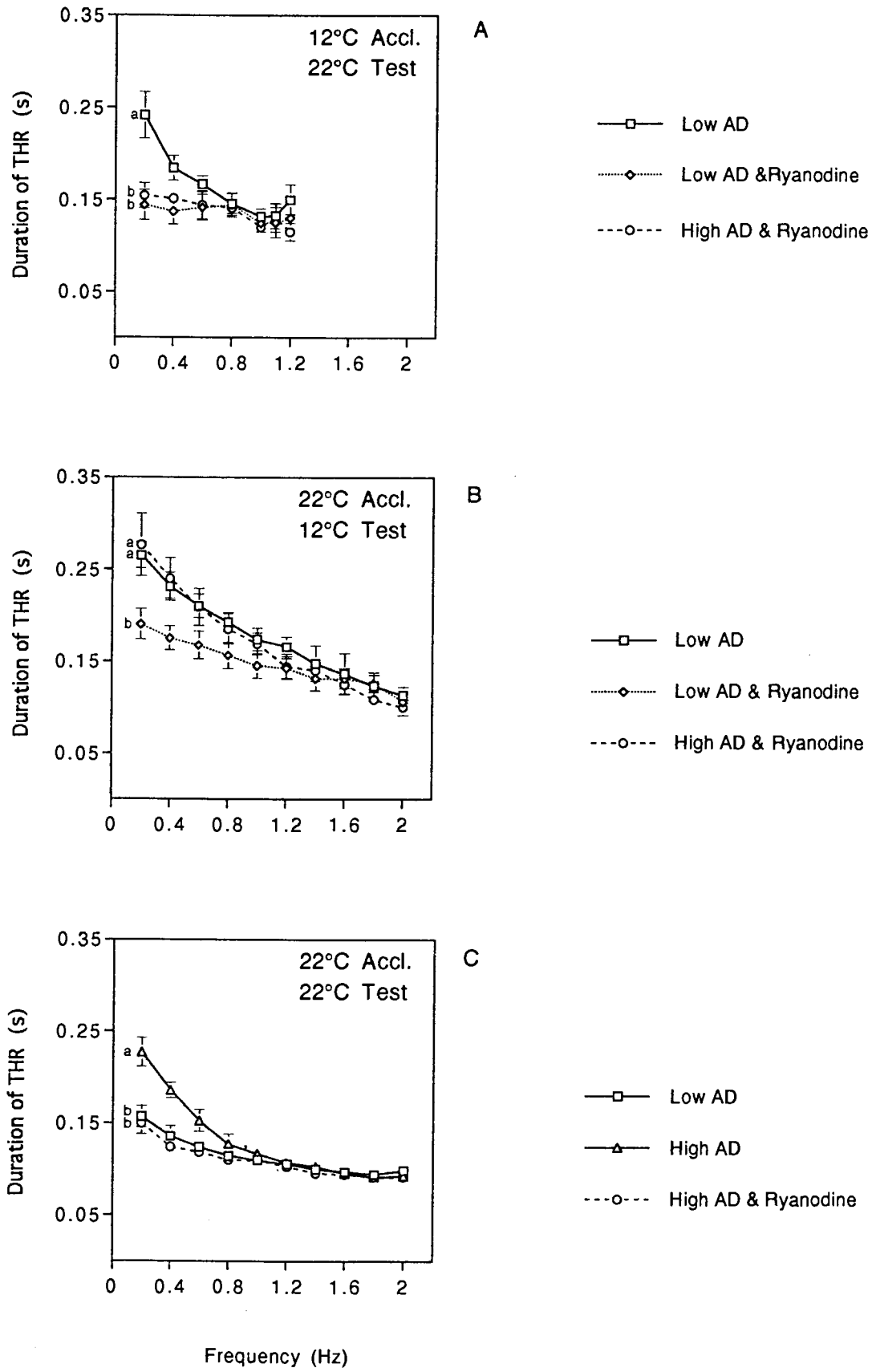

12°C
A
22°C
B
Low AD
Low AD & Ryanodine
High AD & Ryanodine
Peak Tension (% of maximal response at Low AD and 0.2 Hz)
Frequency (Hz)
C
D
High AD

Figure 2.2 Effects of increased pacing frequency and drug treatment on duration of time to half relaxation (THR) in ventricular trabeculae from rainbow trout. Test temperature and acclimation temperature are indicated in the upper right corner of each graph. (A) & (B) are protocol 1, (C) is protocol 2 as expressed in the figure legends. Values are group means. Vertical bars represent ±1 S.E.M. Within each frequency, significant differences ($p<0.05$) between drug treatments are denoted by dissimilar letters. Within each drug treatment, significant differences exist between 0.2 Hz and 1.2 Hz but not between 1.2 Hz and 2.0 Hz.


A
12°C Accl.
22°C Test
Duration of THR (s)
0.35
0.25
0.15
0.05
0
0.4
0.8
1.2
1.6
2
a
b
b
Low AD
Low AD &Ryanodine
High AD & Ryanodine
B
22°C Accl.
12°C Test
Duration of THR (s)
a
a
b
Low AD
Low AD & Ryanodine
High AD & Ryanodine
C
22°C Accl.
22°C Test
Duration of THR (s)
a
b
b
Low AD
High AD
High AD & Ryanodine
Frequency (Hz)

high AD stimulation overshadowed the ryanodine effects and increased peak tension beyond that observed under tonic AD stimulation (Fig. 2.3A).

In protocol 2, increasing the AD concentration from 10 nM to 10 μM caused a significant increase in peak tension at 0.2 Hz (Fig. 2.3C). Associated with this positive inotropy was a significantly longer THR at 0.2 Hz (not shown), but there were no other significant changes. Addition of ryanodine under high AD stimulation significantly decreased peak tension at 0.2 Hz but not at 1.2 Hz or 2.0 Hz (Fig. 2.3C), again demonstrating that ryanodine effects are only prevalent at low (0.2 Hz) (sub-physiological) pacing frequencies when tested at low (12°C) temperatures. This limited ryanodine response, especially at low pacing frequencies, is irrespective of warm (22°C) or cold (12°C) acclimation.

Acclimation to 22°C: Tests at 22°C

As with the 12°C acclimated fish tested at 22°C, contractility of the 22°C-acclimated fish was different when tested at 22°C compared with 12°C. With a tonic level of AD, peak tension was 182 $mg \cdot mm^{-2}$ at 0.2 Hz which was significantly lower than the 255.5 $mg \cdot mm^{-2}$ observed at the 12°C test temperature (Table 2.1). TPT was 55% shorter than the 12°C tests at 0.2 Hz, 35% shorter at 1.2 Hz and 20% shorter at 2.0 Hz. THR was also significantly shorter at all frequencies when compared with the 12°C test temperature (Table 2.1).

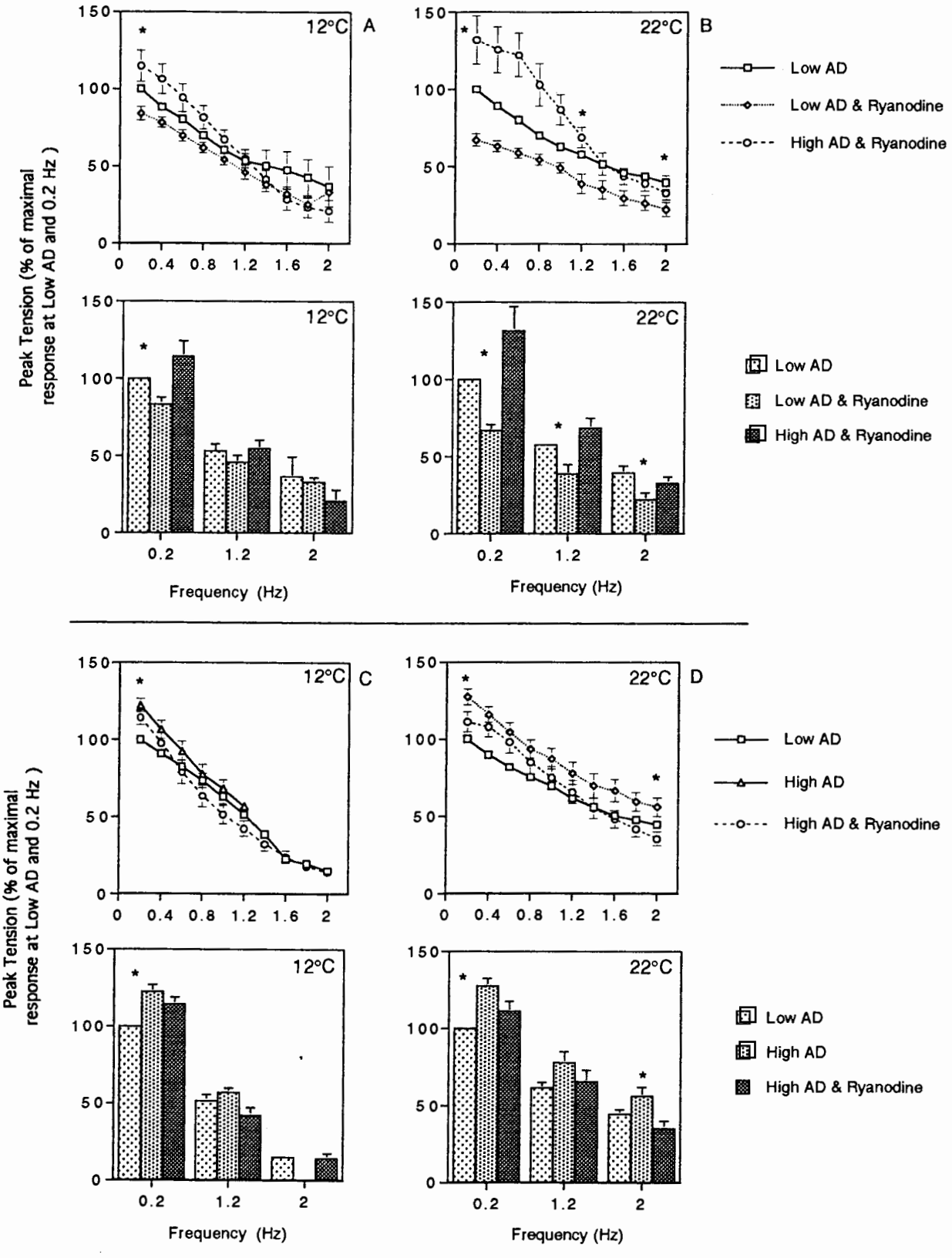
As with all other test groups a negative force-frequency relationship was observed in response to increased pacing frequency. With low AD, peak tension was decreased by 55% as pacing frequency was increased from 0.2 Hz to 2.0 Hz. (Table 2.1, Fig. 2.3A-D). TPT and THR were also significantly decreased by increased pacing frequency. TPT fell from 0.28 s at 0.2 Hz to 0.21 s at 1.2 Hz but no further at 2.0 Hz. THR fell from 0.17 s at 0.2 Hz to 0.11 s at 1.2 Hz, but no further at 2.0 Hz.

With tonic AD, application of ryanodine in protocol 1 significantly reduced peak tension at 0.2 Hz, 1.2 Hz and 2.0 Hz (Fig. 2.3B). This is in contrast to the 12°C test temperature which showed ryanodine effects only at low pacing frequencies. Thus induction of a significant ryanodine response at physiological pacing frequencies (1.2-2.0 Hz) is dependent upon a warm test temperature. Ryanodine incubation also caused significantly shorter THR at 0.2 Hz but not at 1.2 Hz or 2.0 Hz (not shown), and was without significant effects on the other variables. As in the previous test, increasing the AD concentration to 10 μM reversed these ryanodine effects, restoring tension at 0.2 Hz, 1.2 Hz and 2.0 Hz. However, the adrenaline-mediated increase in tension was less at 1.2 Hz than at 0.2 Hz, and lesser still at 2.0 Hz (Fig. 2.23B). This suggests that the ability of adrenaline to compensate for the inhibition of the SR, is reduced as frequencies increase. At 0.2 Hz but not at 1.2 Hz or 2.0 Hz, this improvement in peak tension was associated with a significantly higher rate of relaxation (not shown).

In protocol 2, increasing the AD concentration from 10 nM to 10 μM, significantly increased peak tension at both 0.2 Hz and 2.0 Hz (Fig. 2.3D). This is in contrast to the 12°C test where the positive inotropic effects of AD could only be seen at low stimulation frequencies (Fig. 2.3C). THR was significantly increased at 0.2 Hz by 10 μM AD (Fig. 2.2D), but other measured variables were not significantly changed.

Application of ryanodine, in the presence of high AD, significantly decreased peak tension at both 0.2 Hz and 2.0 Hz (Fig. 2.3D) which again, is in contrast to the 12°C tests where ryanodine effects where observed only at 0.2 Hz (Fig. 2.3C). This lends further credence to the suggestion that in trout ventricular muscle, the responsiveness to ryanodine at physiological pacing frequencies is increased after acute exposure to warm temperature.

Figure 2.3 Force-frequency relationships from ventricular trabeculae from rainbow trout normalized to the Low AD and 0.2 Hz treatment for fish acclimated to 22°C, at each test temperature (upper right corner) and each protocol (protocol 1 A&B, top panel; protocol 2 C&D, lower panel). Results are displayed as line graphs to show progressive changes with increased frequency and as bar graphs to highlight drug effects at high and low frequencies. Values are group means. Vertical bars represent ±1 S.E.M. * denotes significant differences ($P<0.05$) between all drug treatments within each frequency. Within each drug treatment, significant differences were found between pacing frequencies (0.2 Hz, 1.2 Hz and 2.0 Hz).


12°C
A
22°C
B
Low AD
Low AD & Ryanodine
High AD & Ryanodine
Peak Tension (% of maximal response at Low AD and 0.2 Hz)
Frequency (Hz)
C
D
High AD

Accompanying this ryanodine-mediated decrease in peak tension, were significant decreases in THR (Fig. 2.2C).

Effects of Temperature Acclimation

Under tonic AD stimulation peak tension was significantly increased (by 65% at 0.2 Hz and by 125% at 1.2 Hz) by 22°C acclimation. Additionally, 22°C acclimation allowed consistent pacing at higher frequencies (2.0 Hz). However, the overall reduction in peak tension which occurred when pacing frequency was increased from 0.2 Hz to the frequency at which contractions became irregular, remained approximately the same (60-70%) at both acclimation temperatures (Table 2.1).

Acclimation to 22°C also influenced the responsiveness of the muscles to ryanodine. With tonic AD and high AD stimulation, reductions in peak tension were observed at high and low frequencies (Fig. 2.3) after 22°C acclimation but only at low frequencies with 12°C acclimation (Fig. 2.1). Nonetheless, test temperature, rather than acclimation temperature seemed to be the predominant influencing factor in the high frequency pervasiveness of the ryanodine response. This is shown by the significant reductions in peak tension observed at both high (1.2 Hz or 2.0 Hz) and low (0.2 Hz) frequencies when tested at 22°C, irrespective of acclimation temperature.

The second protocol revealed that muscles from the 12°C acclimation group were more responsive to high AD stimulation. This was demonstrated by 50% increases in peak tension at both 0.2 Hz and 1.2 Hz (Fig. 2.1C&D) after 12°C acclimation, compared with approximately 25% increases at the same frequencies after 22°C acclimation (Fig. 2.3C&D). This increased AD sensitivity was also observed in the first protocol where AD mediated increases in tension after ryanodine treatment was greater after 12°C acclimation. This may be related to the acclimation-induced change in adrenaline sensitivity reported by

Keen *et al.*, (1993) which suggests that fish cardiac muscle exhibits greater AD sensitivity after cold acclimation.

Of the other variables measured, only THR was significantly effected by acclimation. These effects however were only prevalent at the low pacing frequencies (Fig. 2.2). Warm temperature acclimation caused a significantly shorter THR (Table 2.1). The muscle's response to ryanodine, as assessed by THR, was also affected by acclimation temperature. This was shown by significant reductions in THR at 0.2 Hz under 22°C acclimation at both test temperatures (Fig. 2.2B&C) but only at the 22°C test temperature under 12°C acclimation (Fig. 2.2A). The compensatory effect of high AD on THR duration was observed only after 22°C acclimation and only at 22°C test (Fig. 2.2B). High AD stimulation in the second protocol caused a significant increase in THR at both test temperatures under 22°C acclimation. However, ryanodine-induced reduction at high AD stimulation, was only evident at 22°C test (Fig. 2.2C). These results suggest that THR at 0.2 Hz is profoundly influenced by temperature, being most responsive to both AD and ryanodine when tested at 22°C after 22°C acclimation. However, because all responses where observed only at 0.2 Hz, it is questionable whether these results have physiological relevance.

DISCUSSION

Force-frequency response

This study demonstrated a consistent decline in peak tension with increased pacing frequency irrespective of adrenaline concentration, ryanodine treatment or temperature change (Table 2.1). This negative force-frequency response or negative staircase, is consistent with the "typical" teleost force-frequency response reported by Driedzic and Gesser (1988), Hove-Madsen (1992) and Matikainen and Vornanen (1992). However, though the negative force-frequency response is considered typical for fish, other studies

have suggested the shape of the response curve to be both temperature and ryanodine dependent. Hove-Madsen (1992) reported a monophasic decline in force with increased frequency from trout tested at 25°C which is in agreement with the results presented in this paper, however, when testing trout strips at 15°C, they found a biphasic response with a minimum at 0.2 Hz. Additionally, they noticed a reversal of the negative force-frequency response after ryanodine treatment at 25°C. Matikainen and Vornanen (1992) report a change in the force-frequency response with acclimation. In their study, cold-acclimated (5°C) fish show the typical negative force-frequency relationship, whereas the warm-acclimated (15°C) fish demonstrate a positive force-frequency relationship. In this study, neither ryanodine nor temperature converted the negative force-frequency response to a positive or biphasic one, but did however, significantly alter inotropic force development. It is possible that these differences relate to the fact that, unlike this study, none of the earlier studies used a tonic level of adrenergic stimulation though Keen *et al.*, (1993) have clearly shown that adrenergic sensitivity and receptor density in rainbow trout heart is temperature dependent. Thus, in the absence of tonic AD, SL- Ca^{2+} flux during excitation may vary significantly with temperature acclimation.

Peak tension

Peak tension is a reliable indicator of the inotropic capabilities of the heart. This study illustrated the ability of temperature, adrenaline and ryanodine to alter peak tension with the effectiveness of each treatment varying with pacing frequency. This work demonstrates that trout ventricular muscle is responsive to ryanodine, which indicates SR involvement in supplying the contractile elements with Ca^{2+}. However, it is evident that at low temperatures (12°C) the ryanodine response is observed only at sub-physiological pacing frequencies (0.2 Hz). Thus, in agreement with Driedzic and Gesser (1988), Keen *et al.*, (1994) and Vornanen (1996), it is concluded that at low temperatures the SR is probably not important in contributing Ca^{2+} to routine contractions, even in the presence of tonic AD.

A novel finding is that at 12°C, high AD concentrations overwhelm the negative inotropic effects caused by ryanodine and increase force beyond control levels. These results suggest that at 12°C, the trout heart can nearly double force generating ability by increasing Ca^{2+} influx via SL channels, negating the necessity of intracellular (SR) contribution. This increased force associated with high AD at 12°C may reflect an increased adrenergic sensitivity of the myocardium and heighten SL ß-receptor density (Keen *et al.*, 1993). However, though increasing Ca^{2+} influx via SL channels may compensate for the loss of SR-Ca^{2+} at lower frequencies, it may not be able to do so high frequencies, especially at high temperatures. This was demonstrated by the adrenaline-mediated amelioration of the negative inotropic effect of ryanodine at both acclimation temperatures and test temperatures between 0.2 Hz and 1.2 Hz only. At pacing frequencies beyond 1.2 Hz, despite high AD stimulation, peak tension fell equal to or below those obtained with tonic AD.

The positive inotropic response observed after adrenergic stimulation is due to increased transsarcolemmal Ca^{2+} influx mediated by the ß-adrenergic signal transduction pathway. Adrenaline may also induce positive inotropy via an α-adrenergic pathway. Stimulation of the α-adrenoreceptor in mammals increases the Ca^{2+} sensitivity of the myofilaments and can increase intracellular Ca^{2+} concentrations through the actions of inositol 1,4,5-triphosphate (IP_3) (Benfey, 1990). IP_3 induces the release of Ca^{2+} from the SR in smooth muscle cells, but has been ruled out as the primary catalyst for SR-Ca^{2+}-release in mammalian cardiac cells because the rate and degree of IP_3-induced Ca^{2+}-release is significantly lower than Ca^{2+}-induced Ca^{2+}-release (Kentish *et al.*, 1990). However, the role of the IP_3 in E-C coupling in fish, is yet unknown. Indeed, though α-receptors may be present on the SL membrane, they do not mediate positive inotropy or chronotropy in trout (Ask *et al.*, 1983; Farrell *et al.*, 1986) flounder, (Ask *et al.*, 1983), and carp, (Vornanen, 1989) but may in eel (Peyraud-Waitzenegger *et al.*, 1980) and perch, (Tirri and Lehto, 1984). Additionally, it is possible, that similar to rainbow trout hepatocytes,

myocyte cytosolic IP_3 is not modulated by adrenergic stimulation of the α-adrenoreceptor (Fabbri *et al.*, 1995).

Other experimental approaches, such as specific ß-agonists, could have been used in this study to avoid the possibility of activating both α- and ß-adrenoreceptors. Additionally, increasing extracellular Ca^{2+} levels could increase transsarcolemmal Ca^{2+} influx without activating either signal transduction pathway. However, though both of these approaches, in conjunction with ryanodine application, would be useful in determining the relative importance of intracellular and extracellular Ca^{2+} flux during E-C coupling, for the purpose of this study, realistic levels of adrenergic stimulation were used in an effort to best assess the physiological relevance of each potential source of activator Ca^{2+}.

This study is the first to demonstrate that at 22°C, ryanodine is capable of depressing peak tension at physiologically relevant pacing frequencies in a temperate fish species. This implies that at warm test temperatures (irrespective of acclimation temperature), the SR contributes a significant, albeit small, proportion of activator Ca^{2+} for routine (>0.6 Hz) contractions. This appears to be a novel finding. Previous studies (with the skipjack tuna as a notable exception (Keen *et al.*, 1992)), have demonstrated ryanodine sensitivity at warm test temperatures but not at physiologically relevant pacing frequencies (Keen *et al.*, 1994; Hove-Madsen, 1992). The difference between the results from this study and past studies may be related to the failure of previous studies to utilize a tonic level of AD. The use of tonic adrenaline increases the attainable peak tension at control levels which may facilitate observations of changes in force which were undetectable in previous work.

The finding that a warm test temperature, more so than acclimation temperature amplifies the ryanodine response, is consistent with results from Keen *et al.* (1994). At a test temperature of 8°C, Keen *et al.* (1994) were unable to demonstrate a significant effect of

ryanodine regardless of acclimation temperature and pacing frequency. This thermal dependence of the ryanodine response is also consistent with the known gating and conductance properties of the SR-Ca^{2+}-release channel in the mammalian myocardium, where the open state probability of the channel increases with temperature reductions (Sitsapesan *et al.*, 1991). Our results are also in accordance with observations of the effects of rapid-cooling on Ca^{2+} release from the SR in mammals (Bers, 1987, 1989). Rapid cooling of mammalian cardiac tissue to 1°C results in a prolonged contracture that has been equated with the release of Ca^{2+} from SR stores (Bers, 1987, 1989). If this is a general property of all vertebrate cardiac tissue, then, as suggested by Keen *et al.* (1992) and Tibbits *et al.* (1991), low ambient temperatures experienced by ectotherms would promote the open probability of the SR-Ca^{2+}-release channel, reducing the effectivness of the SR in sequestering and releasing Ca^{2+} during contraction. This rationale can be used to explain the absence of a ryanodine effect at physiological pacing frequencies in our 12°C groups. The acquisition of a ryanodine response at 0.2 Hz in the 12°C group, may be attributed to the fact that temperatures much lower than 12°C are necessary for complete inactivation of the SR-Ca^{2+}-release-channels in fish. Therefore, a sufficient number of channels remain functional at 12°C such that, at slow contraction rates (0.2 Hz), the SR maybe able to accumulate enough Ca^{2+} between depolarizations to contribute significantly to force development. However, *in vivo*, and at cold temperatures, this SR contribution is probably insignificant at physiological heart rates and is easily overwhelmed by adrenergic stimulation. In contrast, when trout explore water temperatures near their upper thermal tolerance level, SR-Ca^{2+} plays a secondary role (25%) in cardiac contractility at physiological heart rates. Because this involvement is independent of temperature acclimation, and since salmonids behaviourally exploit thermoclines (Brett, 1971), SR-Ca^{2+} release may become an important factor when trout move from the colder depths of a lake, to feed in the warmer upper waters as well as for fish routinely living in warm water. The increased involvement of SR-Ca^{2+} in E-C coupling at warm temperatures may also be

related to the reduced adrenergic sensitivity of the myocardium. Regardless of test temperature and adrenergic sensitivity, maximal adrenergic stimulation (i.e. increasing the SL Ca^{2+} influx) can overwhelm the SR-Ca^{2+} signal, but more at low frequencies than high frequencies.

Contraction Kinetics

TPT and THR provide information on the kinetics of each contraction. This study demonstrates changes in the duration of both variables with changes in test temperature, acclimation temperature and pacing frequency as summarized in Table 2.1. However, only THR was significantly affected by drug treatment and then, only at low pacing frequencies. In all cases addition of ryanodine caused a reduction in THR at 0.2 Hz, having the most significant effects where ryanodine-induced reductions in force were most significant (22°C test temperature after 12°C acclimation and at both test temperatures after 22°C acclimation). The most probable explanation for the faster relaxation in the presence of ryanodine is that $[Ca^{2+}]_i$ is lower (as determined by tension loss in ryanodine-treated muscle) and as such, Ca^{2+} extrusion is faster. Additionally, the relatively large time interval between depolarizations at low frequencies, may allow Ca^{2+} to leak out of the SR and be extruded by either SL Ca^{2+}-pump, SR Ca^{2+}-pump or the Na^+/Ca^{2+} exchanger. Locking the SR-Ca^{2+}-release-channel in a closed state with ryanodine, may prevent or slow Ca^{2+} leakage from the SR and as such allow extrusion mechanisms to be entirely devoted to removal of activator Ca^{2+} and thus decrease THR.

Mammalian studies have demonstrated faster rates of relaxation under adrenergic stimulation due to increased activity of the SR-Ca^{2+}-pump and stimulation of the Na^+-pump, which ultimately increases Ca^{2+} efflux via Na^+/Ca^{2+} exchange (Bers, 1991). However, these responses have yet to be demonstrated in fish and the effect of temperature acclimation on these processes is unknown. In contrast to mammalian responses, this

study showed slower rates of THR and longer THR after adrenergic stimulation at 22°C. Further, after the reduction in THR by ryanodine, high AD increased THR to pre-ryanodine levels. Thus the combination of warm temperature and adrenaline seem to slow relaxation (Fig. 2.2B&C). Studies assessing the temperature dependency of both the Na^+/Ca^{2+} exchanger (between 4°-15°C) (McKnight *et al.*, 1989) and the SR Ca^{2+}-pump (between 0°-20°C) (for skeletal muscle, Toledo *et al., 1995*) for rainbow trout, suggest little variation in activity with temperature change. As such, variable extrusion capabilities between acclimation temperatures does not seem a probable explanation the slowed relaxation at 22°C after adrenergic stimulation. Therefore, perhaps an explanation can be formed based on intracellular Ca^{2+} levels. After 22°C acclimation, when peak tension is higher and relaxation is faster than after 12°C acclimation (Table 2.1), the Ca^{2+} extrusion mechanisms may be operating near to maximal, and as such, cannot sustain rapid relaxation when $[Ca^{2+}]_i$ is increased by high adrenaline, resulting in prolonged THR. Clearly, further studies are needed to better elucidate the effect of adrenaline and ryanodine on the kinetics of myocardial relaxation in fish.

Cardiac Pumping Capacity

Pumping capacity, the product of heart rate and peak tension was used to estimate the power or pumping ability of the isolated muscle (Fig. 2.4). The measure of pumping capacity provides a more integrative look at the effects of frequency, temperature and drug treatment on ventricular performance. Moreover, it provides a means to evaluate the pacing frequency at which pumping capacity is highest. Consistent with the conclusions drawn from the inotropic responses, pumping capacity is reduced by ryanodine application and increased by adrenergic stimulation. After 12°C acclimation, the effect of ryanodine on pumping capacity is more pronounced at low levels of adrenergic stimulation but these effects are not statistically resolvable. Pumping capacity was very sensitive to temperature acclimation with superior pumping ability after 22°C acclimation (note the different scales in

Figure 2.4 Pumping capacity (product of frequency and peak tension) for isolated ventricular trabeculae from rainbow trout. 12°C acclimation are graphs A&B; 22°C acclimation are graphs C&D. Note that the scales for each acclimation temperature are different. Test temperature is indicated in the upper left corner of each graph. Values are group means. Vertical bars represent ±1 S.E.M. Significance ($p \leq 0.05$) was established for graph D: there is a significant effect of ryanodine treatment at both Low AD and High AD at 1.6, 1.8 and 2.0 Hz. The effect of High AD treatment was significant at all frequencies.

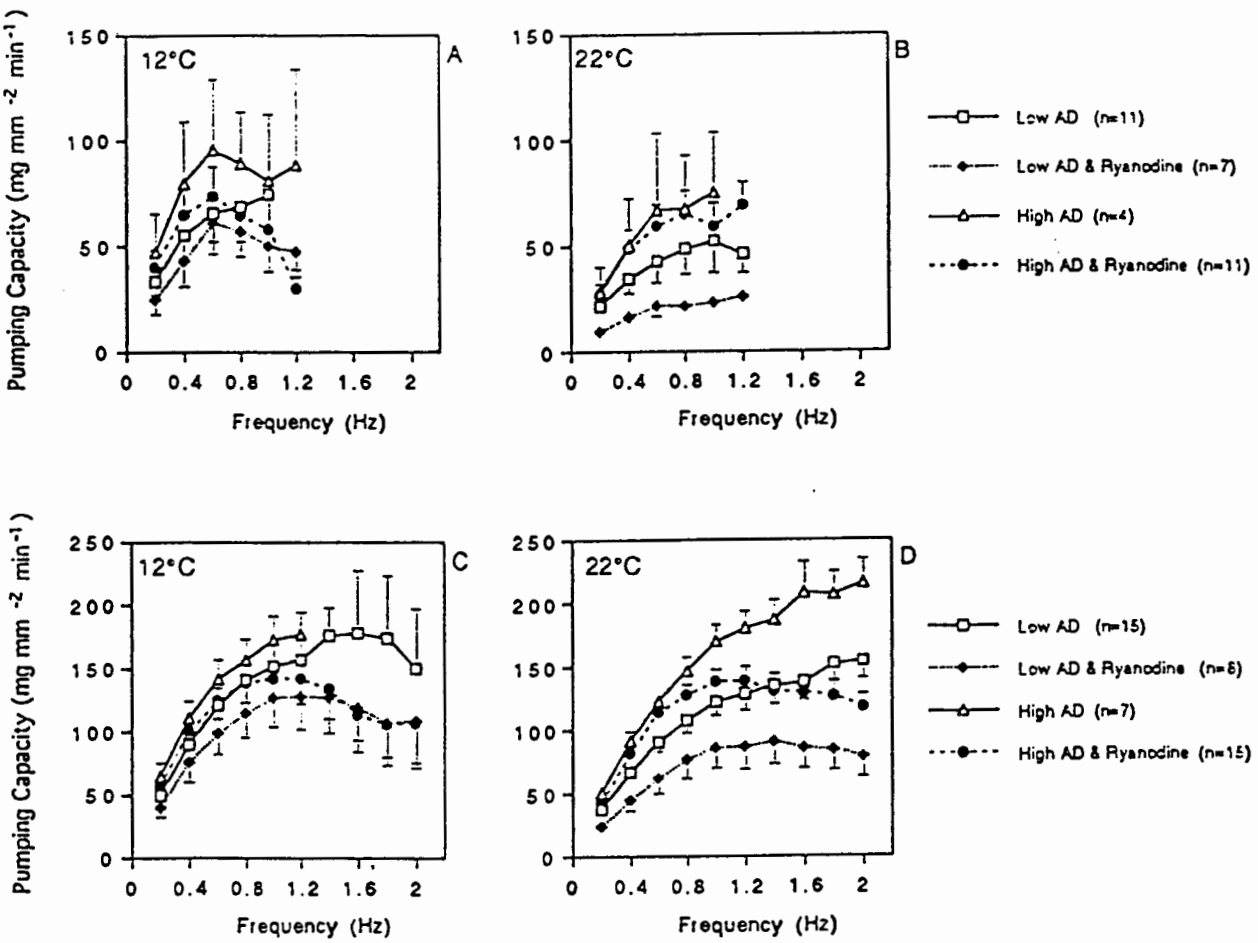


Fig. 2.4) which is in accordance with findings from crucian carp which report that warm acclimation (20°C) confers superior heart rate, isometric force development and pumping capacity compared with cold acclimation (4°C) (Matikainen and Vornanen, 1992). Additionally, pumping capacity is maintained at higher frequencies after 22°C acclimation than after 12°C acclimation, but is particularly sensitive to ryanodine. Ryanodine reduces the ability to pump at high frequencies, and tends the apex of the curves to the left (Fig. 2.4). Furthermore, it is evident that though high levels of adrenaline can compensate for the ryanodine-induced loss of power, it can only do so up to 0.8-1.0 Hz. Afterwhich, pumping capacity falls presumably suffering from lack of intracellular Ca^{2+} contribution. This suggests that blocking Ca^{2+} release from the SR reduces the pumping power of the ventricle at high frequencies.

SUMMARY

The Ca^{2+} requirement for E-C coupling in rainbow trout, as in most other teleosts, is primarily derived from transsarcolemmal Ca^{2+} influx. However, at warm temperatures (22°C) the SR can contribute a significant proportion of Ca^{2+} to force development, the magnitude of which is frequency dependent (~40-60% at 0.2 Hz, ~25-35% at 1.2 Hz and ~20% at 2.0 Hz). Adrenergic modulation of the SL significantly alters extracellular Ca^{2+} influx as increases in peak tension were consistently observed upon maximal adrenergic stimulation. This increased SL Ca^{2+} influx decreases the importance of SR Ca^{2+} contribution, though at high levels of adrenergic stimulation, the SR is still contributing a small amount (~10%) of activator Ca^{2+}. Nevertheless, SL Ca^{2+} influx dominates E-C coupling in the trout heart at both warm and cold temperatures. Interestingly, at high pacing frequencies (>1.2 Hz) adrenergically stimulated positive inotropy can not compensate for the negative effect of ryanodine, which suggests that the ability to maintain force at high frequencies may involve recruitment of SR stores. The slight left-ward shift of the optimum frequency for pumping capacity after ryanodine treatment at high

temperatures lends support to this contention. Although these results are not entirely conclusive, it is tempting to suggest that warm temperatures allow fish to utilize SR-Ca^{2+} stores which lay dormant at low temperatures due to the temperature-dependency of the SR-Ca^{2+}-release channel. This line of thought can be furthered by suggesting that this ability to utilize SR stores, places the source of Ca^{2+} (SR) in closer proximity to the contractile elements, thereby allowing for faster activation and de-activation with each pulse, ultimately increasing the rate at which the heart can contract. Additionally, the fact the adrenergic sensitivity of the myocardium is reduced after warm-temperature acclimation (Graham and Farrell, 1989; Keen *et al*, 1993; Gamperl *et al.*, 1994,1996), invites the speculation that an increased dependence on the SR corresponds to the reduced ability of the muscle to increase Ca^{2+} availability via adrenergic modulation of the SL.

Thus the interplay between SR and SL Ca^{2+} flux appears to be frequency, and temperature dependent, with the SL playing the dominant role in delivering and removing Ca^{2+} during contraction in trout ventricular muscle at temperatures around the preferred. However, as temperature approaches the UILT, sole dependence on SL Ca^{2+} flux may limit the ability of the fish to maintain contractility at the elevated contraction frequencies concomitant with elevated temperatures. Under such conditions the relative importance of the SR may increase allowing the heart to maintain pumping ability at warm temperatures and elevated heart rates.

CHAPTER 3

Using the work loop technique to re-examining the relationship between SR and SL Ca^{2+} contribution to force development in isolated ventricular trabeculae from rainbow trout

INTRODUCTION

The isometric estimation of power output termed "pumping capacity" provided insight into the effects of modulating SR and SL function with temperature and adrenaline respectively, under isometric conditions. Frequency modulation of pumping capacity could also be estimated from the muscles performing isometric contractions. The work loop technique provides information on the actual work and power output of muscles *in vitro* allowing insight into the performance capabilities of the heart under conditions closely resembling those experienced *in vivo*. In the work loop technique, a muscle is subjected to cyclic length change (strain) and is stimulated to contract and do work while shortening. Net work output is measured as the area of the loop formed when muscle force is plotted against muscle length over the full range of length change (Syme, 1993). Mean power output ($W \cdot kg^{-1}$) is calculated as the product of net work and cycle frequency. This determination of power may provide greater insight into the modulating effects of adrenaline and temperature on cardiac function. More specifically by subjecting the muscle to a physiologically realistic pattern of cyclical contractions, this preparation will test the validity of the frequency response achieved under isometric conditions, since contraction and relaxation processes may be affected by length change. Indeed, any step in the activation process may depend upon muscle length. Specifically, action potential duration (APD), the rapid rise and fall of cytoplasmic free Ca^{2+} concentrations (the Ca^{2+} transient) and the Ca^{2+} sensitivity of the myofibrils have all been demonstrated to be sensitive to changes in muscle length (Allen and Kentish, 1985). By oscillating the trabeculae around

the length optimized for maximal work (L_{opt}), it is conceivable that length-dependent mechanisms, absent under isometric conditions, become operative.

The purpose of this series of experiments is to determine whether the responses achieved with ventricular trabeculae under isometric conditions are repeatable using the more physiological oscillating muscle preparation. It is hypothesized that unless length-dependent changes in the activation processes occur, responses will not vary between the two preparations.

MATERIAL AND METHODS

Fish origin and maintenance.

Untrained rainbow trout (mean weight 2494 ± 99 g) of both sexes were obtained from Alma Aquaculture Research Station, Alma, Ontario and held in a flowthrough fiberglass tank receiving aerated, dechlorinated tap water. Tank was maintained at 12°C (± 2°C) throughout the 12°C acclimation and test period. A second group of fish from the same source was acclimated to 22°C but the temperature control was not reliable, as such results from the 22°C acclimation group are not reported. Fish were exposed to a neutral photoperiod (12L:12D) and were offered food three times a week.

Tissue Preparation.

The tissue was prepared as in Chapter 2. One end of the muscle was attached to the movable arm of an ergometer (Cambridge Technology) and the other end was tied to the fixed arm of an isometric force transducer (Harvard Apparatus). Mean muscle length was 0.30 ± 0.012 cm and mean wet weight was 0.006 ± 0.001g.

The preparation was then lowered into an organ bath containing physiological saline of the following ionic composition (in mM): Na^+, 136.80; K^+, 3.08; Ca^{2+}, 2.52; Mg^{2+}, 0.93;

SO_4^{2-}, 0.93; Cl^-, 129.66; CO_3^-, 2.74 and 10.00 mM of pyruvate as substrate. Solutions were buffered with TES acid, 3.49; TES salt 6.37 mM. Temperature was controlled at either 12° or 22°C. Extracellular pH was controlled with a Wostoff pump that maintained the ratio of CO_2:O_2 such that extracellular pH was 7.58 at 25°C.

Settings for Oscillatory Measurements

Preparation was stimulated via platinum plate electrodes positioned on either side of the muscle. Stimulus voltage (3 V) was adjusted to 1.2x that required to produce maximal isometric twitch force. Muscle length was adjusted to maximize work (L_{opt}), in contrast to the isometric experiments which maximized isometric force (L_{max}). Studies with rat papillary muscle (Layland *et al.*, 1995a, 1995b) and frog ventricular muscle (Syme, 1995) show that work is optimized between, 85% and 95% of L_{max}. Each stimulus train consisted of 8 biphasic pulses at a pulse width of 1.0 ms. Stimulus train duration was 16 ms. All measurements were recorded to disk with a Nicolet digital storage oscilloscope for later analysis. To measure work and power, a sinusoidal signal was used to cycle muscle length about L_O. The amplitude of the length change cycle (strain) was 0.05 (5%) (i.e. L_O ± 0.025 (2.5%)) for all trials. The cycle frequency (frequency of the imposed length change) was varied from 0.2 Hz to 2.0 Hz in 0.2 Hz steps. The phase of stimulation refers to the timing of the start of the stimulus train compared with the start of the imposed length change. In the present study, phase of stimulation is given as a percent of the length change cycle; muscle shortening occurs from 25% to 75%. Phases of stimulation that produced maximum power were different at different cycle frequencies. At 0.2 Hz, phase of stimulation was 35%, 23% at 0.4 Hz, 16% at 0.6 Hz, 10% at 0.8 Hz, 7% at 1.0 Hz, 5% at 1.2 Hz, 3% at 1.4 Hz, 2% at 1.6 Hz, 1% at 1.8 Hz, and 0 at 2.0 Hz.

Displacement, the change in muscle length, was measured with the ergometer and force was measured with the isometric transducer. Work, the product of force and displacement,

was calculated by integration of the force-displacement curve and was expressed as net work, the difference between positive work (work done by muscle during shortening) and negative work (work done on the muscle to lengthen it).

Prior to experimentation, the muscle was allowed to stabilize at L_O for 30 min under basal stimulation (0.2 Hz) before being subjected to either protocol 1 or protocol 2. The protocols are explained in detail in Chapter 2, Methods and Materials. Briefly, in protocol 1, the muscle was stimulated with low AD and subjected to a force-frequency trial to establish control. Afterwhich, the effect of ryanodine (10 μM) was tested under tonic (10 nM) AD stimulation. The muscle was then stimulated with (10 μM) AD to observe the effect of high AD on ryanodine-treated muscle. The purpose of protocol 2 was to determine whether ryanodine effects were discernible at high levels of AD. A first force-frequency trial with low AD established control performance, against which two further trials, one with high AD, and the other with high AD after ryanodine were evaluated.

Temperature Trial

Due to the inability to generate substantial work and power at the 22°C test temperature (see results) a single experiment was conducted to examine the effect of acute temperature change on work and power production. In this trial, the muscle was stimulated with 10 μM AD at 12°C and subjected to a force-frequency trial where pacing frequency was increased from 0.4 Hz to 1.4 Hz in 0.2 Hz increments. Bath saline was then replaced and the muscle was allowed to recover at 0.2 Hz while bath temperature was increased by 2°C. The preparation was left to stabilize at the new temperature for 15 min, after which new saline (containing 10 μM AD) was added and the force-frequency trial was repeated. This procedure was repeated for 16, 18, 20 and 22°C.

Standardization, Drugs and Statistics

The standardizing procedure, drug origin and statistical analysis are as given in Chapter 2, Methods and Materials.

RESULTS

Tests at 12°C

Frequency Effects

At 12°C, with a tonic level of AD, the effect of increasing stimulation frequency from 0.2 Hz to 1.6 Hz was a 70% reduction in peak tension from 358 $mg \cdot mm^{-2}$ to 97 $mg \cdot mm^{-2}$ (Table 3.1, Fig. 3.1A). Similarly, TPT decreased with increased pacing frequency from 0.41 s at 0.2 Hz to 0.21 s at 1.6 Hz. THR also decreased with increased frequency from 0.21 s at 0.2 Hz to 0.14 s at 1.6 Hz (Table 3.1). Additionally, the rates of contraction and relaxation increased with stimulation frequency (Table 3.1). Net work first increased with increased pacing frequency from 1.20 $J \cdot kg^{-1}$ at 0.2 Hz to 1.57 $J \cdot kg^{-1}$ at 0.4 Hz, but then gradually declined below the 0.2 Hz value to 1.08 $J \cdot kg^{-1}$ at 1.0 Hz and further to 0.26 $J \cdot kg^{-1}$ at 1.6 Hz resulting in an 80% reduction in net work from 0.2 Hz to 1.6 Hz (Table 3.1). Similarly, power demonstrates a biphasic response with a minimum of 0.24 $W \cdot kg^{-1}$ at 0.2 Hz, a peak between 0.8 and 1.2 Hz at 1.18 $W \cdot kg^{-1}$ (±1 S.E.M.), followed by a decline, reaching 0.42 $W \cdot kg^{-1}$ at 1.6 Hz (Table 3.1, Fig. 3.2).

Ryanodine and Adrenaline Effects

Addition of ryanodine in the presence of tonic AD decreased peak tension by a small but significant amount (~10%) at 0.2 Hz, 1.2 Hz and 1.6 Hz (Fig. 3.1A). Other measured variables were not significantly effected by ryanodine. Increasing the AD concentration to 10 μM in the presence of ryanodine, induced a positive inotropic response at low frequencies (0.2 Hz) but not high frequencies (>0.6 Hz), resulting in a reversal of the ryanodine effect and improving peak tension beyond that observed under tonic AD

Table 3.1 The effect of test temperature and pacing frequency on measured variables from ventricular trabeculae from rainbow trout under control conditions (low AD). Values are group means; sample size and S.E.M. are indicated below each value (n; S.E.M.). Within each test temperature, dissimilar letters represent significant difference ($P<0.05$) between frequency trials. * in the 12°C test temperature indicates significant difference from 22°C test temperature within each frequency trial.

	12°C Test				22°C Test				
Variables	0.2 Hz	0.8 Hz	1.2 Hz	1.6 Hz	0.2 Hz	0.8 Hz	1.2 Hz	1.6 Hz	2 Hz
PT ($mg \cdot mm^{-2}$)	^{a*} 358 (12; 50)	^{b*} 197 (12; 30)	^{b*} 152 (9; 20)	^{b*} 97 (6; 10)	^{a} 171 (12; 20)	^{b} 90 (12; 10)	^{b} 75 (12; 01)	^{b} 57 (12; 10)	^{b} 48 (10; 10)
% peak tension	^{a*} 100 (12; 0)	^{b*} 54.3 (12; 2.7)	^{c*} 36.6 (12; 1.8)	^{d*} 20.9 (8; 1.0)	^{a} 100 (12; 0)	^{b} 56.4 (12; 1.5)	^{c} 43.7 (12; 1.6)	^{d} 33.3 (12; 1.5)	^{e} 25.9 (12; 1.4)
TPT (s)	^{a*} 0.41 (12; 0.01)	^{b*} 0.31 (11; 0.01)	^{c*} 0.27 (10; 0.01)	^{d*} 0.21 (6; 0.01)	^{a} 0.23 (12; 0.0)	^{b} 0.2 (11; 0.0)	^{b} 0.18 (12; 0.0)	^{c} 0.16 (12; 0.0)	^{c} 0.15 (9; 0.0)
THR (s)	^{a*} 0.21 (12; 0.01)	* 0.17 (12; 0.01)	^{b*} 0.16 (11; 0.01)	^{b*} 0.14 (6; 0.01)	0.13 (12; 0.01)	0.12 (12; 0.01)	0.11 (12; 0.01)	0.14 (12; 0.01)	0.12 (10; 0.01)
dfdt to PT ($mg \cdot s^{-1}$)	^{a} 321 (12; 40)	254 (11; 30)	205 (10; 30)	^{b} 149 (6; 30)	^{a} 254 (12;50)	150 (12; 20)	155 (12; 30)	128 (12; 20)	^{b} 117 (12; 20)
dfdt to HR ($mg \cdot s^{-1}$)	^{a} -323 (12; 40)	-187 (11; 50)	-192 (11; 20)	^{b} -126 (4; 30)	^{a} -259 (10; 50)	-155 (12; 30)	^{b} -120 (12; 30)	^{b} -102 (12; 20)	^{b} -82 (10; 20)
work ($J \cdot kg^{-1}$)	* 1.201 (12; 0.20)	^{a*} 1.359 (12; 0.27)	* 0.844 (12; 0.18)	^{b} 0.261 (6; 0.15)	0.073 (12; 0.05)	0.049 (12; 0.06)	0.07 (12; 0.08)	0.069 (12; 0.08)	0.067 (9; 0.10)
power ($W \cdot kg^{-1}$)	^{a} 0.24 (12; 0.04)	^{b} 1.03 (12; 0.21)	1.18 (11; 0.25)	^{b} 0.42 (6; 0.23)	0.01 (12; 0.1)	0.04 (12; 0.05)	0.09 (12; 0.09)	0.11 (12; 0.14)	0.03 (10; 0.67)

stimulation (Fig. 3.1A). The contractions often became irregular at frequencies greater than 1.2 Hz with the high AD and ryanodine treatment. At frequencies greater than 1.0 Hz, power was reduced by high AD stimulation but could not be resolved statistically (Fig. 3.2A). Other variables were unaffected by high AD.

The addition of 10 μM of AD in the second protocol did not significantly effect peak tension. This was unexpected and is commented on in the discussion. Net work and power (Fig. 3.2C) increased at all frequencies under high AD stimulation but the increases were not statistically resolvable. The addition of ryanodine caused significant decreases in peak tension at 1.6 Hz, indicating the ability of ryanodine to depress tension even after high AD stimulation (Fig. 3.1C). Furthermore, at frequencies >0.6 Hz, both work (not shown) and power (Fig. 3.2C) are compromised by ryanodine treatment, though this could not be statistically resolved.

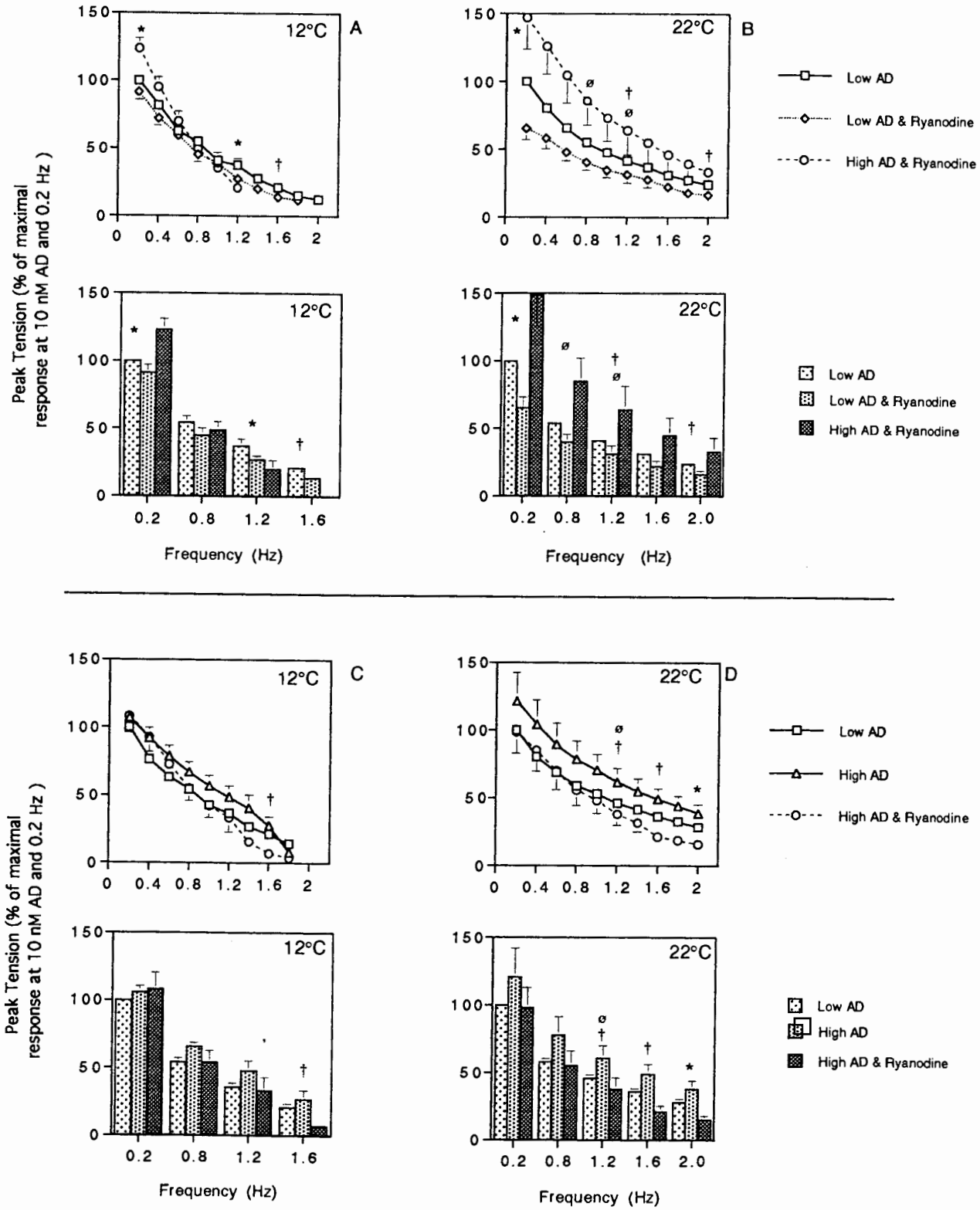
Tests at 22°C

Frequency Effects

Increasing the test temperature by 10°C significantly altered ventricular contractility. Peak tension was 50% lower at 0.2 Hz and 1.2 Hz in the 22°C tests compared with the 12°C tests (Table 3.1). At 22°C, preparations maintained regular contractions at a stimulation frequency of 2.0 Hz. TPT and THR were significantly faster at all frequencies when tested at 22°C, as were the rates of contraction and relaxation (Table 3.1). The 10°C increase in test temperature reduced work and power by 95% at all frequencies suggesting severe impairment of cardiac performance upon acute warm temperature exposure (see *'Temperature Trial'* results for explanation).

Increasing the pacing frequency from 0.2 Hz to 2.0 Hz resulted in a negative force-frequency curve, similar to that observed in the 12°C tests, with a 70% reduction in peak

Figure 3.1 Force-frequency relationships normalized to the Low AD and 0.2 Hz treatment for ventricular trabeculae from rainbow trout acclimated to 12°C, tested at 12°C or 22°C (indicated in upper right corner) and subjected to a drug treatment protocol (Protocol 1 A&B; protocol 2 C&D). Results are displayed as line graphs .to show progressive changes with increased frequency and as bar graphs to highlight drug effects. Values are group means. Vertical bars represent ±1 S.E.M. Significance is indicated as follows; * denotes significant difference between all drug treatments within each frequency, ø denotes significant effect of AD treatment within each frequency, † denotes significant effect of ryanodine treatment within each frequency.


A
12°C
B
22°C
Low AD
Low AD & Ryanodine
High AD & Ryanodine
Peak Tension (% of maximal response at 10 nM AD and 0.2 Hz)
Frequency (Hz)
C
D
High AD

tension, from 171 $mg \cdot mm^{-2}$ at 0.2 Hz to 48 $mg \cdot mm^{-2}$ at 2.0 Hz (Table 3.1, Fig. 3.1B). TPT was also significantly reduced as frequency increased, from 0.23 s at 0.2 Hz to 0.15 s at 2.0 Hz (Table 3.1). Likewise, the rate of contraction was decreased with increased pacing frequency from 254 $mg \cdot s^{-1}$ at 0.2 Hz to 120 $mg \cdot s^{-1}$ at 2.0 Hz. The THR remained unchanged, but the rate of relaxation decreased from -259 $mg \cdot s^{-1}$ at 0.2 Hz to - 82 $mg \cdot s^{-1}$ at 2.0 Hz, probably as a result of the decrease in cytoplasmic Ca^{2+} levels at high frequencies. Work and power were unchanged by pacing frequency (Table 3.1).

Ryanodine and Adrenaline Effects

Under tonic AD stimulation, application of ryanodine significantly reduced peak tension by ~40% at 0.2 Hz, and ~10% at 1.2 Hz and 2.0 Hz. This suggests that the ryanodine-induced depression of peak tension is more prominent, and apparent over a greater range of frequencies at 22°C than at 12°C (Fig 3.1B compared with Fig. 3.1A). At 22°C net work was minimal, and was not affected by drug treatment. Likewise, power was compromised by the warm test temperature and was unresponsive to either ryanodine or adrenaline treatment (Fig. 3.2B). Increasing the AD concentration to 10 μM, after ryanodine incubation, induced a positive inotropic response, restoring peak tension to values beyond those obtained under tonic AD stimulation (Fig. 3.1B). Work and power remained unaffected (Fig. 3.2B).

Increasing the AD concentration from 10 nM to 10 μM, in the second protocol, caused a positive inotropic response in peak tension which was statistically significant at 1.2 Hz and 1.6 Hz (Fig. 3.1D). Other measured variables remained unchanged. Addition of ryanodine depressed peak tension at 1.2 Hz, 1.6 Hz and 2.0 Hz again indicating that the negative inotropic effects of ryanodine can be observed under high adrenaline stimulation. Other measured variables remained unchanged.

Figure 3.2 Effects of increased pacing frequency and drug treatment on power (product of work and pacing frequency) from ventricular trabeculae from rainbow trout. Test temperature is indicated in the upper right corner of each graph. A&B are protocol 1, C&D are protocol 2 as expressed in the figure legends. Values are group means. Vertical bars represent ±1 S.E.M. Statistical differences could not be resolved for this variable.

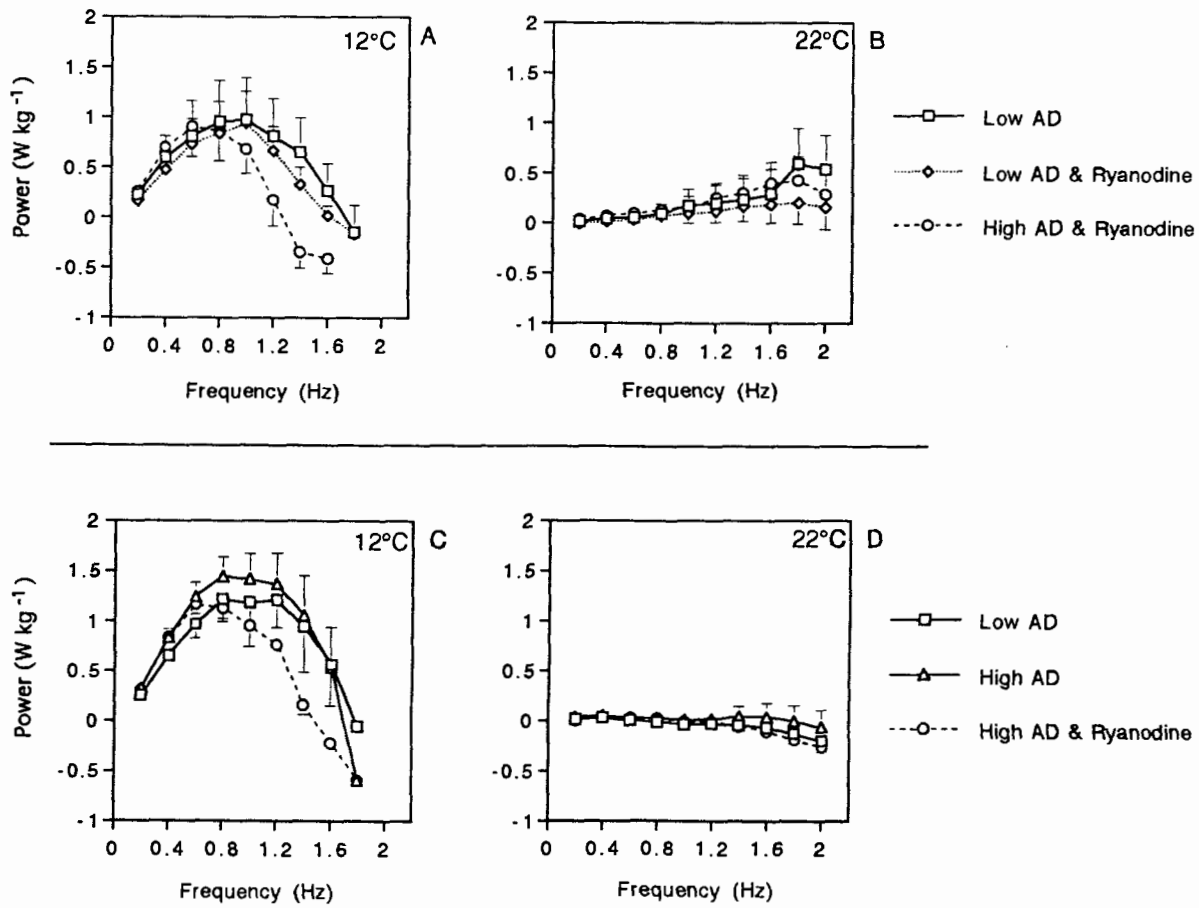
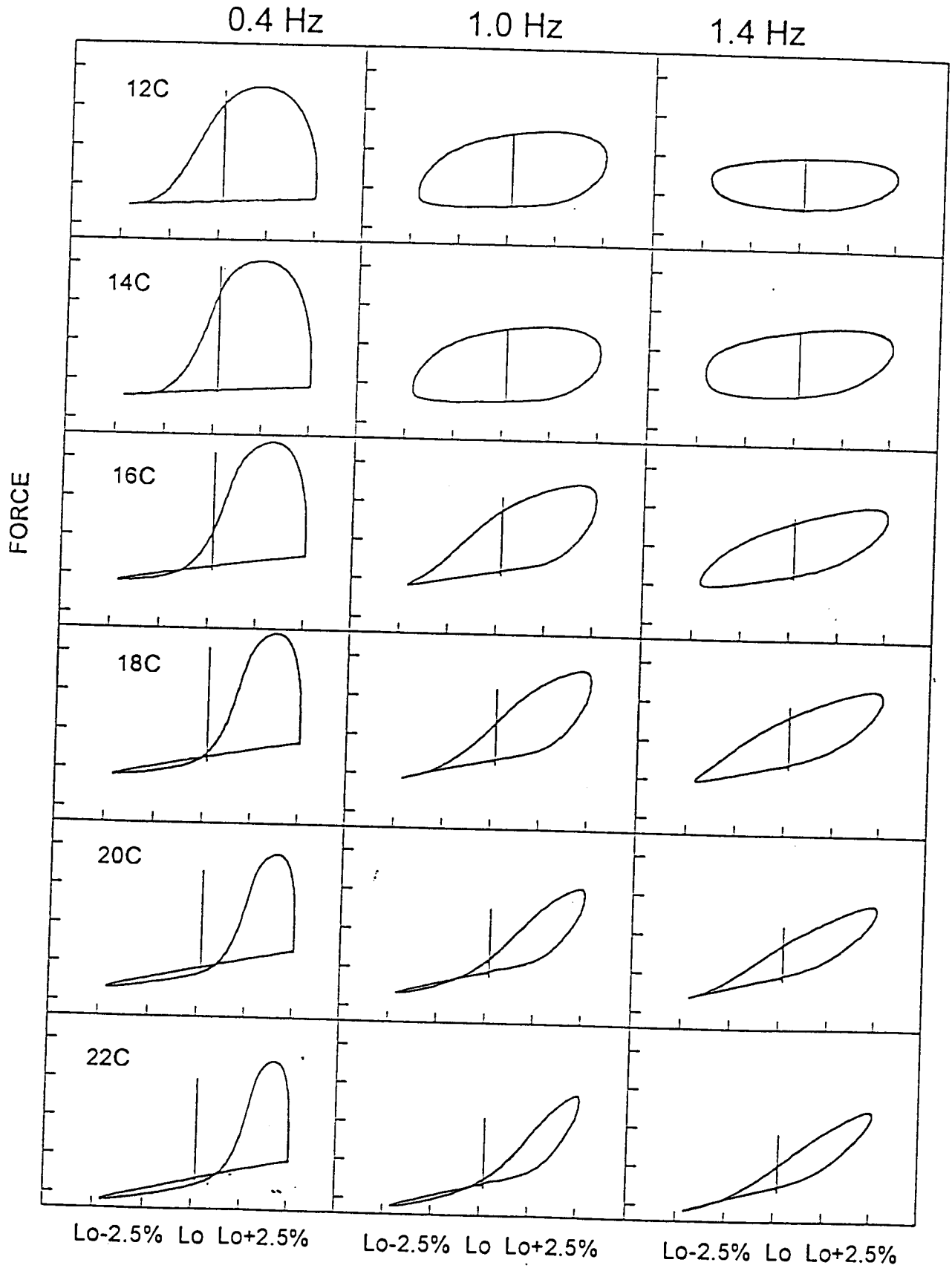


Figure 3.3. Work loop traces of an individual trabecular muscle from rainbow trout under high adrenergic stimulation, contracting at 3 frequencies (indicated by the columns) and at increasing temperatures (indicated in the rows). This figure is to show the relative change in positive vs. negative work at increasing temperature and frequency therefore force and length have undefined units. The perpendicular line at L_o in each graph is an isometric contraction.


0.4 Hz
1.0 Hz
1.4 Hz
12C
14C
16C
18C
20C
22C
FORCE
Lo-2.5% Lo Lo+2.5%
Lo-2.5% Lo Lo+2.5%
Lo-2.5% Lo Lo+2.5%

Temperature Trial

Because of the substantial loss in both work and power at high temperatures, a single experiment was conducted to observed the shape of the work loop at increasing temperature and frequency. The results indicate that as temperature was increased the muscle relaxed faster such that relaxation was completed during the shortening phase of the length change cycle. (Fig. 3.3). This reduced the positive work done by the muscle during shortening resulting in a decrease in net work (difference between positive (shortening) work and negative (lengthening) work.

DISCUSSION

Frequency Responses

The purpose of this series of experiments was to test whether the responses found under isometric conditions were repeatable using the oscillatory preparation. The lack of a 22°C acclimation group under oscillatory conditions limits the comparisons to the 12°C acclimation temperature. The response to increased frequency was consistent between the two preparations at both test temperatures, though the oscillating muscles were able to maintain regular contractions at higher frequencies than the isometric muscles (i.e. > 1.2 Hz). In all cases a negative force-frequency response was observed. The absolute values for peak tension under the oscillatory preparation were greater than 2-fold higher than the absolute values for the isometric preparation (compare Table 2.1 and 3.1). This is probably a result of the larger size (and thus cross-sectional area) of the trabeculae used in the oscillating preparation (6 mg vs. 2 mg), though a standardizing procedure was used in an attempt to minimize such differences. However, by normalizing peak tension to control levels and expressing the frequency and drug effects as a percent change, comparisons between the two experiments was possible. Peak tension from muscle performing isometric contractions at 12°C at 1.2 Hz were 36% of there value at 0.2 Hz, likewise peak tension was 39% of the 0.2 Hz value under oscillatory contractions (compare Table 2.1 & 3.1).

Similar reductions were observed at the 22°C test temperature with a 65% reduction in peak tension from 0.2 Hz to 1.2 Hz measured isometrically and 57% reductions over the same frequency range measured with oscillatory contractions. Additionally the effect of frequency on the kinetics of contraction were comparable with approximately 30% reductions in TPT between from 0.2 Hz to 1.2 Hz in both preparations at 12°C (Table 2.1 & 3.1). THR was more varied with 45% and 25% reductions in isometric and working preparations, respectively. The responses at 22°C similar to those at 12°C. Further, the estimations of rate from the isometric data set compare well with the actual rate measurements obtained in the working muscle, with 35-45% decreases in rate of TPT and 30-40% decrease in rate of THR in both preparations. Thus, the effect of frequency and acute temperature change on inotropic force development and contraction kinetics obtained isometrically, are strengthened and substantiated by their compatibility with those obtained from oscillatory working muscle.

Both force and work declined with increased frequency in all preparations (Table 3.1). The work-frequency relationship has been studied by Layland *et al.* (1995a), who also found that work decreased with increased cycle frequency in rat papillary muscle. They attributed this response to velocity-dependent factors, since velocity of shortening must be faster at high frequencies than at low frequencies. Hill (1938) dictates that the force achieved by a contracting muscle is reduced with increased shortening velocity, which would result in the negative force-frequency response and the reduction in work observed at high pacing frequencies in this study.

Ryanodine Effects

The response to ryanodine incubation in the working muscle (30% reductions at 0.2 Hz at 22°C) was less than that observed under isometric conditions (60% reductions at 0.2 Hz at 22°C) (compare Fig. 2.1 and 3.1). The reason for this is unclear. This is the first study to

examine the interplay between SL and SR-Ca^{2+} flux using a work loop. It is possible that by subjecting the muscle to a length change cycle, length dependent activation processes may be altered such that the response to ryanodine is weakened (a possibility that is explored in more detail in the next section concerning the effect of AD). However, regardless of the slightly reduced amplitude, the frequency dependence of the response to ryanodine, and the effect of temperature on the relative size of the response was consistent between the two preparations, with ryanodine effects being most prominent at low frequencies and at 22°C. No effect of ryanodine was observed at 12°C after high AD stimulation (Fig. 3.1C). This is also in agreement with the isometric experiments where ryanodine had no significant effect under these conditions. This is not surprising, as the interplay between SL and SR-Ca^{2+} flux would highly favour transsarcolemmal Ca^{2+} influx as the principle source for activator Ca^{2+} at 12°C and under high AD stimulation.

Adrenaline Effects

The responses to AD were, for the most part, consistent, though smaller, in the working muscle compared with those observed in the isometric muscle preparation (compare Fig. 2.1 and 3.1). High levels of AD increased peak tension and ameliorated the negative effect of ryanodine, especially at frequencies below 0.6 Hz. The lack of a dramatic increase in force with application of 10 μM AD at 12°C, in Fig. 2.1C was unexpected. Indeed, the response to adrenergic stimulation in the entire series of experiments was less than what was obtained isometrically. This may be a result of either subjecting the muscle to a length change cycle, or differences in the fish used in each experiment. Most likely, it is the fish, rather than the experimental preparation that is responsible for the weaken adrenergic response. There exists a large degree of individual variability between strains of rainbow trout (E.D.Stevens, pers. com.) which may partially account for the varying drug responses observed between data sets. In addition to the two groups of trout being of different origin, there was a difference in size and reproductive status between the animals.

The mean fish weight was 1224 ± 102 g in the isometric experiments and 2494 ± 99 g in the working muscle experiments. Additionally fish from the oscillatory group were spawning during the experimental period. Though size and reproductive status may not alone fully explain the different responses to adrenergic stimulation, it is possible that these conditions increased the stress levels of the animals during holding and acclimation. In mammals, chronic *in vivo* catecholamine exposure results in the desensitization of tissues through down-regulation or uncoupling of ß-receptors (Vatner *et al.*, 1989). If this occurs in fish, it would undermine the ability of circulating catecholamines to maintain tonic levels of cardiac stimulation, to protect cardiac performance during stress and, most importantly for this study, it would reduce the ability of catecholamines to maximally stimulate cardiac performance (Gamprel *et al.*, 1994).

However, it is also possible that the length change itself effected the response of the muscle to AD. Of the length dependent activation processes, the increase Ca^{2+} sensitivity of the myofilaments with increased length (Allen and Kentish, 1985) provides a plausible explanation for a reduced response to AD (and perhaps to ryanodine). If by stretching the muscle during the contraction cycle increased the muscle length sufficiently to increase the Ca^{2+} sensitivity of the myofilaments, then activation may have already been maximal, such that increasing $[Ca^{2+}]_i$ via adrenaline (or decreasing $[Ca^{2+}]_i$ with ryanodine) would have less of an effect when compared with the stationary muscle under isometric conditions. However, this argument is weakened by the fact that muscle length was optimized for work (L_{opt}) in the oscillatory muscle experiment, which is ~10% shorter than L_{max} which optimizes peak isometric force. Therefore, the muscle length was probably not longer (even while being stretched) in the working muscle than at L_{max} in the isometric preparation. Clearly, more studies on length-dependent mechanisms of activation in fish heart are needed to clarify these relationships.

Power

Power output is a measure of the ability to do work at a particular frequency and is calculated as the product of net work and cycle frequency ($W \cdot kg^{-1}$). The apex of the power curve indicates the frequency at which power production is maximal and is refereed to as the optimum frequency. The power curves generated from our study suggest that at 12°C an optimum frequency for power production exists between 0.8 Hz and 1.2 Hz (Fig. 2.2A&C). This agrees with the 55 bpm (~1.0 Hz) *in vivo* heart rate measured in trout at 12°C (Wood *et al.*, 1979). Moreover, ryanodine application tends to move the apex of the curve to the left. This suggests that without involvement of the SR, the power production of the muscle is reduced at a given frequency. However, as these results could not be resolved statistically, therefore, further studies are needed to better clarify these relationships. It was surprising that AD did not effect the power curve. The only other study to observe the effect of AD on an oscillatory cardiac muscle preparation (rat papillary muscle; Altringham, pers. comm), found that the power curve was shifted significantly up and to the right with adrenergic stimulation. The lack of such an effect in this study may be the result of either a stress-induced or length-dependent reduction in adrenergic response as discussed above.

The ability of ventricular trabeculae from trout to do work or to produce power is greatly influenced by temperature. This is demonstrated by the negligible power production at 22°C (Fig. 3.2B&D). The mechanisms behind this response were investigated by a single temperature trial experiment (Fig. 3.3). The results of this experiment suggested that at 22°C the rate of contraction and relaxation are elevated such that the muscle is fully relaxed during the shortening phase of the strain cycle, especially at frequencies < 1.0 Hz. This results in a reduction in the amount of positive work, the work done by muscle during shortening, such total positive work becomes proportional to negative work (work done on muscle to lengthen it) and the net work production approaches zero. This phenomena can

also be thought of in terms of total contraction duration. Because the duration of contraction determines how long the force is maintained during the shortening period of the cycle, which in turn influences the amount of work produced at a given cycle frequency (Layland *et al.*, 1995a) at high temperatures, when contraction duration is reduced, work and power decline. Because of this phenomena, power output at 22°C is approximately zero (Fig. 2.3). It is important to note that this situation would not occur *in vivo*, as contraction frequency increases with temperature such that relaxation would only just be completed before the next contraction was initiated. The loss of power at high frequencies (> 1.0 Hz) at 22°C is most likely due to the fact that 22°C is near the UILT for this species causing contractility to decrease at maximal contraction rates. This is in agreement with results from Farrell *et al.* (1996) which demonstrate that the maximum power output of the *in situ* trout heart decreases at temperatures > 18°C.

SUMMARY

The purpose of this investigation was to determine whether oscillating ventricular muscle displayed a response similar to that observed in a isometrically contracting muscle. The results indicate that the main difference between the two preparations involved the magnitude of the response to adrenergic stimulation and ryanodine treatment, both being less under oscillating conditions. The reasons for this difference remain unclear. The additional information provided by this study was that, like other animals (rat, Layland *et al.*, 1995a; crab, Stokes and Josephson, 1988), the contraction frequency of the trout ventricle produced maximal power output at frequencies close to its routine operating frequency. Although the results from the isometric series of experiments suggest that the SR does not play a major physiological role in modulating maximum force development at 12°C, power curves generated from the working muscle preparation suggest that the optimum frequency for power production is ryanodine sensitive. Thus, at 12°C, recruitment of SR-Ca^{2+} may play a role in reaching optimum frequency for performance as

it may facilitate faster Ca^{2+} handling at high frequencies. However, more experiments are needed to support this contention, without which, this conclusion remains entirely speculative.

CHAPTER 4

Major Findings and Conclusions

The purpose of this thesis was to investigate the effect of temperature and adrenergic stimulation on the interaction between Ca^{2+} released from the SR, and Ca^{2+} entering the cell across the SL. The major findings, presented in relation to the initially stated objectives are as follows:

Objective 1.

To determine the effect of temperature and temperature acclimation (of 12°C and 22°C) on the relative importance of the SR-Ca^{2+}-release channel in contributing Ca^{2+} to force development at physiologically realistic pacing frequencies under tonic adrenergic stimulation in isolated ventricular trabeculae from rainbow trout. (Chapter 2)

Findings:

1. The utilization of a tonic level of adrenergic stimulation in all preparations ensured physiologically relevant SL Ca^{2+} influx (i.e. better approximates the *in vivo* condition) under the varying temperatures and frequencies used in these experiments. Additionally, tonic AD increased attainable force under control conditions, which may have allowed changes in force to be observed in this study, that were undetectable in previous studies.

2. The SR-Ca^{2+}-release channel in the ventricle of rainbow trout is temperature-dependent, being more sensitive to acute temperature change than to acclimation, and more responsive at 22°C than at 12°C.

3. Under all conditions tested, SL Ca^{2+} influx is the primary source of Ca^{2+} for contraction. However, at 22°C the SR can contribute a significant proportion of activator Ca^{2+} at physiological pacing frequencies as indicated by the ~25-35% reduction in force observed after ryanodine treatment.

Objective 2.

To examine the muscle's ability to maintain tension after blocking SR-Ca^{2+} release with ryanodine, over a range of pacing frequencies, under temperature and adrenergic modulation. (Chapter 2)

Findings:

1. At 12°C with tonic adrenergic stimulation, ryanodine reductions in peak tension could only be observed at unphysiological pacing frequencies, independent of previous temperature acclimation, indicating that SL Ca^{2+} influx is the primary source of activator Ca^{2+} at cold temperatures.

2. At 22°C, ryanodine caused significant reductions in peak tension over the entire range of stimulation frequencies (0.2 Hz to 2.0 Hz), with the effects slightly more pronounced after 22°C acclimation. This suggests that the SR may be physiologically important in contributing Ca^{2+} to force production at warm temperatures.

3. Reductions in peak tension after ryanodine treatment were also observed under maximal adrenergic stimulation, at both temperatures and all frequencies suggesting that despite large increases in extracellular Ca^{2+} influx, a small (~10%) proportion of activator Ca^{2+} is still derived from intracellular stores.

Objective 3.

To examine the effect of modulating SL Ca^{2+} influx with maximal adrenergic stimulation on the relative importance of SR-Ca^{2+} contribution to excitation-contraction coupling at physiologically realistic pacing frequencies at 12°C and 22°C. (Chapter 2)

Findings:

1. Maximal adrenergic stimulation overwhelmed the Ca^{2+} contribution from the SR presumably by increasing SL Ca^{2+} influx. This adrenaline effect was more pronounced at low frequencies (<1.2 Hz) than at high frequencies.

Objective 4.

To re-examine objective (1), (2) and (3) using an oscillatory muscle preparation, to substantiate the isometric results, and to assess modulatory effects of temperature and adrenaline on power production. (Chapter 3)

Findings:

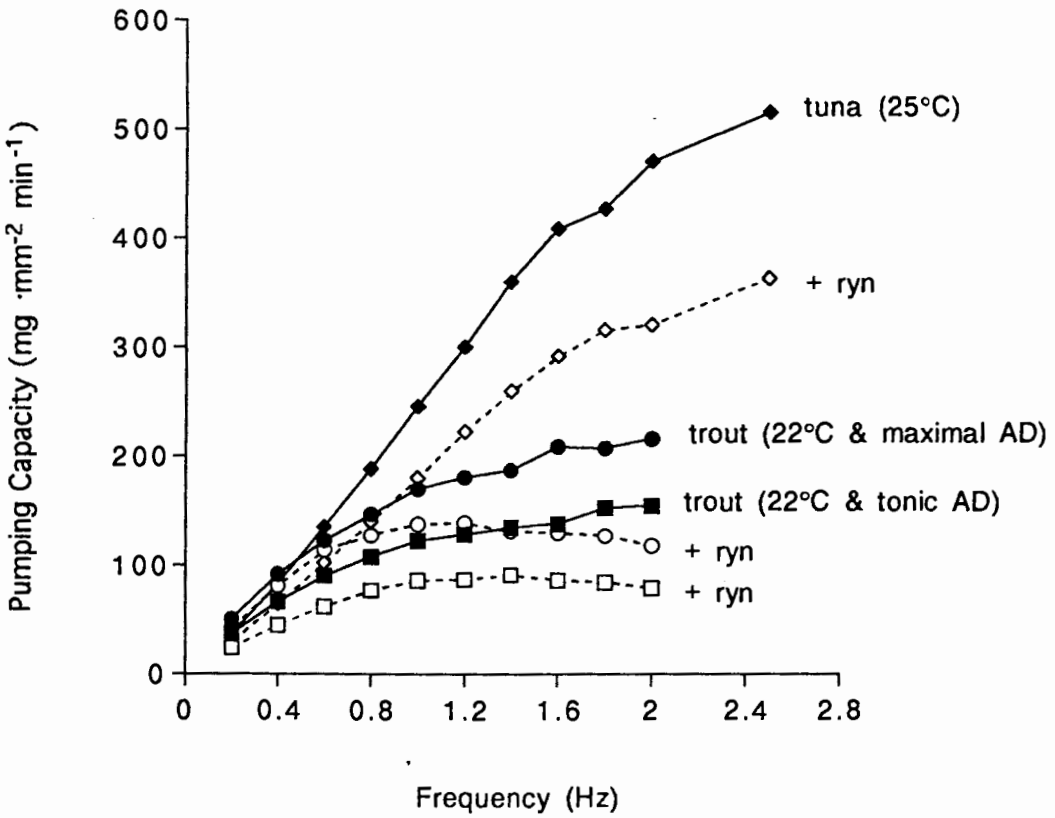
1. The oscillating muscle preparation substantiates the effects observed in the isometric preparations. However, the magnitude of the adrenaline and ryanodine responses were reduced, and it is not clear whether this is due to the status of the fish or the oscillatory muscle preparation itself.

2. At 12°C, an optimum frequency for power production exists between 0.8 and 1.2 Hz, which may be ryanodine sensitive.

This research is the first to establish a significant role for the SR in contributing Ca^{2+} to force development at physiologically realistic contraction frequencies and temperatures in a temperate-water fish. Using physiologically realistic modulators (temperature and adrenaline) to examine the interaction between Ca^{2+} influx across the SL and Ca^{2+} released from the SR, this study provides indirect evidence for interplay between the relative importance of these two sources of activator Ca^{2+}. At the trout's preferred temperature, the interplay between SR and SL Ca^{2+} is likely small, with SL Ca^{2+} influx dominating Ca^{2+} handling for E-C coupling. However, as temperature increases towards the UILT, the ability of the trout to utilize SR-Ca^{2+} stores may be of vast biological importance, since heart rate is elevated concomitant with increased temperature. Evidence from this thesis suggests a limited ability of SL Ca^{2+} flux to maintain force in the absence of SR-Ca^{2+} contribution at 22°C at high frequencies, even under maximal adrenergic stimulation. Thus, it may not be coincidental that the reduced ability to increase SL Ca^{2+} flux via adrenergic stimulation, coincides with the increased activity of the SR-Ca^{2+}-release channel at warm temperatures. Though many more studies are required to be conclusive, it is tempting to suggest that a fish who is able to exploit both sources of Ca^{2+} during cardiac contraction, may be better able to maintain contractility at high frequencies in the face of environmental temperature change.

Figure 4.1 illustrates the pumping capacity of the skipjack tuna, a teleost possessing both the greatest reliance on SR Ca^{2+}, and the highest heart rates recorded to date. From this figure it is obvious that both the power generating ability at any frequency, and the maximal attainable heart rate of the tuna eclipse those of the trout. Conceivably, it is the ability to recruit SR-Ca^{2+} for routine contractions in tuna that results in such disparity between these species.

Figure 4.1 A comparison of the pumping capacity of rainbow trout at 22°C after 22°C acclimation and skipjack tuna at 25°C (estimated from the data collected by Keen *et al.*, 1992). The tuna data is derived from atrial strips and the trout data is derived from ventricular trabeculae therefore no true comparisons are possible, however, the figure is useful to illustrate the increased dependence on the SR at high pacing frequencies in both species. Species and drug treatment is labeled on the graph.



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