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**Effects of Extracellular Calcium Concentrations on Cardiac Muscle in Selected Vertebrates**

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## Abstract

Hypercalcaemia was induced artificially in American eels, *Anguilla rostrata* LeSueur, by infusing  $\text{CaCl}_2$ . The treatment did not have any significant effect on recorded blood pressure or heart rate. Considering these data I concluded that, at least *in vivo*, the eel heart is apparently insensitive to physiological increases in plasma ultrafiltrable calcium concentration. The effects of changes in external calcium concentrations on the strength of contraction of isolated electrically-paced heart strips were also measured in rats, frogs, rainbow trout and American eels. This was done to test the hypothesis that eel cardiac tissue demonstrates a relatively reduced sensitivity to changes in extracellular calcium concentration. The data obtained, however, showed that contrary to the hypothesis, the eel cardiac strips were generally more sensitive than those from the other animals tested. As a result the null hypothesis that eel hearts do not have reduced sensitivity to changes in extracellular calcium concentrations *in vitro* was accepted. In fact, on the basis of the *in vitro* data, one should conclude that eel cardiac tissue is very sensitive to extracellular calcium concentrations. This conclusion is confounded by the observation that the uptake rate of  $^{45}\text{Ca}$  measured in contracting and non-contracting eel heart muscle strips did not differ, an observation that suggests that the eel heart relies more on internal than on external calcium stores for the development of tension. We are thus left with an indeterminate situation. That is, whereas eel cardiac tissue is very sensitive to changes in extracellular calcium concentrations *in vitro*, *in vivo* the heart is able to mitigate the effects of alterations in extracellular (plasma) calcium concentrations. Future studies should focus on how this mitigation, or at least the attenuation, of the effect of extracellular calcium on cardiac

function *in vivo* is obtained.

## Introduction

### A. Overview

Eels and other teleosts are equipped with an excellent capacity to regulate their extracellular calcium concentration over a wide range of environmental calcium concentrations (Chan *et al.*, 1967; Urasa and Wendelaar Bonga, 1987; Wendelaar Bonga *et al.*, 1976). Indeed, whether in low calcium fresh water or high calcium sea water, eels (*Anguilla sp.*) can maintain a relatively stable plasma calcium concentration (Chan and Chester Jones, 1968; Hanssen *et al.*, 1992). This is not surprising as most vertebrates are remarkably sensitive to alterations in the concentration of extracellular calcium (Hurwitz, 1996; Wendelaar Bonga and Pang, 1992) which often results in substantial physiological changes and even death if normal calcium levels are not restored quickly (Guyton, 1991). Eels can survive, and for extended periods of time, a variety of experimental procedures that induce levels of frank hypercalcaemia which would kill most vertebrates (Bailey and Fenwick, 1975; Butler and Oudit, 1994, 1995; Fenwick and Brasseur, 1991; Guyton, 1991). Further, the eels survived with minimal observed physiological perturbations other than a tendency to show some rigor. These observations raise an interesting question. How do eels escape the normal deleterious effects of hypercalcaemia?

The most obvious way of escaping the effects of hypercalcaemia is avoidance. That is, to prevent an excessive rise in extracellular calcium concentration. And to this end eels, like all teleosts studied to date, have a well developed system for preventing excessive increases in extracellular calcium concentration. In short, possibly by reducing renal tubular resorption of calcium (Butler, 1969; Fenwick, 1974), as well as reducing calcium uptake (Cano *et al.*, 1994; Fenwick and Brasseur, 1991; Perry *et al.*, 1989) via the gut (Takagi *et al.*, 1985) and gills (So

and Fenwick, 1979; Lafeber *et al.*, 1988; Lafeber and Perry, 1988; Perry *et al.*, 1992; Verboost *et al.*, 1993b). Because of these very effective mechanisms in bony fish, the plasma calcium concentration of eels probably rarely undergoes major alteration in nature (Chan and Chester Jones, 1968). But eels can be subject to hypercalcaemia by experimental manipulations and perhaps also during the final stages of sexual maturation (Bailey, 1957; Björnsson *et al.*, 1986; Norberg *et al.*, 1989).

In vertebrates in general, extracellular calcium fluctuations rapidly affect cardiac tissue (Tibbits *et al.*, 1991). Typically, high and low levels of calcium have, respectively, positive and negative inotropic actions (Keen *et al.*, 1992; Driedzic and Gesser, 1988; Hansen and Gesser, 1987; Orchard *et al.*, 1991, Yee and Jackson, 1984). But eels experiencing high plasma levels of  $\text{Ca}^{2+}$  appear to function almost normally although they do show some modifications in blood pressure and flow pattern (Bailey and Fenwick, 1975; Butler and Oudit, 1994, 1995). Because of these observations, the hypothesis to be tested in this thesis is that eel (*Anguilla rostrata* LeSueur) cardiac tissue is less sensitive to changes in plasma calcium levels than is the cardiac tissue of other vertebrates.

## **B. Background**

The concentration of intracellular calcium must be, and is, closely regulated (Rosenberg, 1991). In large part this is accomplished by membrane associated calcium pumps which maintain free cytosolic calcium concentrations in the normal resting range of approximately  $10^{-7}$  M by pumping calcium out of the cytosol into the extracellular fluids or into internal calcium storage areas such as the endoplasmic reticulum or the mitochondria. But the efficient

maintenance of intracellular calcium depends to a degree upon the maintenance of normal extracellular calcium concentrations. Thus there must be tight regulation of extracellular calcium levels. And indeed, all vertebrates studied to date are capable of hormonally regulating extracellular calcium levels (for review see Wendelaar Bonga and Pang, 1992). How this is achieved, however, is affected by the normal habitat of the animal in question.

Air breathing vertebrates can only obtain calcium via the ingestion of foods and thus have episodic availability to calcium. Therefore, when ingestion is curtailed they must rely on internal sources of calcium. Under these potential hypocalcaemic conditions the dominant hormone is parathyroid hormone or parathormone (PTH) (Wendelaar Bonga and Pang, 1992) although vitamin D<sub>3</sub> also plays a role in the maintenance of calcium homeostasis (Wendelaar Bonga and Pang, 1992). PTH increases extracellular calcium levels by enhancing the release of calcium from bone and by reducing Ca<sup>2+</sup> excretion from the kidney.

Conversely, fish have continuous access to the virtually limitless supply of calcium dissolved in their aquatic medium. Fish can access calcium through their diet, but for the most part, the bulk of the calcium taken in is obtained directly from their ambient medium (Wendelaar Bonga and Pang, 1992). In fresh water, most of the calcium is taken up by the gills and little enters via the gut because fresh water fish do not drink (Dacke, 1979). In sea water, fish drink copiously (Dacke, 1979) and so have little difficulty obtaining sufficient calcium. But even in fresh water, the ability of teleosts to take up calcium from the ambient medium makes large quantities of calcium available and perhaps even renders them susceptible to hypercalcaemia.

In seawater, where Ca<sup>2+</sup> levels are approximately eight times that of extracellular fluids, the threat of hypercalcaemia is exacerbated by the fact that the fish must drink and then

desalinate sea water in order to replace the body water which is continually lost via osmosis. But even in freshwater, where typical blood plasma  $\text{Ca}^{2+}$  concentrations are up to ten times that of the water there still appears to be a potential for excessive calcium uptake. Calcium uptake occurs passively through the apical membrane of the specialized mitochondrial-rich chloride cells in the branchial epithelium of gills because the cytoplasmic  $\text{Ca}^{2+}$  levels are at least 100 times lower than the environment (Wendelaar Bonga and Pang, 1992). Under these conditions both holostean and teleostean fishes regulate their homeostatic levels of plasma free  $\text{Ca}^{2+}$  through the action of the anti-hypercalcaemic hormone, stanniocalcin (STC) (Wendelaar Bonga and Pang, 1992). STC, for example rainbow trout STC, is a 54 kDa glycoprotein (Lafeber *et al.*, 1988a) which is synthesized and released from the corpuscles of Stannius (CS) in teleost fish (Verbost *et al.*, 1993a). Although present in other vertebrates (Chang *et al.*, 1995; Haddad *et al.*, 1996), STC is known in physiological circles as the main calcium regulating hormone in bony fish (Wagner, 1993). STC inhibits  $\text{Ca}^{2+}$  influx from the water via the gut (Sundell *et al.*, 1992; Takagi *et al.*, 1985) and more importantly the gills (So and Fenwick, 1979; Lafeber *et al.*, 1988; Lafeber and Perry, 1988; Perry *et al.*, 1992; Verbost *et al.*, 1993b).

In amphibians, which may be either fully terrestrial, fully aquatic, or a mixture of the two, control of calcium metabolism also reflects their environmental habitat. Purely aquatic species lack parathyroid glands (Wendelaar Bonga and Pang, 1992), an observation also made in fish (Pang, 1973). In fish, the absence of stanniocalcin results in an increase in the extracellular levels of this ion (Fenwick and Brasseur, 1991). This explains why a potent hypercalcaemic hormone such as PTH is not present in fish which predominantly regulate calcium levels via the hypo- or anti-hypercalcaemic hormone, STC. On the other hand, parathyroid glands are present

in partially or fully terrestrial anurans (Wendelaar Bonga and Pang, 1992). Therefore, when these animals are faced with hypocalcaemia, a lowering of extracellular  $\text{Ca}^{2+}$ , PTH is released. Being partially aquatic, anurans such as the leopard frog, *Rana pipiens*, must also be capable of quickly reducing their  $\text{Ca}^{2+}$  levels as their skin is in intimate contact with water and calcium uptake could pose a hypercalcaemic threat. This regulation is accomplished primarily by calcitonin (CT) (Robertson, 1970) which lowers plasma calcium levels by stimulating bone calcium uptake.

Most vertebrate species require uninterrupted heart functioning to survive, which means that the concentration of calcium ions in the blood must be regulated within a narrow physiological range. Ever since the seminal studies of Ringer (1883) and McLean and Hastings (1935) which revealed the extreme sensitivity of frog hearts to changes in extracellular calcium concentrations, it has been widely accepted that this may be one of the principal reasons for which vertebrates regulate their levels of plasma free calcium so tightly. Numerous studies followed which investigated the effect of varying calcium concentrations on cardiac physiology in many vertebrate species. It was shown that an increase in extracellular  $[\text{Ca}^{2+}]$  had a positive inotropic effect (Keen *et al.*, 1992; Meissner *et al.*, 1996; Vornanen, 1996; Yee and Jackson, 1984), wherein the strength of contraction increased up to five-fold (Driedzic and Gesser, 1988). Conversely, a decrease in extracellular  $[\text{Ca}^{2+}]$  caused an opposite response, with the strength of contraction being reduced (Orchard *et al.*, 1991). All of these studies were carried out on individual heart strips where the calcium concentration in the bathing medium was changed. These results confirm the initial findings by Ringer and make an obvious statement as to the profound sensitivity of both ventricular and atrial tissues to  $\text{Ca}^{2+}$ .

Extracellular calcium represents less than 0.1% of the total body calcium in vertebrates. As mentioned earlier, in the American eel (*Anguilla rostrata*) as well as other teleostean and holostean fishes, the maintenance of homeostatic levels of plasma free  $\text{Ca}^{2+}$  is regulated by the anti-hypercalcaemic hormone stanniocalcin (Fenwick, 1985; Verbost *et al.*, 1993b) secreted from the corpuscles of Stannius (CS) in response to hypercalcaemia (Perry *et al.*, 1989).

The removal of the corpuscles of Stannius, paired glands found on the ventral surface of the posterior functional kidney in eels, causes acute hypercalcaemia which can last for six weeks or more. Hyponatremia, hyperkalemia and hypomagnesia (see reviews by Hirano, 1989 and Wagner, 1993) have also been reported. This increase in extracellular  $[\text{Ca}^{2+}]$  can be remedied either by re-implantation of the glands or by injection of CS extracts (see review by Fenwick, 1982).

Butler and Oudit (1994, 1995) observed a decrease in blood pressure, cardiac output and stroke volume following stanniectomy (STX) in eels. Heart rate did not change. But experimentally induced hypercalcaemia by the intravenous infusion of  $\text{CaCl}_2$  did not cause similar alterations in cardiovascular functioning in the freshwater North American eel (Butler and Oudit, 1995). These results suggest that the presence of intact CS masks the effect of hypercalcaemic challenge on cardiovascular functioning or that the cardiovascular effects of stanniectomy are not the result of the subsequent hypercalcaemia. But this leaves the question as to why these animals show no cardiovascular response to elevated extracellular calcium levels.

In their natural environment, eels would improbably experience very large variations in their plasma free calcium concentrations as can be induced in the laboratory. Unlike terrestrial vertebrates, fish plasma  $\text{Ca}^{2+}$  levels fluctuate between, as well as within species (Wendelaar

Bonga and Pang, 1992; Dacke, 1979). The North American eel is an euryhaline species which migrates from seawater to freshwater and vice versa, thus encountering waters with different levels of  $\text{Ca}^{2+}$ . Thus, although eels do not normally experience wide fluctuations in plasma  $[\text{Ca}^{2+}]$ , at least not to the extent seen in terrestrial vertebrates, fish cells must still have very strict control over calcium entry. This changing environment causes some variations in plasma electrolyte concentrations which are rapidly compensated for (Pang *et al.*, 1980), although no significant long-term changes are observed (Fenwick, unpublished observations). Calcium concentrations in the plasma tend to rise during sexual maturation (Lopez *et al.*, 1976) and reproduction.

The fact that the eel can withstand large fluctuations in extracellular calcium concentrations for long periods of time (Hanssen *et al.*, 1989) without suffering from cardiac dysfunction (Butler and Oudit, 1995) seems to place this animal in a class by itself. One possible explanation may be that its cardiac tissue is less sensitive to calcium changes than that of other vertebrates or, that something is protecting the heart from the extracellular fluctuations. The perplexing thing about these observations is that as eels probably do not experience extracellular calcium increases in their natural environment that are as large as can be imposed in the laboratory, why would they have a regulating mechanism which would enable them to have proper heart functioning under these conditions? Perhaps the development of the corpuscles of Stannius, and hence stanniocalcin, came later on the evolutionary line and these animals developed a cardiac regulating mechanism to allow their survival prior to this.

### C. Goals of the Investigation

The general aim of my thesis is to determine the sensitivity of eel cardiac tissue to extracellular calcium concentrations.

An *in vitro* comparative study was carried out to determine the relative sensitivity of isolated eel hearts to various  $\text{Ca}^{2+}$  concentrations when compared to other vertebrate species. Atrial and ventricular strips from rat, leopard frog, rainbow trout and American eel were exposed to both increasing and decreasing extracellular calcium concentrations, and strength of contraction was recorded. Differences arising between species will give insight on the calcium sensitivity of these tissues and may in turn enlighten the path to understanding the protective mechanism underlying proper heart functioning in a hypercalcaemic environment.

To determine the source of calcium responsible for the generation of force in eel cardiac muscle, an uptake study was carried out. Contracting and non-contracting atrial and ventricular strips were exposed to  $^{45}\text{Ca}$  and the uptake was determined by measuring the radioactivity of the tissues. This may give some insight as to the dependence of these tissues on extracellular calcium concentrations.

An *in vivo* study was also carried out wherein eels were subjected to hypercalcaemia via an intravenous injection of  $\text{CaCl}_2$ . Under these conditions, dorsal aortic blood pressure, heart rate and plasma ultrafiltrable calcium concentrations were measured. Experimental hypocalcaemia was also attempted by intravenous phosphate loading. The results arising from this set of experiments should determine the extent to which the cardiac functioning of the eel is affected by extracellular calcium changes *in vivo*.

The hypothesis of my thesis is that eel cardiac tissue has a low sensitivity to extracellular

calcium fluctuations. Specifically, I predict that isolated heart strips from the eel will undergo a smaller increase in the strength of contraction when exposed to high levels of calcium than the other vertebrates it is compared to. Also, I predict that no marked changes will occur in cardiac function during the *in vivo* elevation of extracellular calcium. Low calcium, in both the *in vitro* and *in vivo* experiments, may cause changes in cardiac function since these animals are not readily exposed to hypocalcaemia in their natural environment as there exists an almost limitless supply of the ion in water.

## Materials and Methods

### Experimental Animals

#### A. Eels

Freshwater American eels, *Anguilla rostrata* LeSueur, purchased from Bainsville Fisheries (Cornwall, Ontario, Canada), were transported to the University of Ottawa in ice-cooled boxes and left to acclimate for a minimum of 2 weeks before use. The eels used in the *in vitro* experiments had an average weight of  $559 \pm 37$ g (N=25), whereas the fish used in the *in vivo* experiments weighed  $425 \pm 11$ g (N=5). The eels were held in large fibreglass tanks supplied with aerated, recirculated and dechloraminated Ottawa tap water ( $[Na^+] = 0.10$  mM,  $[Cl^-] = 0.15$  mM,  $[Ca^{2+}] = 0.35-0.40$  mM,  $[K^+] = 0.03$  mM, pH = 7.8) maintained at 13°C. Fish were not fed and were kept at a photoperiod of 12h light : 12h dark.

#### B. Trout

Rainbow trout, *Oncorhynchus mykiss*, were purchased from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada) and were transported to the University of Ottawa in oxygenated water. The trout were maintained under the same conditions as the eels except that they were fed commercial trout pellets three times per week to satiation. The trout had an average weight of  $566 \pm 74$ g (N=11).

#### C. Frogs

Leopard frogs, *Rana pipiens*, weighing  $22 \pm 2$ g (N=16) were purchased from Anilab (Ste-Foy, Québec, Canada). The animals were fed mealworms three times per week and

maintained at 18° - 20° C in a flow-through tank with a dry end. The frogs were exposed to a photoperiod of 12h light : 12h dark and were left to acclimate for one week.

#### **D. Rats**

Male albino Sprague-Dawley rats, *Rattus norvegicus*, weighing  $233 \pm 16$ g (N=5) were purchased commercially (Charles River, Massachusetts, USA). They were kept in cages at room temperature (21°C), were fed a commercial rat diet *ad libidum* and had free access to water. The rats were exposed to a 12h light : 12h dark constant photoperiod and were left to acclimate to their surroundings for one week.

### **Surgical Procedures**

#### **A. Preparation of Heart Strips**

Eels were anaesthetized in a solution containing 2 g L<sup>-1</sup> ethyl-m-aminobenzoate (MS 222; Syndel Laboratories; Vancouver, B.C., Canada) buffered with 4 g L<sup>-1</sup> NaHCO<sub>3</sub>. The normal time for anaesthetization was about 10 minutes. Trout were killed with a blow to the head, frogs were double pithed and rats were exposed to a lethal dose of CO<sub>2</sub>. In each case the hearts were removed from the animal as quickly as possible and were placed in the appropriate aerated physiological saline solution at a suitable temperature. Both ventricular and atrial cardiac muscle were used to compare strength of contraction and sensitivity to varying calcium concentrations.

The heart was divided into atria and ventricle(s) and the muscles were cut parallel to the long axis of the chamber to a length of approximately 8 mm and a width of approximately 2

mm. The thickness of the muscle was approximately 1 mm for the atria and 2 mm for the ventricles. Heart clips were attached to both ends of the heart strip. The lower clip was attached to a supporting wire with ligature thread (#00). The strip was then immersed in a 30 ml tissue bath containing a physiological saline solution aerated with the appropriate gases (eel and trout, 99% O<sub>2</sub> and 1% CO<sub>2</sub>; frog and rat, 95% O<sub>2</sub> and 5% CO<sub>2</sub>). The tissue bath was maintained at physiological temperatures (eel and trout, 13°C; frog, 23°C; rat, 37°C) by placing it in a temperature controlled water jacketed chamber linked to a water pump. A thread was attached to the upper clip and then tied to a Narco Bio-Systems isometric force transducer (F-60). A stimulating electrode was placed in the tissue bath next to, but not touching, the heart strip. All heart strips were electrically stimulated to contract at a frequency ranging from 0.3 to 0.5 Hz. The stimulus strength required to obtain regular and consistent contractions was equal to 1.5 times the threshold value with a 2 ms duration. The muscle strips were allowed a 30 minute recovery period from the setting-up procedure before they were stimulated or exposed to any experimental treatments.

## **B. Cannulation in the American eel**

Eels were anaesthetised as described above.

### **i) Blood Pressure Measurements**

The pneumogastric artery was exposed by a ventro-lateral incision about 2 cm in length and 1 cm lateral to the ventral midline in the caudal region of the liver. The artery was then cannulated with polyethylene tubing 1.5 times the length of the animal (PE50; Intramedic, Clay-Adams; Parsippany, New Jersey, USA) filled with eel Ringer containing 50 units of sodium heparin per ml. The abdominal cavity was then sutured shut using #00 silk thread. The

cannula was wrapped around the eel onto its back where it was secured to the dorsal fin with two surgical staples. This procedure prevented the eels from dislodging the cannula which was subsequently used for blood pressure measurements.

## **ii) Injection and Sampling**

Following the cannulation of the pneumogastric artery a 3 cm incision was made along the lateral line, approximately 2/3 of the way down the body of the eel, to expose the caudal vein. Cotton swabs (Q-tips) were used to gently tease away the muscle until the spine was reached. The incision was clamped open and bent forceps were used to get underneath a spine which was then snapped off and removed revealing the caudal vein. This was repeated until a 2 cm length of the vein was visible (2 to 3 spines). The vein was then cannulated using PE60 tubing (Intramedic, Clay-Adams). The rest of the procedure was as mentioned for the pneumogastric cannulation above. The cannula was used to infuse the calcium and phosphate loads into the animal and to take blood samples.

The entire surgical procedure took approximately 30 minutes. After the animals were cannulated they were placed in black plexiglass boxes supplied with aerated, flowing and dechloraminated Ottawa tap water at 13°C. They were left to recover from the surgery for 24 hours before being tested further.

## **Preparations**

### **A. Salines (all values are given as millimoles per litre)**

#### **i) Eel**

Normal eel Ringer was used in both the *in vivo* and *in vitro* experiments (NaCl, 112.8;

KCl, 4.2; NaHCO<sub>3</sub>, 13.1; KH<sub>2</sub>PO<sub>4</sub>, 0.4; Na<sub>2</sub>HPO<sub>4</sub>, 1.0; CaCl<sub>2</sub>, 1.3; MgSO<sub>4</sub>, 1.2; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1; pH 7.4). In the *in vitro* experiments, glucose was added as a substrate (5.6).

#### **ii) Trout**

Cortland Ringer was used for the trout (NaCl, 124; KCl, 5.1; CaCl<sub>2</sub>, 1.62; MgSO<sub>4</sub>, 0.9; NaHCO<sub>3</sub>, 11.9; NaH<sub>2</sub>PO<sub>4</sub>\*2H<sub>2</sub>O; glucose, 5.6 ; pH 7.4).

#### **iii) Frog**

The frog Ringer used was previously described by Zarrow *et al.* (1964).

#### **iv) Rat**

The rat physiological saline used was a modified Tyrodes (NaHCO<sub>3</sub>, 25; KCl, 5.14; NaH<sub>2</sub>PO<sub>4</sub>, 1.0; MgSO<sub>4</sub>, 0.94; NaCl, 125; CaCl<sub>2</sub>, 1.25; glucose, 5; pH 7.4).

Glucose was added just prior to *in vitro* experiments for all animals.

### **B. Calcium Solutions**

#### **i) *In vitro***

Three physiological salines were used in the *in vitro* heart strip preparations for both atrial and ventricular tissue. One solution had normal [Ca<sup>2+</sup>] (rat, 1.25; frog, 1.08; trout, 1.62; eel, 1.3 mM), another had low (rat, 1.0; frog, 0.54; trout, 0.81; eel, 0.65 mM) and the last had high [Ca<sup>2+</sup>] (rat, 1.5; frog, 2.16; trout, 3.24; eel, 2.6 and 1.95 mM). The high calcium solution contained double the normal [Ca<sup>2+</sup>] for the trout, frog and eel. The low calcium solution had half the normal [Ca<sup>2+</sup>] for the same animals. For the eel an additional high calcium solution

containing 50% more calcium than the normal solution was also used. Because the mammalian heart is known to be very sensitive to extracellular calcium concentrations the high and low calcium solutions used for the rat contained, respectively, only 20% more and 20% less calcium than the normal saline. These solutions were chosen as full recovery could be obtained once the strips were again exposed to normal calcium saline. The total osmotic strength of the salines was maintained by increasing or decreasing the NaCl concentration.

### *ii) In vivo*

In the *in vivo* experiments, the extracellular calcium concentration in eels was increased by 50% *via* the injection of an appropriate volume of 40 mM Ca<sup>2+</sup> solution (pH 7.4). To calculate this volume, the extracellular space was estimated to be equal to 15% (Holmes and Donaldson, 1969) of the total body weight of the animal. The concentration of the injection was calculated to give a 50% increase with an injection of approximately 1 ml, without excessively increasing the blood volume. Taking into account the average 400g weight of the eels, and that 0.04 mmol of calcium would be required to raise the plasma [Ca<sup>2+</sup>], approximately 1 ml of this solution was required. The final volume of the injection was determined by the following formula (X = volume; ml):

$$X = \frac{\text{weight of fish}}{400}$$

### **C. Phosphate Solution**

In an attempt to reduce the extracellular calcium concentration in the eels during the *in vivo* experiments, a solution containing 10 mg of inorganic phosphate per 100g of fish in 1 ml

ddH<sub>2</sub>O (Wagner *et al.*, 1997) was injected. A 1M stock solution of P<sub>i</sub> was made using monobasic and dibasic anhydrous sodium phosphate in a 1:5 ratio to obtain a pH of 7.4. Therefore, each ml contained 31 mg of P<sub>i</sub>. The formula used to determine the final volume of injection was:

X = volume and,

amount of P<sub>i</sub> required = wt of fish (g) / (10 mg P<sub>i</sub> / 100 g of fish)

$$X = \frac{\text{amount of P}_i \text{ required}}{31 \text{ mg / ml}}$$

Unfortunately, this procedure did not reduce plasma free calcium levels.

### **Determination of Plasma Free Ca<sup>2+</sup>**

Plasma free calcium (i.e. ultrafiltrable) levels were determined 24 hours post-surgery (T=0) and throughout the calcium and phosphate loading experiments. Blood samples (0.2 ml) were taken at T=0, 15, 75, 135, and 195 minutes in unanesthetized eels. The samples were centrifuged and the plasma retrieved and stored at -80°C until analyzed. A fraction of this plasma was then spun through a 10 kDa cut-off filter and calcium levels were measured in the ultrafiltrate using atomic absorption spectrophotometry (Varian 250 Plus Spectra AA).

### **Experimental Protocols**

#### **A. Effect of Varying Calcium Concentrations on Cardiac Muscle Strips**

For each animal, two atrial and two ventricular strips were mounted at one time. Following a stabilisation period in a normal [Ca<sup>2+</sup>] saline (control), each strip was exposed to

alternating high and low calcium solutions. To determine the effect of the duration of the experiment on the strength of contraction of the muscle strips and to re-establish a control contractile strength, each experimental treatment was followed by a control treatment of saline. The strength of contraction was recorded using a data acquisition system (MP100WSW; Biopac Systems; Goleta, California, USA) and an isometric force transducer (F-60, Narco Bio-Systems). For purpose of comparison all data were normalised to mg tension / 25 mg of wet tissue, as 25 mg was the closest to the actual weight of tissue used. The values were graphically expressed as a % change in contractile strength compared to the strength observed in the normal calcium treatment immediately previous to the experimental (high or low) calcium treatment. The strips were exposed to each concentration until a stable trace was observed. The time required to reach stability varied among species and treatments.

### **B. Calcium Permeability in Eel Cardiac Muscle**

Heart strips from the eel were placed in aerated tissue baths containing normal Ringer. Any strip that was spontaneously beating was discarded. One strip for each tissue type was stimulated to contract at 40 beats per minute (bpm) with a voltage equivalent to 1.5 times the threshold value; a second strip from the same eel was not stimulated. The strips were left to stabilize for 30 minutes. A 100  $\mu\text{L}$  aliquot of  $^{45}\text{Ca}$  ( $5 \mu\text{Ci} \cdot \text{ml}^{-1}$ ) (Amersham; Oakville, Ontario, Canada) was added to each bath and incubated for 15 minutes. The stimulation was stopped and the tissues were rinsed three times by emptying the baths and refilling them with physiological saline to remove any isotope remaining on the muscle. The heart strips were then placed in pre-weighed scintillation vials and weighed to determine the muscle weight. The tissues were

digested by adding 0.5 ml of NCS tissue solubilizer (Amersham; Oakville, Ontario, Canada) and placed in the oven at 60°C overnight. Once the samples had cooled, 15 µL of glacial acetic acid were added to the solution in each scintillation vial to neutralize the base and to avoid excess chemiluminescence. Four ml of OCS scintillation fluid (organic counting scintillant; Amersham; Oakville, Ontario, Canada) were added to each vial. The vials were then well mixed and the radioactivity was measured with a 2500 TR liquid scintillation beta-counter (Packard, Canada).

### **C. Effect of Varying Calcium Concentrations on the Cardiovascular System of the Eel**

The dorsal aortic blood pressure (bp) and plasma calcium levels were measured 24 hours after surgery (time 0) and prior to injecting the eels with approximately 1 ml of a solution of CaCl<sub>2</sub> containing 0.04 mmol of Ca<sup>2+</sup>. The dead space in the cannula was compensated for by a subsequent equivalent saline injection. Cardiovascular measurements (bp and HR) were recorded and blood samples (0.2 ml) were taken at T = 15, 75 and 135 minutes to monitor the effects of high extracellular calcium on the fish. Following this, the eel was injected with approximately 1 ml of a phosphate solution so that it received 10 mg of P<sub>i</sub> per 100 g of fish. Cardiovascular measurements were recorded and a blood sample was taken at T = 195 min. Blood pressure was measured as cm of water using a Narco Bio-Systems 1000B pressure transducer. The blood pressure was recorded using a data acquisition system (MP100WSW, ECG100A; Biopac Systems). The pressure recording apparatus was calibrated against a column of water just prior to each blood pressure recording. Heart rates were determined from the pulse

pressures and were recorded as beats per minute (BPM).

### **Statistical Analysis**

All data are presented as the mean  $\pm$  SEM, unless otherwise indicated. For all heart strip experiments, a Friedman repeated measures ANOVA on ranks was performed to determine if time had an effect on strength of contraction. Where statistical differences were shown, the significance of the differences between individual means was tested by Dunn's pairwise multiple comparison procedure ( $P < 0.05$ ). The effect of high and low extracellular calcium on strength of contraction was tested individually using the Students T-test ( $P < 0.05$ ) by comparing the experimental values to the control values. The blood pressure data was analysed by a one-way ANOVA and where statistical differences occurred, significance was tested by Tukey's least significance difference test ( $P < 0.05$ ). The plasma calcium levels and  $^{45}\text{Ca}$  activity in heart strips were analysed using the Students T-test ( $P < 0.05$ ). All statistical analysis was done on the raw data.

## Results

### A. Heart Strip Experiments

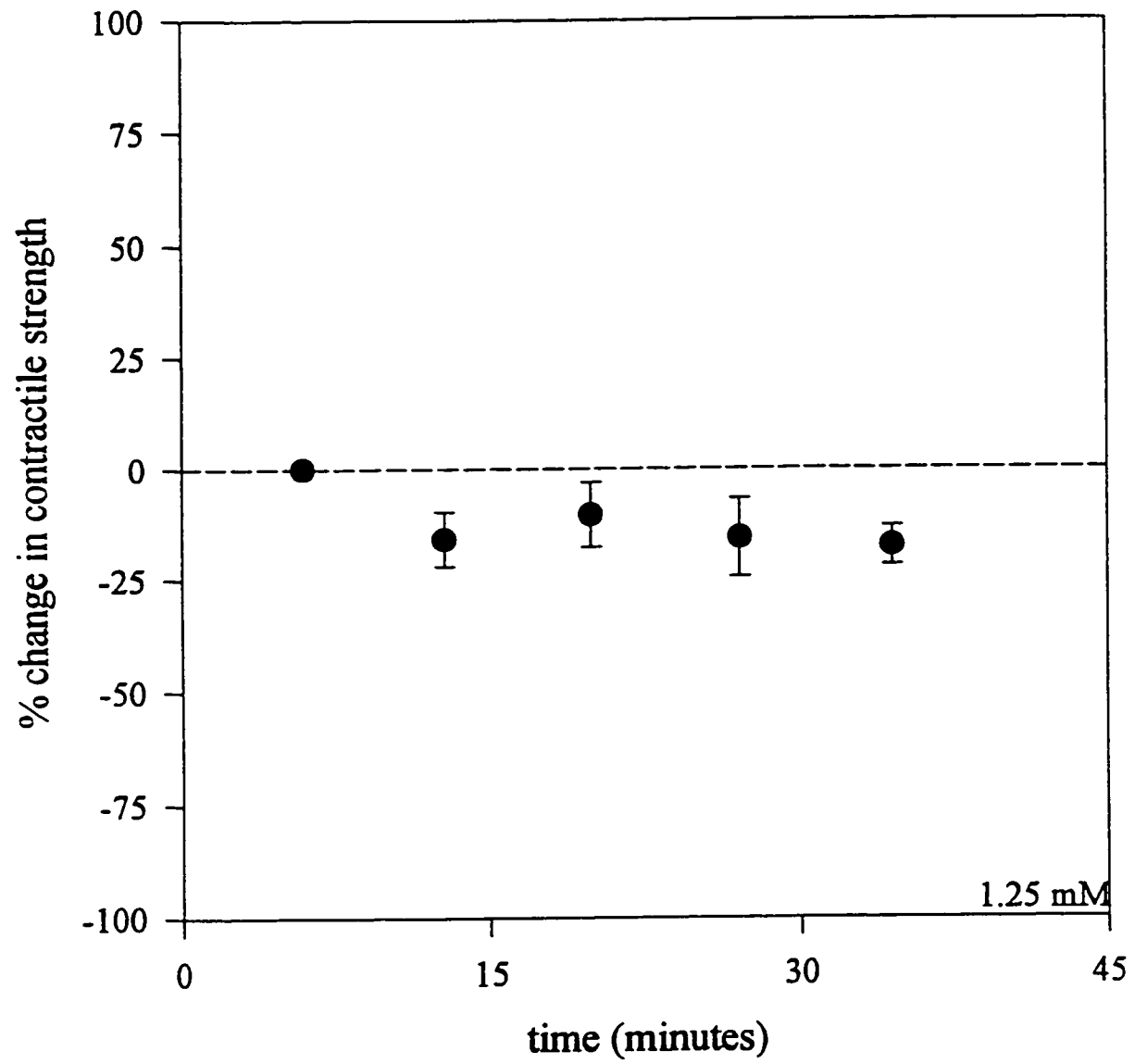
The contractile strength varied with time for some species. For this reason, the experimental data were expressed as the percent change in strength of contraction compared to the control treatment immediately previous to the experimental treatments (high or low).

#### i) Rats

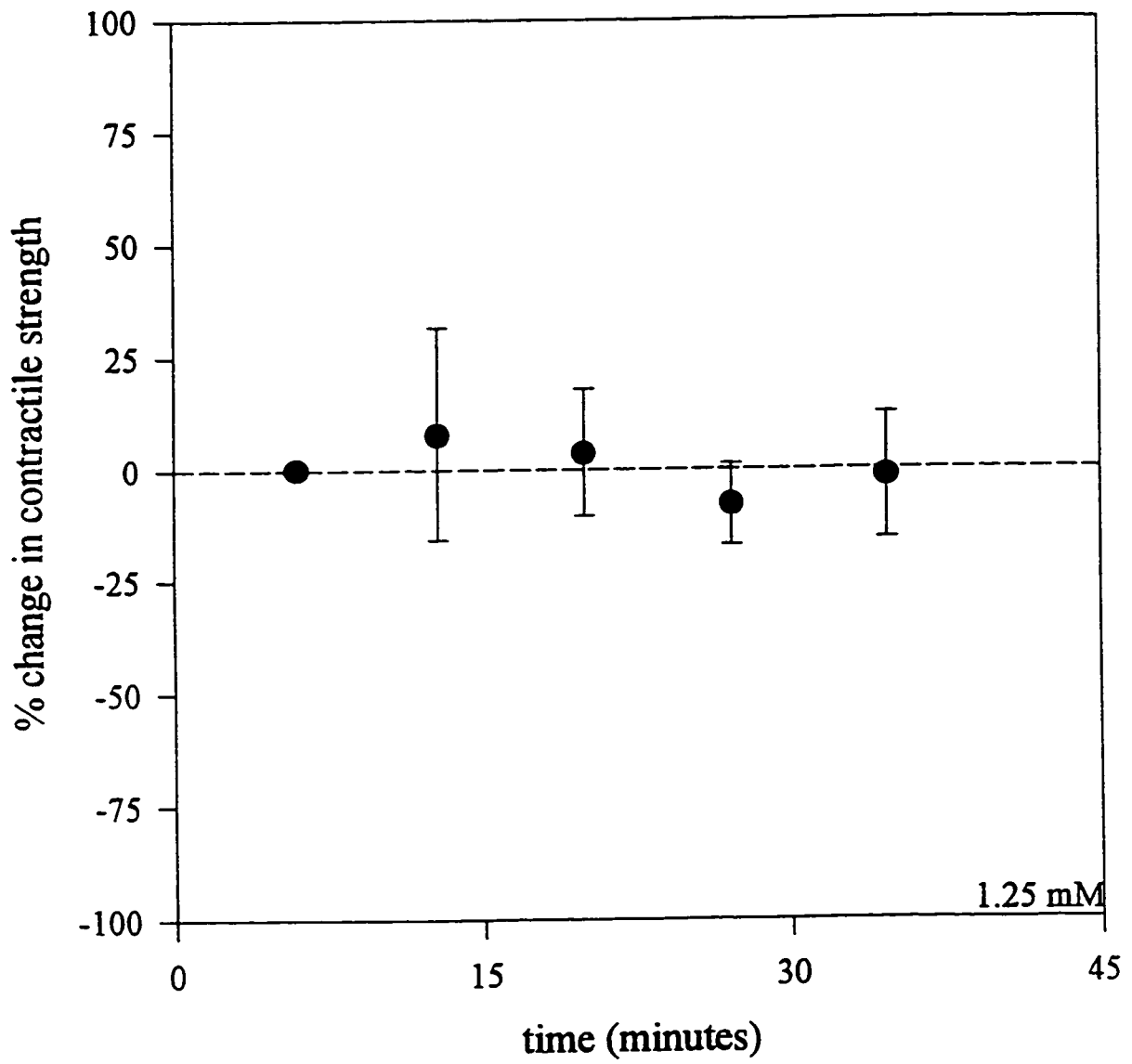
The strength of contraction of rat atrial (Figure 1) and ventricular (Figure 2) muscle exposed to normal  $[Ca^{2+}]$  did not change during the course of the experiment. Figure 3 shows the strengths of contraction attained during exposure of atrial muscle to high (1.5 mM) and low (1.00 mM)  $Ca^{2+}$  concentrations. This figure shows that the strength of contraction of the atrial muscle increased with increasing levels of extracellular calcium. When compared to the immediately previous control (1.25 mM) treatment, a 20% decrease in  $Ca^{2+}$  concentration to 1.00 mM resulted in an average 33% decrease in contractile strength ( $P < 0.05$ ) whereas a 20% increase in extracellular  $[Ca^{2+}]$  produced an average 24% increase in strength of contraction ( $P < 0.01$ ).

Ventricular muscle exposed to changing calcium concentrations showed similar responses to those observed in the atria (Figure 4). A comparison with control (1.25 mM) values indicated a 35% reduction ( $P < 0.05$ ) in contraction strength when extracellular calcium was decreased by 20% and a 74% increase in the strength of contraction following a 20% increase in  $Ca^{2+}$  ( $P < 0.05$ ). When the strengths of contraction of the atrial and ventricular strips were compared on a weight basis, the atrial muscle proved to be approximately twice as strong ( $P <$

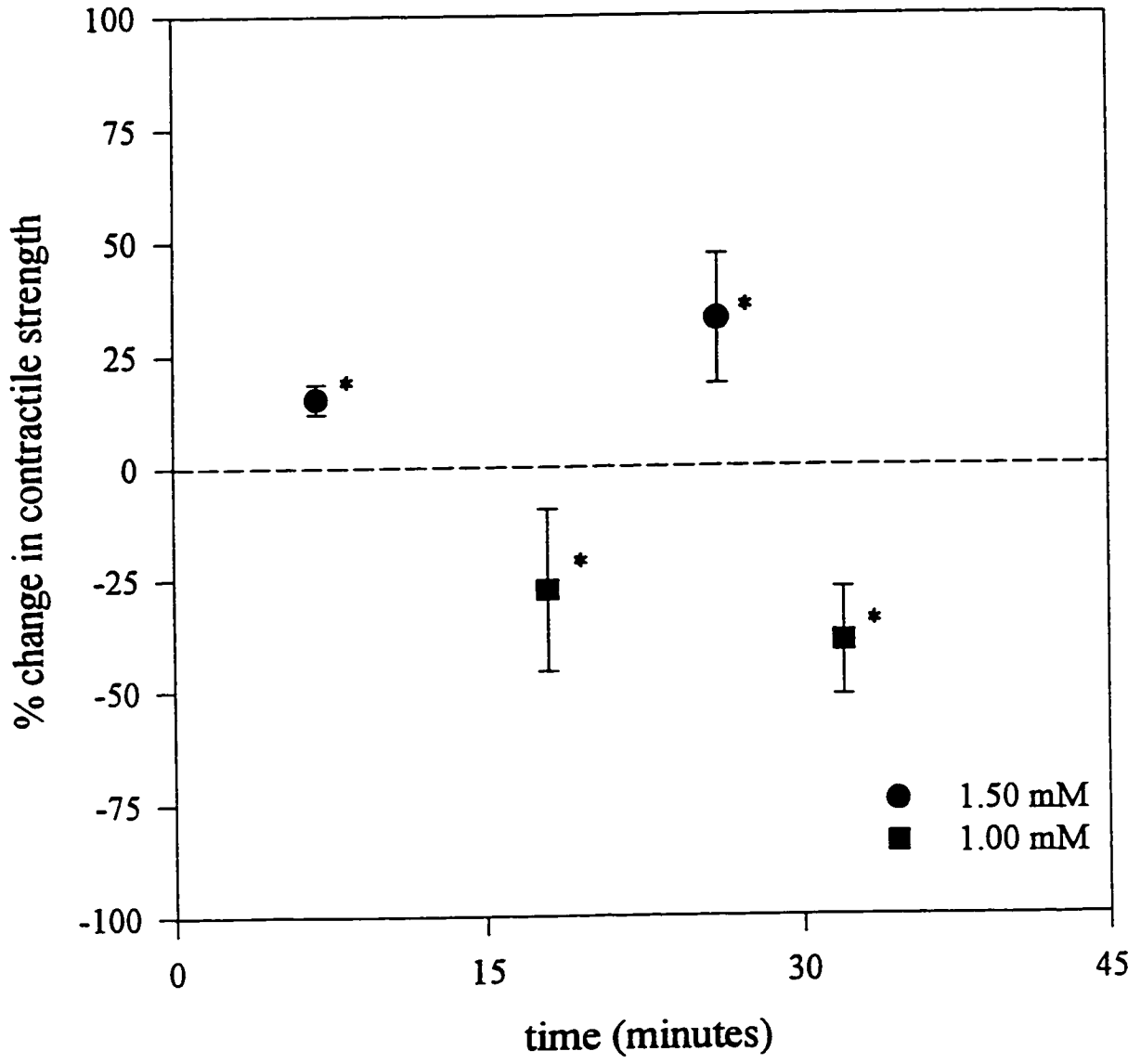
**Figure 1.** Effect of time on the strength of contraction in electrically paced rat (*Rattus norvegicus*) atrial strips exposed to control  $[Ca^{2+}]$  (1.25 mM). Results are expressed as percent change in strength from the initial value (mean  $\pm$  SEM, N = 6). All measurements were made in 1.25 mM  $Ca^{2+}$  before and after experimental treatments (1.5 and 1.0 mM  $Ca^{2+}$ ).



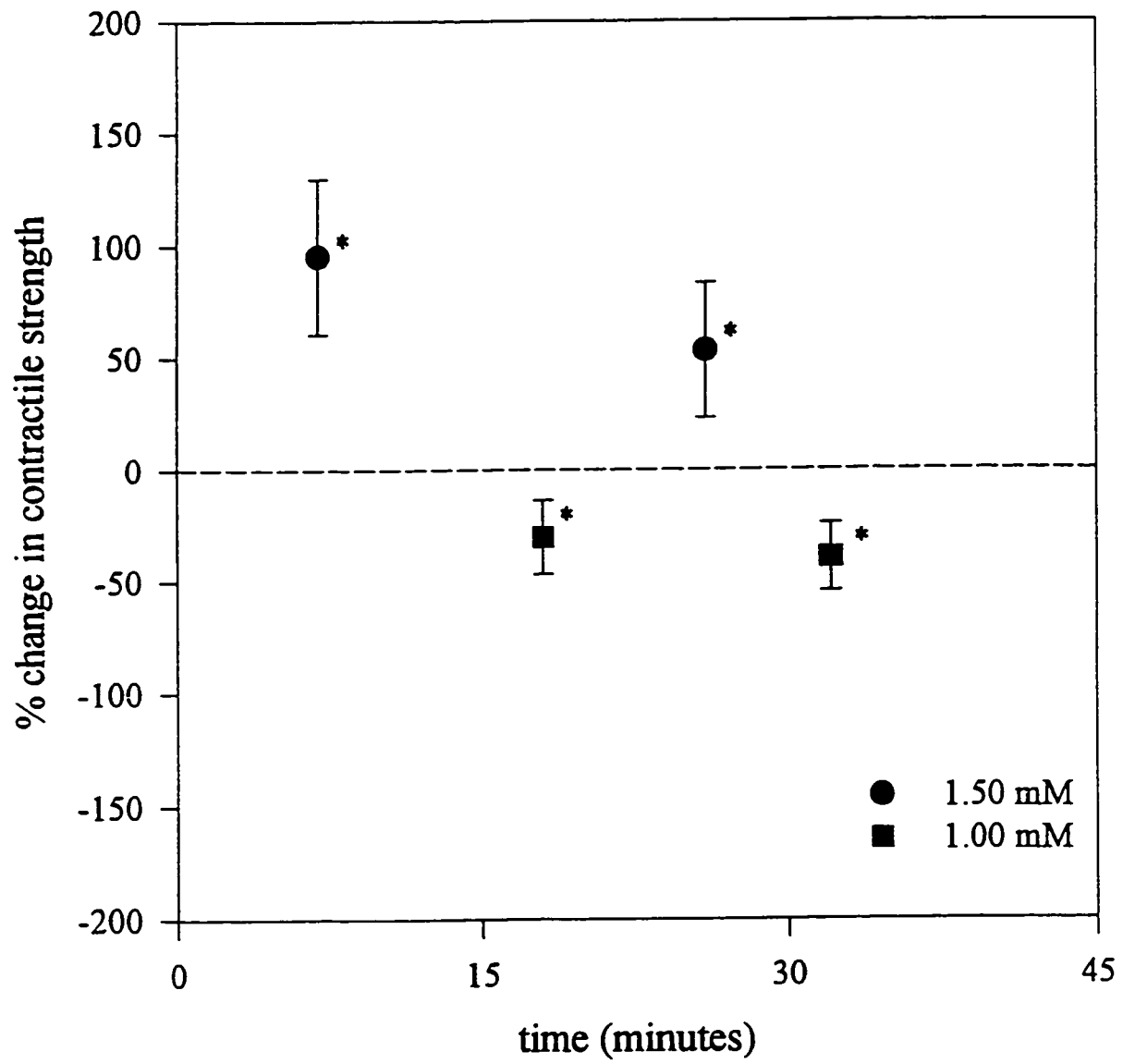
**Figure 2.** Effect of time on the strength of contraction in electrically paced rat (*Rattus norvegicus*) ventricular strips exposed to control  $[Ca^{2+}]$  (1.25 mM). Results are expressed as percent change in strength from the initial value (mean  $\pm$  SEM, N = 5). All measurements were made in 1.25 mM  $Ca^{2+}$  before and after experimental treatments (1.5 and 1.0 mM  $Ca^{2+}$ ).



**Figure 3.** Effect of a 20% increase to 1.5 mM or decrease to 1.00 mM in extracellular  $[Ca^{2+}]$  on the strength of contraction in electrically paced rat (*Rattus norvegicus*) atrial strips. Results are expressed as percent change in contractile strength observed from the immediately preceding normal calcium (1.25 mM) treatment (mean  $\pm$  SEM, N = 6).  
\* indicates a statistically significant difference from the control calcium treatments (P < 0.05) shown in Fig. 1.



**Figure 4.** Effect of a 20% increase to 1.5 mM or decrease to 1.00 mM in extracellular  $[Ca^{2+}]$  on the strength of contraction in electrically paced rat (*Rattus norvegicus*) ventricular strips. Results are expressed as percent change in contractile strength observed from the immediately preceding normal calcium (1.25 mM) treatment (mean  $\pm$  SEM, N = 5). \* indicates a statistically significant difference from the control calcium treatments (P < 0.05) shown in Fig. 2.



0.05) as ventricular muscle (Table 1, raw data). Additionally, a 20% increase in extracellular  $[Ca^{2+}]$  resulted in an overall increase in the strength of contraction of the ventricle by 92%, but only 28% in the atria (Table 1, raw data). The ventricular muscle was also more sensitive to a 20% increase in extracellular  $[Ca^{2+}]$  than to a 20% decrease (Table 1, raw data).

## ii) Frogs

The atrial (Figure 5) and ventricular (Figure 6) strips showed stable contractile strength for the first 40 minutes of the experiment and exhibited only minor changes from the initial value. After 40 minutes there was a significant decrease in the strength of contraction of the ventricle (Figure 6). A similar trend was observed in the atria, but the decrease was not significant. The average decrease in strength of contraction for the atria and ventricle was, respectively, 7% and 14% during the first 40 minutes and 18% and 27% thereafter.

Figure 7 shows the response obtained following exposure of atrial strips to high (2.16 mM) and low (0.54 mM) extracellular calcium. The atrial muscle developed a greater tension in the high calcium medium and a lower tension in the low calcium medium. A doubling of  $[Ca^{2+}]$  resulted in an average 33% increase in strength ( $P < 0.01$ ) and a halving of  $Ca^{2+}$  produced a 43% decrease in strength ( $P < 0.01$ ) when compared to the immediately previous control (1.08 mM) treatment.

The ventricular response to varying calcium concentrations is depicted in Figure 8. A 17% stronger contraction ( $P < 0.01$ ) was observed when  $[Ca^{2+}]$  was doubled to 2.16 mM and a 39% weaker contraction ( $P < 0.01$ ) occurred following a 50% decrease in extracellular calcium to 0.54 mM. Upon comparison of the two cardiac tissues, certain differences become evident. The ventricular muscle is 30% stronger ( $P < 0.05$ ) than atrial muscle under control (1.08 mM)

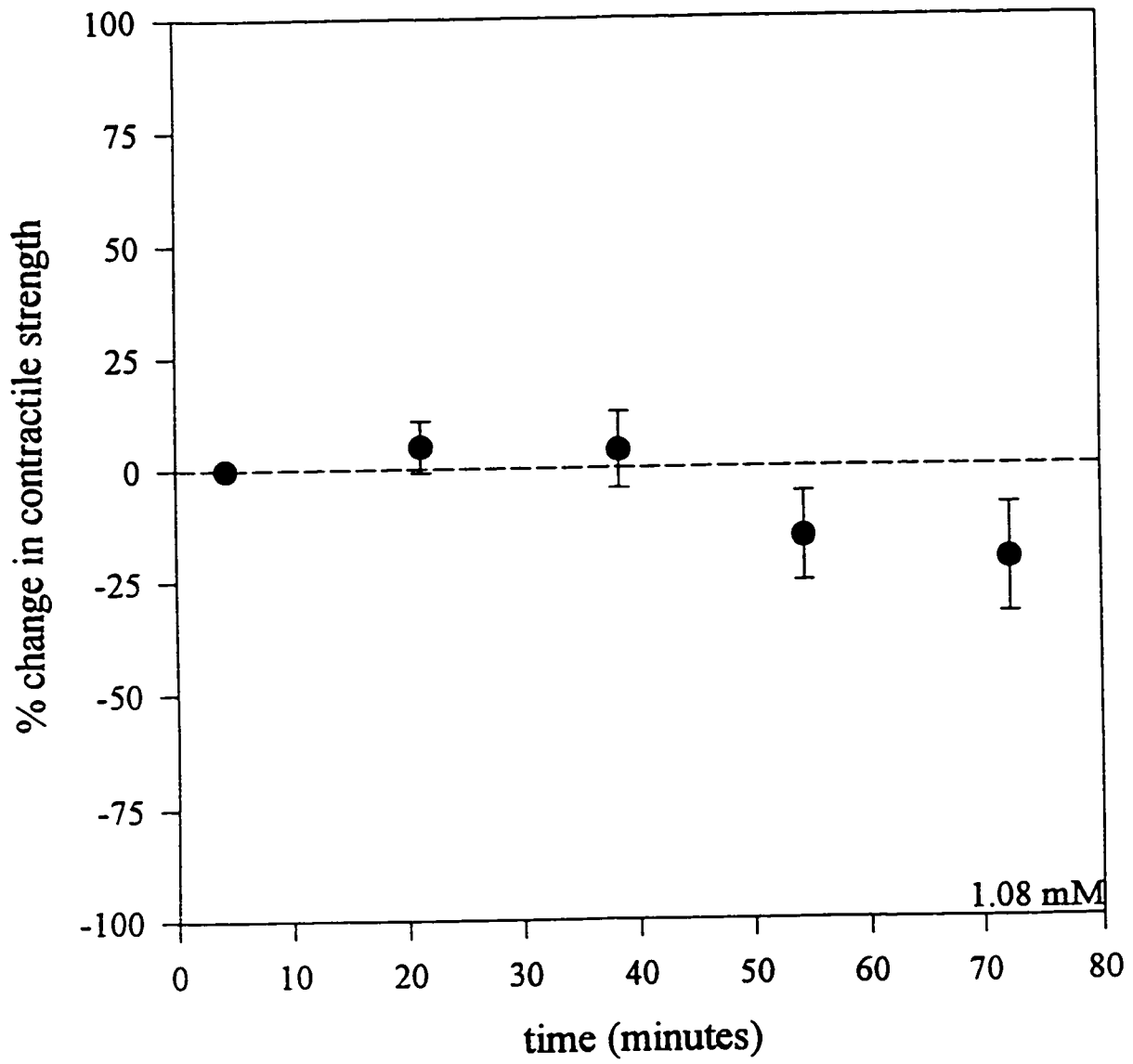
Table 1. Strength of contraction of atrial and ventricular muscle in various vertebrate species following exposure to varying calcium concentrations *in vitro*. The values are expressed as means  $\pm$  SEM. For the calcium concentrations of the individual treatments, refer to page \*\*. The sample sizes are indicated in parentheses. Interspecies comparisons: #, †, ‡ indicates tissues which are not significantly different from each other ( $P > 0.05$ ). Intraspecies comparisons: \* indicates significant difference in strength when compared to ventricle ( $P < 0.05$ ).

Calcium Treatment	Contractile Strength mg tension / 25 mg tissue											
	Rat			Frog			Trout			Eel		
	atrium (6)	ventricle (5)		atrium (12)	ventricle (16)		atrium (11)	ventricle (11)		atrium (13) <sup>a</sup>	ventricle (12) <sup>b</sup>	
high	104 $\pm$ 8	69 $\pm$ 16		3272 $\pm$ 46	3861 $\pm$ 305		2344 $\pm$ 290	1510 $\pm$ 168		972 $\pm$ 203	1557 $\pm$ 128	3213 $\pm$ 193
control	81 $\pm$ 6*	36 $\pm$ 3		2496 $\pm$ 40#*	3240 $\pm$ 209‡		1603 $\pm$ 148#*	1153 $\pm$ 96†		538 $\pm$ 86*	1114 $\pm$ 81†	2827 $\pm$ 179‡
low	51 $\pm$ 9	20 $\pm$ 3		1612 $\pm$ 56	2019 $\pm$ 241		660 $\pm$ 92	731 $\pm$ 112		265 $\pm$ 57	691 $\pm$ 92	—

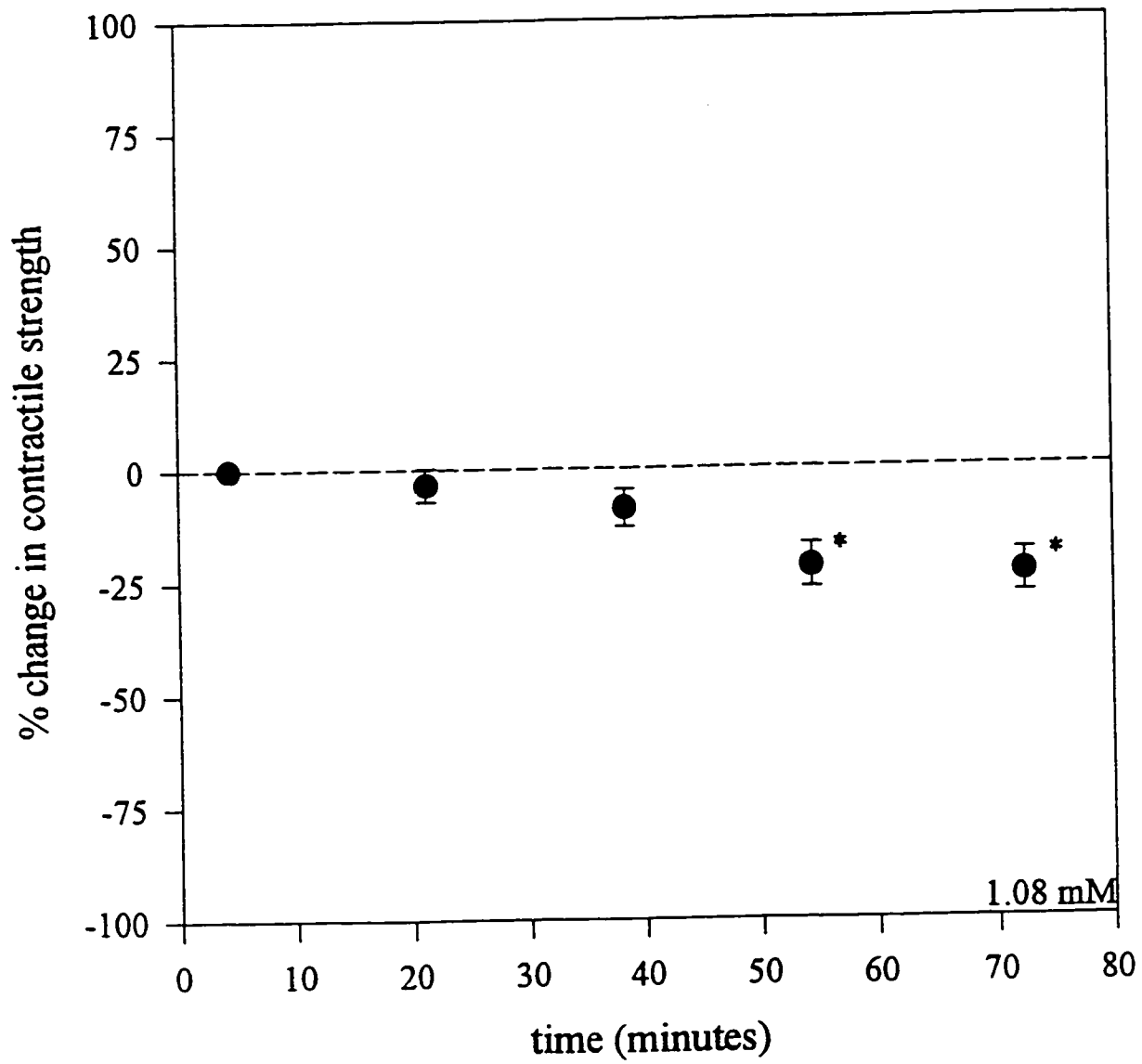
<sup>a</sup> These tissues were exposed to a high treatment that contained double the  $[Ca^{2+}]$  of the normal solution.

<sup>b</sup> The high treatment had a  $[Ca^{2+}]$  50% higher than the normal solution.

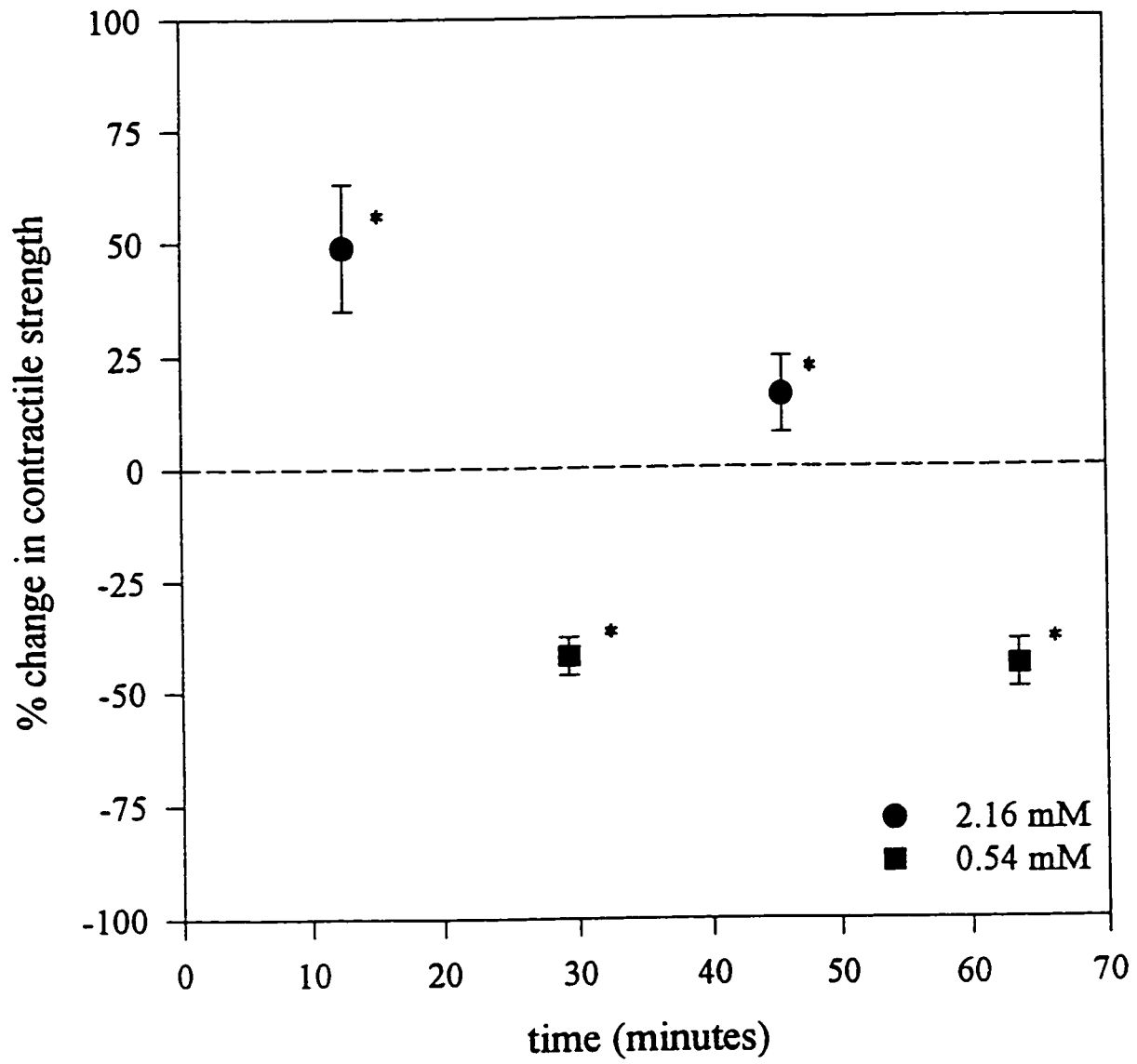
**Figure 5.** Effect of time on the strength of contraction in electrically paced leopard frog (*Rana pipiens*) atrial strips exposed to control  $[Ca^{2+}]$  (1.08 mM). Results are expressed as percent change in strength from the initial value (mean  $\pm$  SEM, N = 12). All measurements were made in 1.08 mM  $Ca^{2+}$  before and after experimental treatments (2.16 and 0.54 mM  $Ca^{2+}$ ).



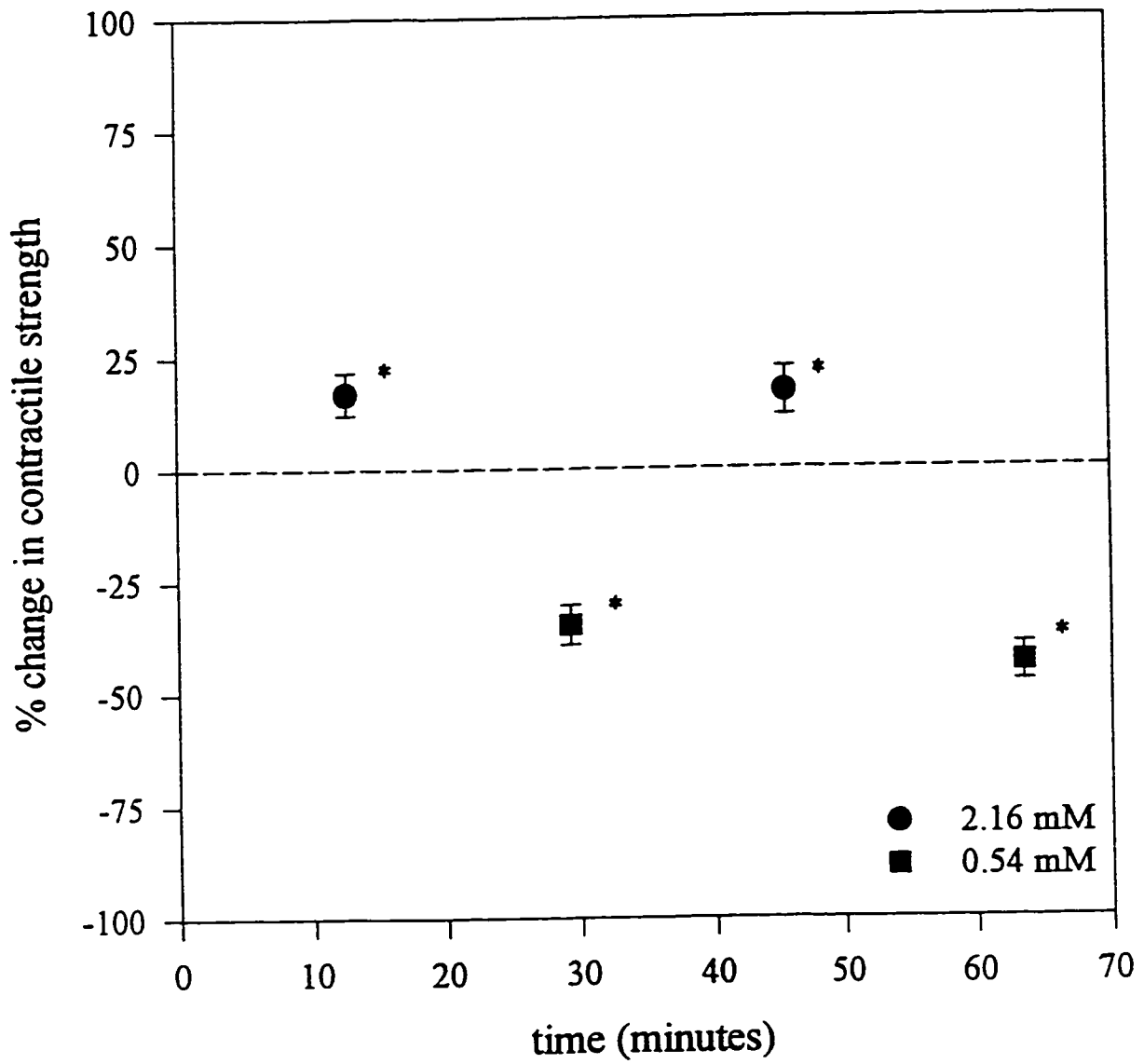
**Figure 6.** Effect of time on the strength of contraction in electrically paced leopard frog (*Rana pipiens*) ventricular strips exposed to control  $[Ca^{2+}]$  (1.08 mM). Results are expressed as percent change in strength from the initial value (mean  $\pm$  SEM, N = 16). All measurements were made in 1.08 mM  $Ca^{2+}$  before and after experimental treatments (2.16 and 0.54 mM  $Ca^{2+}$ ). \* indicates a statistically significant difference from the first control calcium treatment ( $P < 0.05$ ).



**Figure 7.** Effect of a doubling to 2.16 mM and a halving to 0.54 mM of extracellular  $[Ca^{2+}]$  on the strength of contraction in electrically paced leopard frog (*Rana pipiens*) atrial strips. Results are expressed as percent change in contractile strength observed from the immediately preceding control calcium (1.08 mM) treatment (mean  $\pm$  SEM, N = 12). \* indicates a statistically significant difference from the control calcium treatments ( $P < 0.05$ ) shown in Fig. 5.



**Figure 8.** Effect of a doubling to 2.16 mM and halving to 0.54 mM of extracellular  $[Ca^{2+}]$  on strength of contraction in electrically paced leopard frog (*Rana pipiens*) ventricular strips. Results are expressed as percent change in contractile strength observed from the immediately preceding control calcium (1.08 mM) treatment (mean  $\pm$  SEM, N = 16). \* indicates a statistically significant difference from the control calcium treatments (P < 0.05) shown in Fig. 6.



conditions and maintains a stronger contraction under experimental conditions (Table 1). Also, the atrial muscle seems to be more sensitive to increases in extracellular calcium levels where a doubling of  $\text{Ca}^{2+}$  causes a 33% increase in contractile strength whereas only a 17% increase is observed in the ventricle (Figures 7 and 8) while they seemed to have approximately the same sensitivity to decreasing calcium.

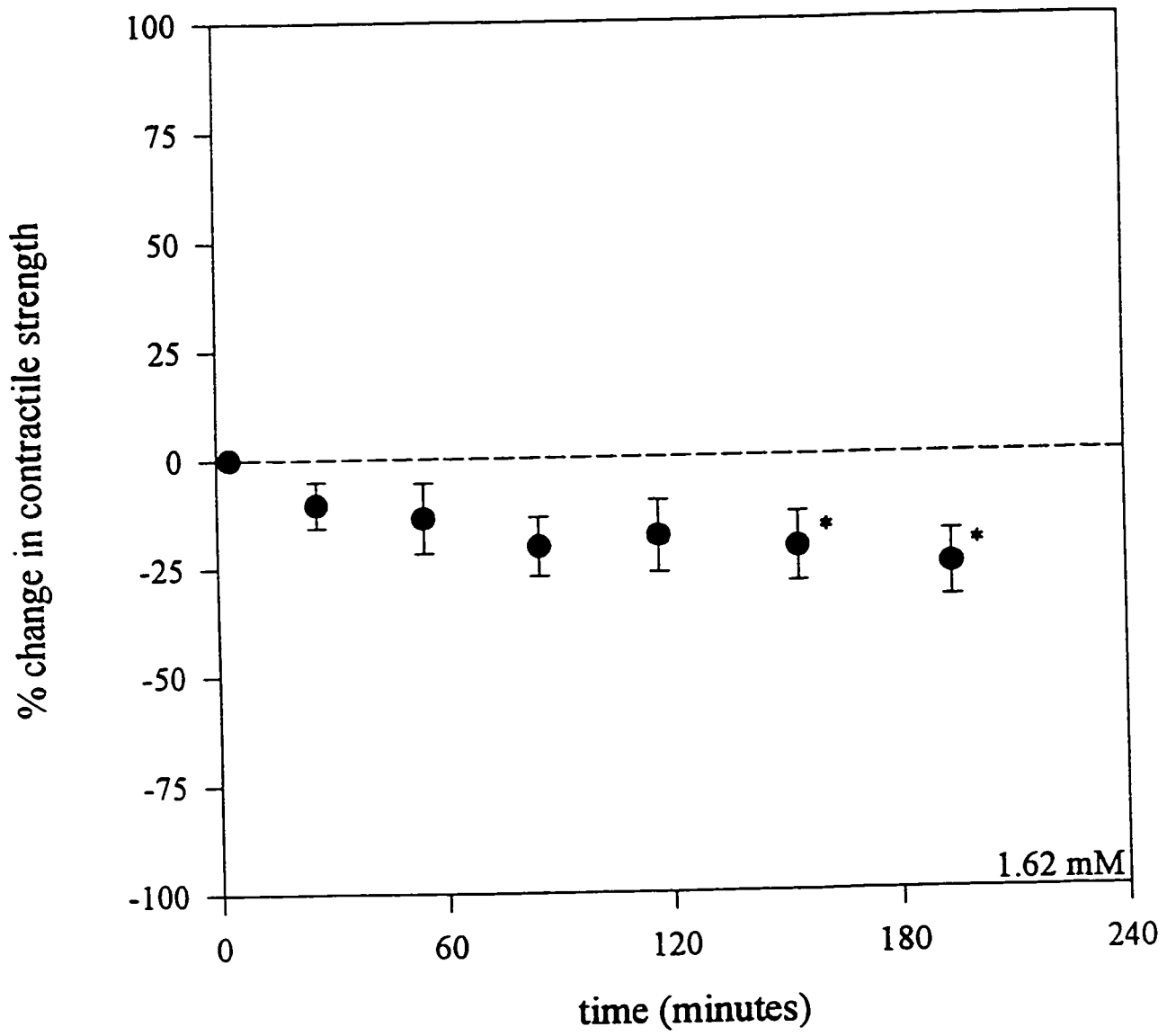
### iii) Trout

Both the atria (Figure 9) and ventricles (Figure 10) showed an immediate decrease in contractile strength following the initial control (1.62 mM) calcium treatment. The mean overall decrease in strength was 18% for the atrial muscle and 22% for the ventricular muscle. This reduction was significant ( $P < 0.05$ ) after 2.5 hours for the atria after which the mean decrease in strength was 23% when compared to the initial value. The ventricular tissue (Figure 10) exhibited a significant 32% decrease ( $P < 0.05$ ) in contractile strength after two hours of experimentation.

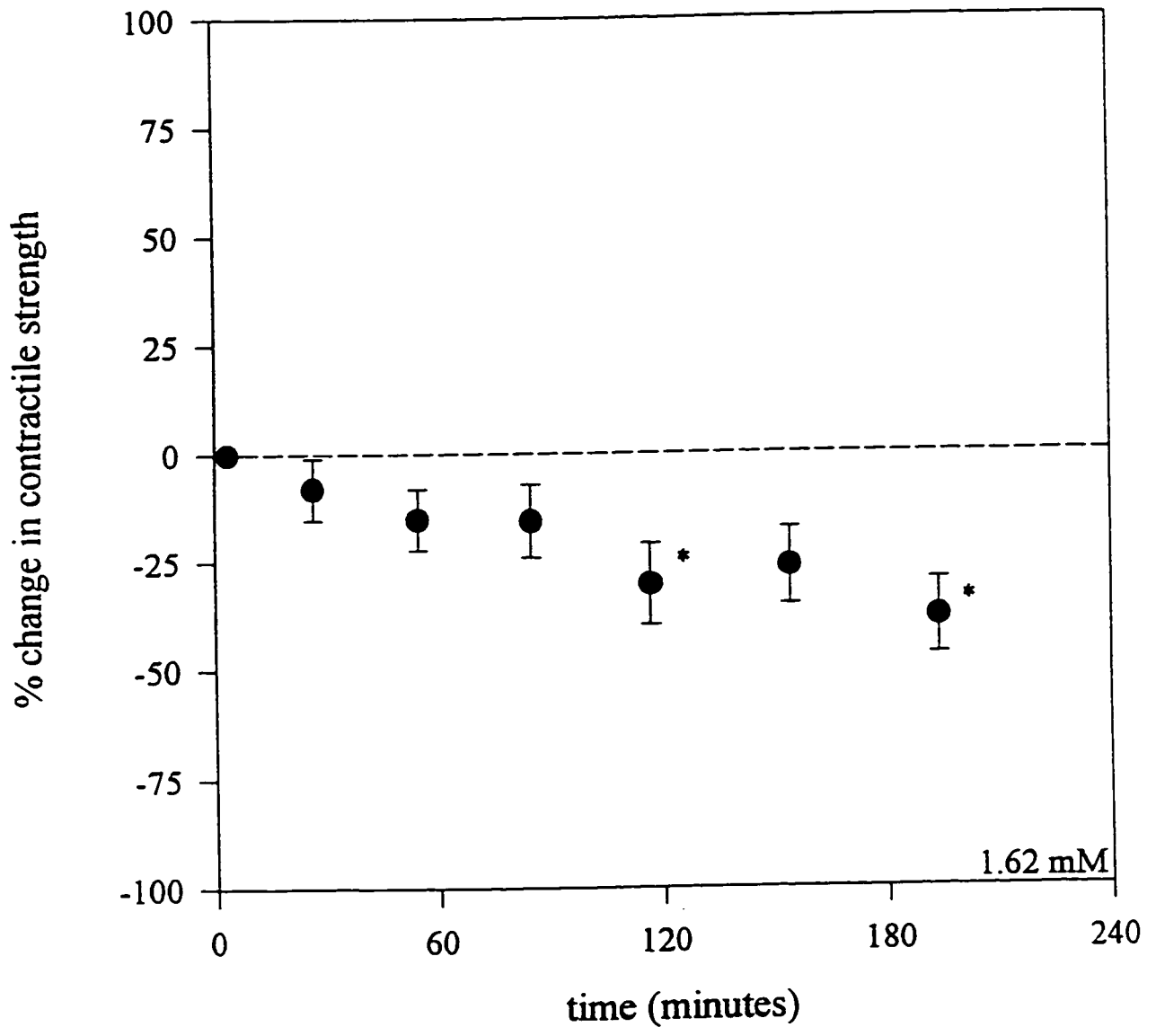
The strength of contraction resulting from the exposure of atrial muscle to high (3.24 mM) and low (0.81 mM) calcium is shown in Figure 11. Once again, increasing the  $[\text{Ca}^{2+}]$  increased the strength of contraction when compared to control values (1.62 mM). A 50% decrease in calcium to 0.81 mM resulted in a 56% decrease ( $P < 0.01$ ) in strength of contraction, and a doubling of  $\text{Ca}^{2+}$  to 3.24 mM resulted in a 53% increase ( $P < 0.01$ ) in the strength of contraction.

Ventricular muscle exposed to changing  $[\text{Ca}^{2+}]$  exhibited the same trend as observed for the atria (Figure 12). A doubling of  $\text{Ca}^{2+}$  concentration resulted in a 33% stronger contraction ( $P < 0.01$ ) when compared to control values and a halving of  $\text{Ca}^{2+}$  caused a 41% decrease ( $P <$

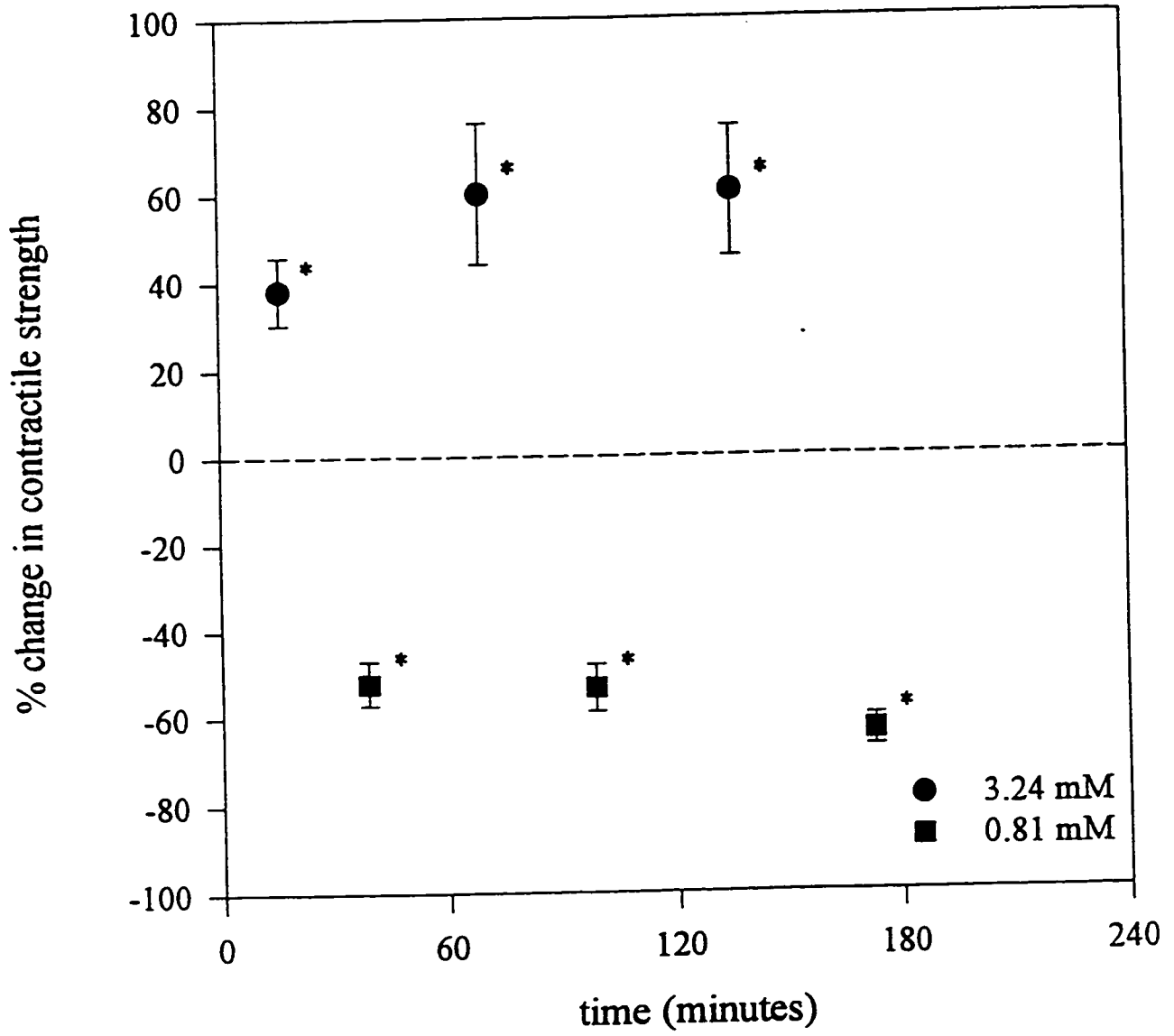
**Figure 9.** Effect of time on the strength of contraction in electrically paced rainbow trout (*Oncorhynchus mykiss*) atrial strips exposed to control  $[Ca^{2+}]$  (1.62 mM). Results are expressed as percent change in strength from the initial value (mean  $\pm$  SEM, N = 11). All measurements were made in 1.62 mM  $Ca^{2+}$  before and after experimental treatments (3.24 and 0.81 mM  $Ca^{2+}$ ). \* indicates a statistically significant difference from the first control calcium treatment (P < 0.05).



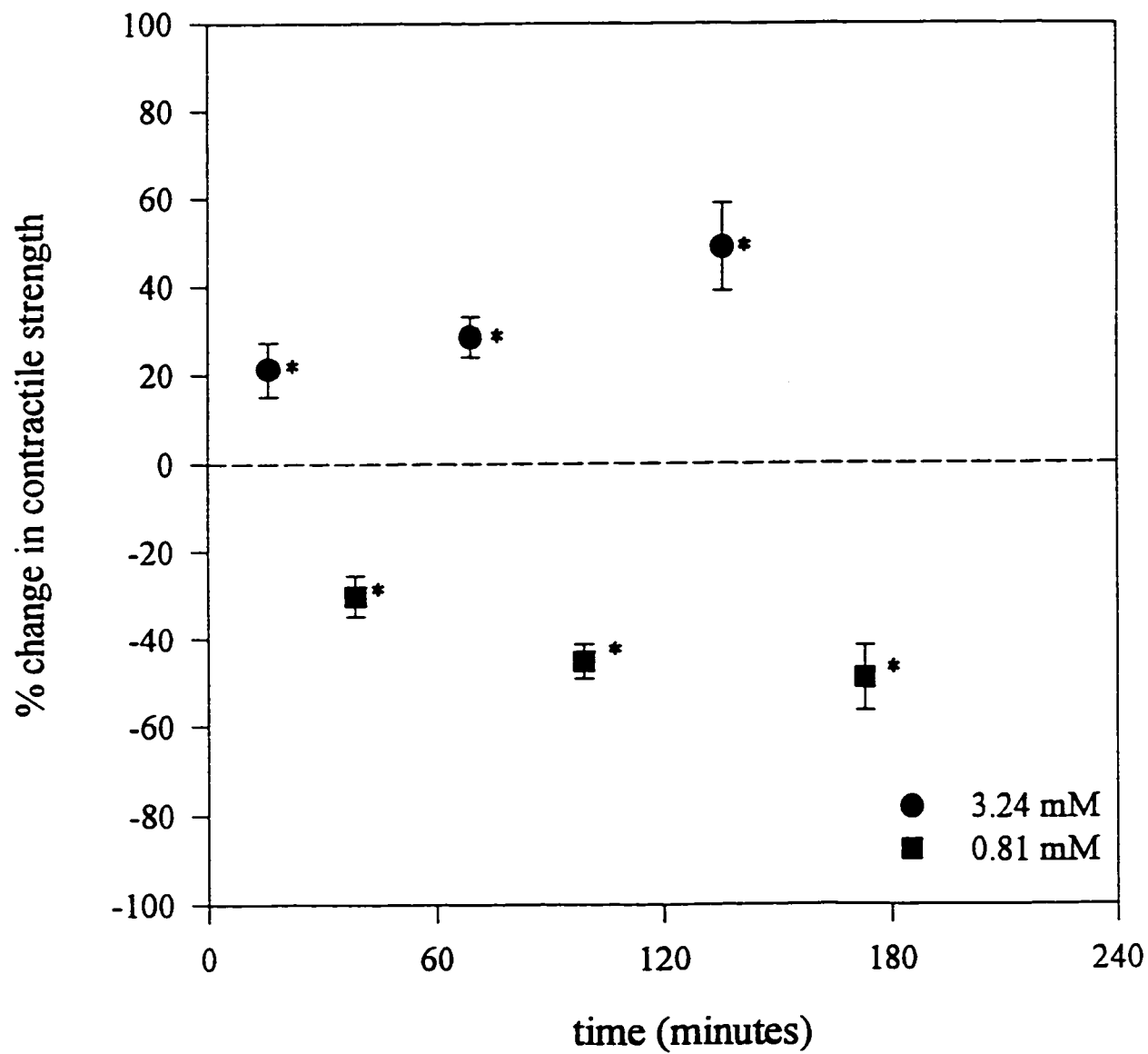
**Figure 10.** Effect of time on the strength of contraction in electrically paced rainbow trout (*Oncorhynchus mykiss*) ventricular strips exposed to control  $[Ca^{2+}]$  (1.62 mM). Results are expressed as percent change in strength from the initial value (mean  $\pm$  SEM, N = 11). All measurements were made in 1.62 mM  $Ca^{2+}$  before and after experimental treatments (3.24 and 0.81 mM  $Ca^{2+}$ ). \* indicates a statistically significant difference from the first control calcium treatment ( $P < 0.05$ ).



**Figure 11.** Effect of a doubling to 3.24 mM and a halving to 0.81 mM of extracellular  $[Ca^{2+}]$  on the strength of contraction in electrically paced rainbow trout (*Oncorhynchus mykiss*) atrial strips. Results are expressed as percent change in contractile strength observed from the immediately preceding control calcium (1.62 mM) treatment (mean  $\pm$  SEM, N = 11). \* indicates a statistically significant difference from the control calcium treatments (P < 0.05) shown in Fig. 9.



**Figure 12.** Effect of a doubling to 3.24 mM and a halving to 0.81 mM of extracellular  $[Ca^{2+}]$  on strength of contraction in electrically paced rainbow trout (*Oncorhynchus mykiss*) ventricular strips. Results are expressed as percent change in contractile strength observed from the immediately preceding control calcium (1.62 mM) treatment (mean  $\pm$  SEM, N = 11). \* indicates a statistically significant difference from the control calcium treatments (P < 0.05) shown in Fig. 10.



0.01) in contractile strength. A comparison between the two types of cardiac tissues reveals that the atrial muscle has a 39% stronger strength of contraction than ventricular muscle ( $P < 0.05$ ) under control (1.62 mM) conditions on a per weight basis (Table 1). Further comparison also indicates a greater response to elevated calcium levels for the atrial tissue where a 53% increase in strength of contraction was observed in 3.24 mM  $\text{Ca}^{2+}$  (Figure 11) whereas the ventricular strength increased by only 33% under the same conditions (Figure 12). The atrial (Figure 11) and ventricular (Figure 12) muscle in trout seemed to have about the same sensitivity to decreasing calcium.

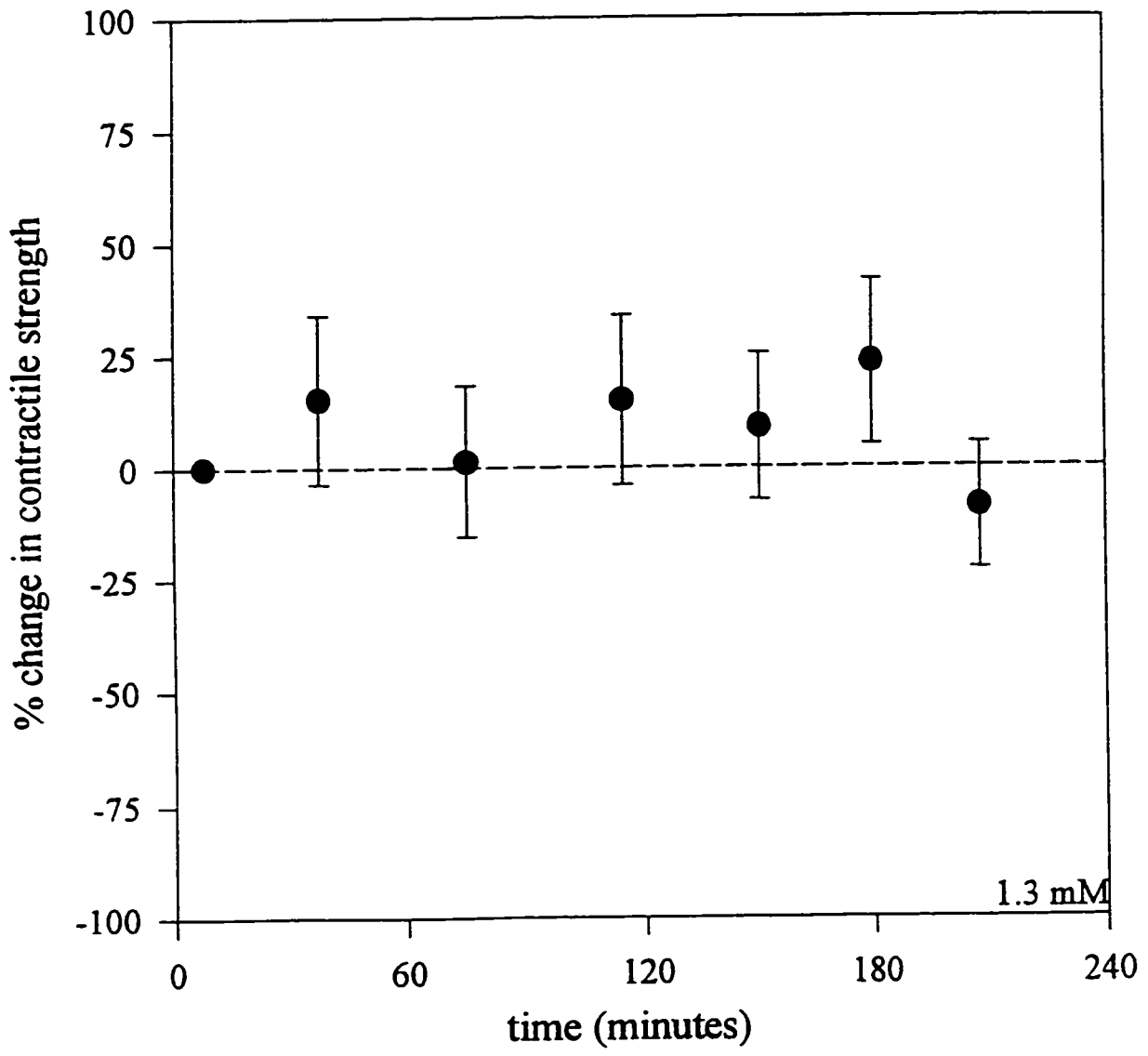
#### iv) Eels

The effect of time on the contractile strength of atrial and ventricular muscle under control conditions (1.3 mM  $\text{Ca}^{2+}$ ) is shown in Figures 13, 14 and 15. In spite of much variability in strength between animals, the atrial muscle showed no significant change throughout the experiment (Figure 13).

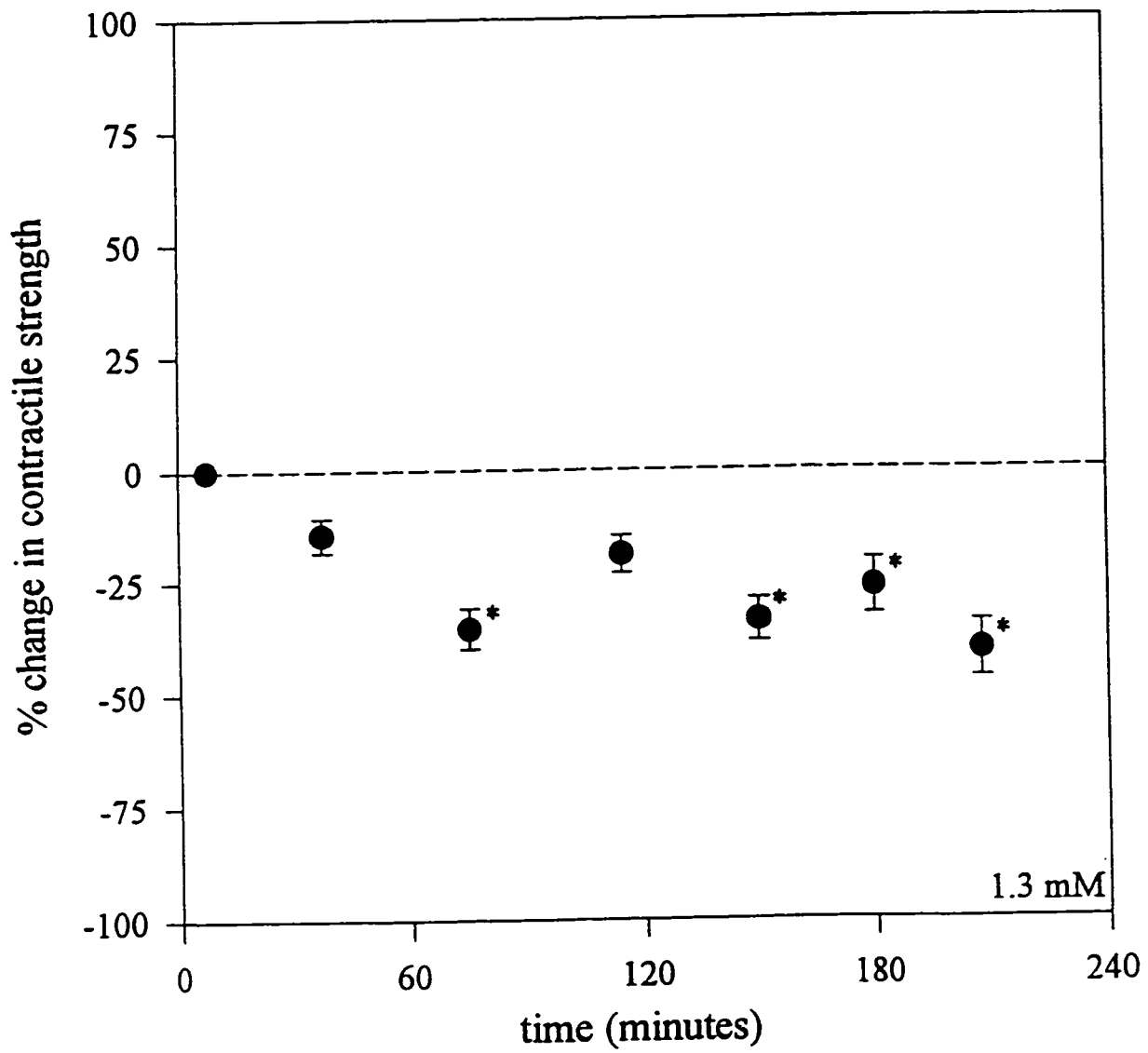
In the case of eels, two groups of ventricles were run using different high calcium solutions and therefore, separate controls were used. The first group (also exposed to 2.6 mM  $\text{Ca}^{2+}$ ), underwent an average 28% decrease in strength during the course of the experiment (Figure 14). A significant reduction was observed after 75 minutes ( $P < 0.05$ ) and was sustained for the rest of the experiment with the exception of the 120 minute measurement. The second group of ventricular tissue was exposed to 1.95 mM  $\text{Ca}^{2+}$  and sustained an initial 12% increase ( $P < 0.05$ ) in contractile strength but recovered by the end of the experiment (Figure 15).

The effect of changing calcium concentrations on eel atrial muscle strips is depicted in Figure 16. This figure shows that an increase in extracellular [ $\text{Ca}^{2+}$ ] is accompanied by an

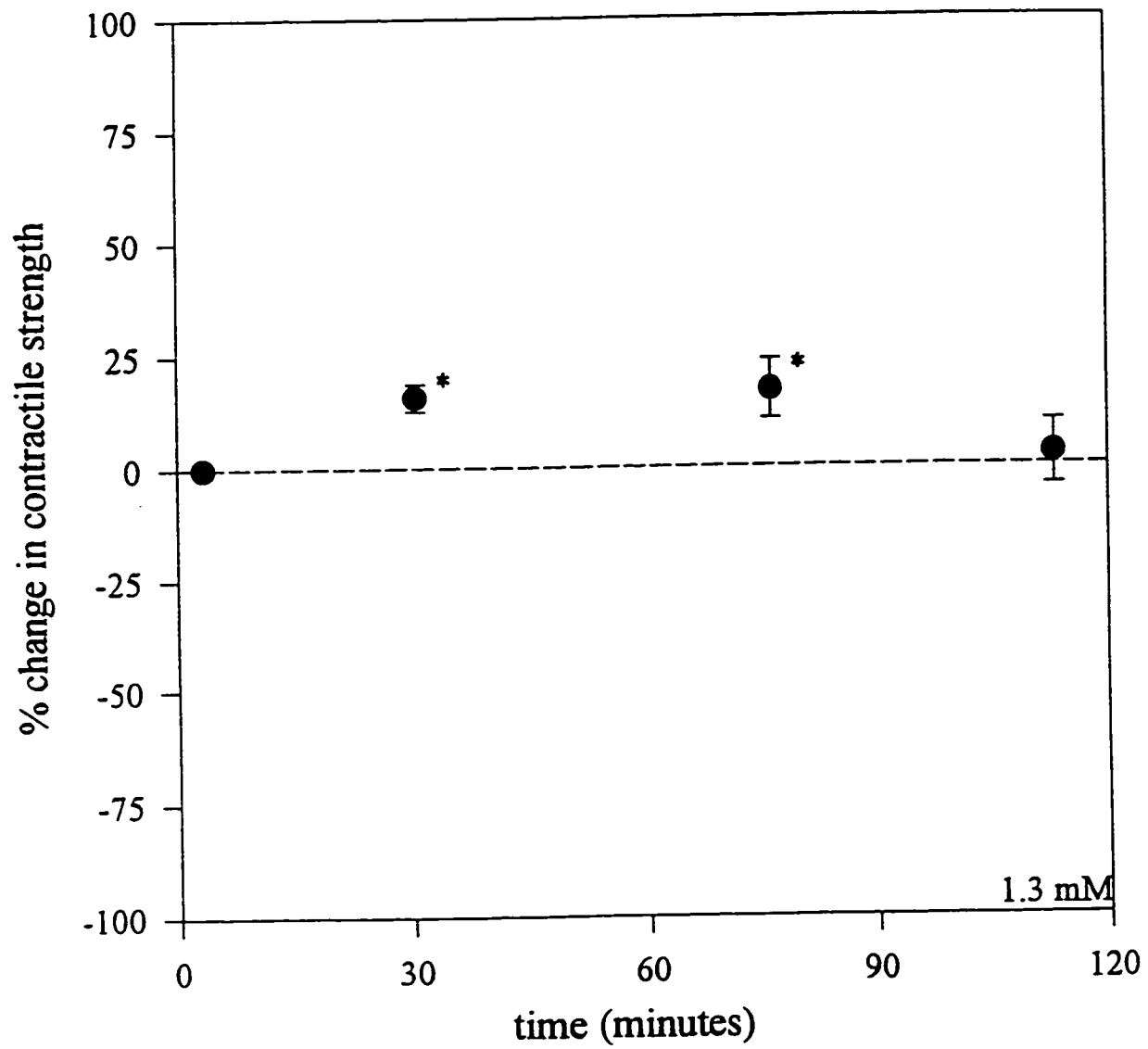
**Figure 13.** Effect of time on the strength of contraction in electrically paced freshwater eel (*Anguilla rostrata* LeSueur) atrial strips exposed to control  $[Ca^{2+}]$  (1.3 mM). Results are expressed as percent change in strength from the initial value (mean  $\pm$  SEM, N = 13). All measurements were made in 1.3 mM  $Ca^{2+}$  before and after experimental treatments (2.6 and 0.65 mM  $Ca^{2+}$ ). \* indicates a statistically significant difference from the first control calcium treatment ( $P < 0.05$ ).



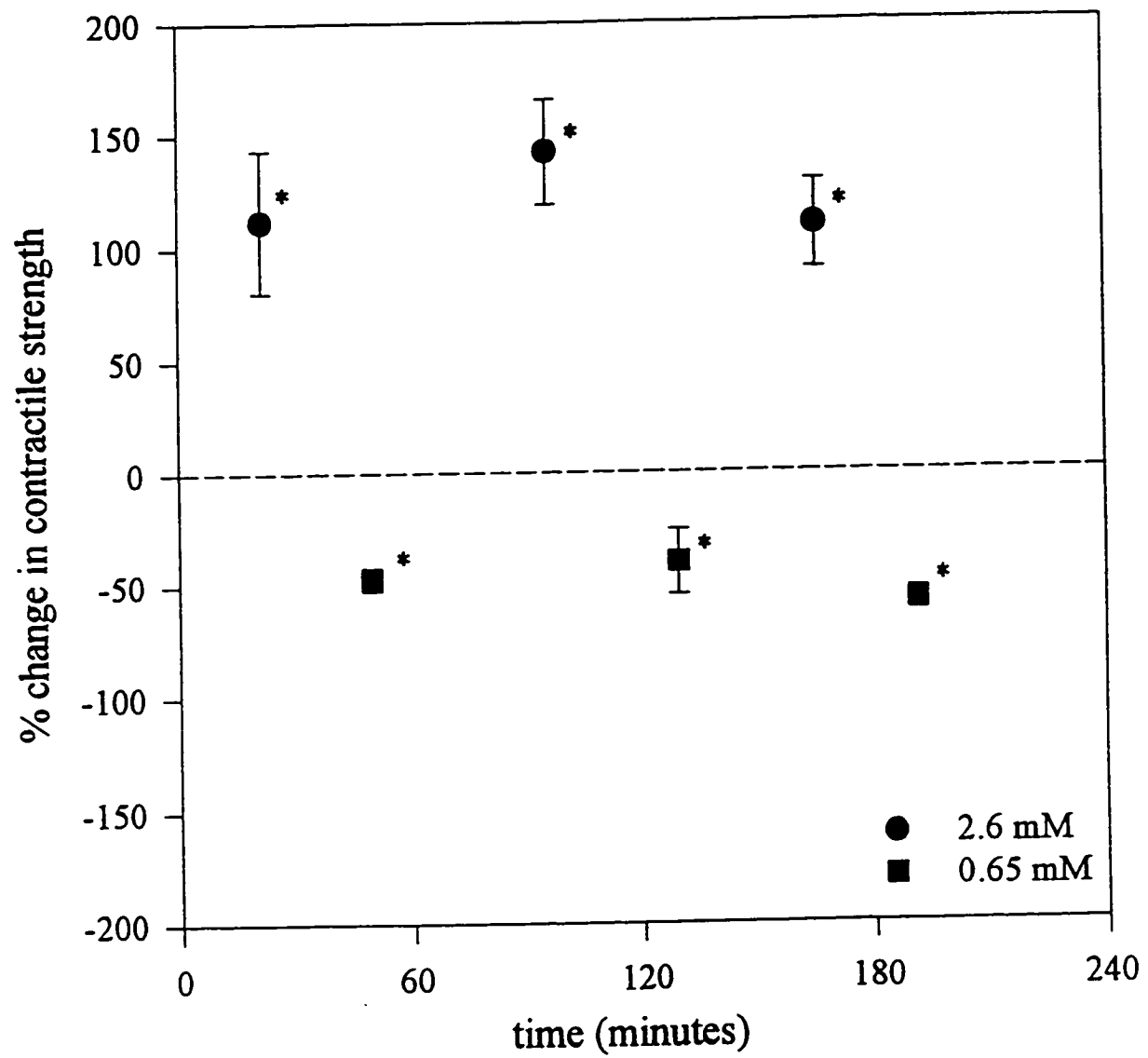
**Figure 14.** Effect of time on the strength of contraction in electrically paced freshwater eel (*Anguilla rostrata* LeSueur) ventricular strips exposed to control  $[Ca^{2+}]$  (1.3 mM). Results are expressed as percent change in strength from the initial value (mean  $\pm$  SEM, N = 13). All measurements were made in 1.3 mM  $Ca^{2+}$  before and after experimental treatments (2.6 and 0.65 mM  $Ca^{2+}$ ). \* indicates a statistically significant difference from the first control calcium treatment (P < 0.05).



**Figure 15.** Effect of time on the strength of contraction in electrically paced freshwater eel (*Anguilla rostrata* LeSueur) ventricular strips exposed to control  $[Ca^{2+}]$  (1.3 mM). Results are expressed as percent change in strength from the initial value (mean  $\pm$  SEM, N = 12). All measurements were made in 1.3 mM  $Ca^{2+}$  before and after experimental treatments (1.95 mM  $Ca^{2+}$ ). \* indicates a statistically significant difference from the first control calcium treatment (P < 0.05).



**Figure 16.** Effect of a doubling to 2.60 mM and a halving to 0.65 mM of extracellular  $[Ca^{2+}]$  on the strength of contraction in electrically paced freshwater eel (*Anguilla rostrata* LeSueur) atrial strips. Results are expressed as percent change in contractile strength observed from the immediately preceding control calcium (1.30 mM) treatment (mean  $\pm$  SEM, N = 13). \* indicates a statistically significant difference from the control calcium treatments ( $P < 0.05$ ) shown in Fig. 13.



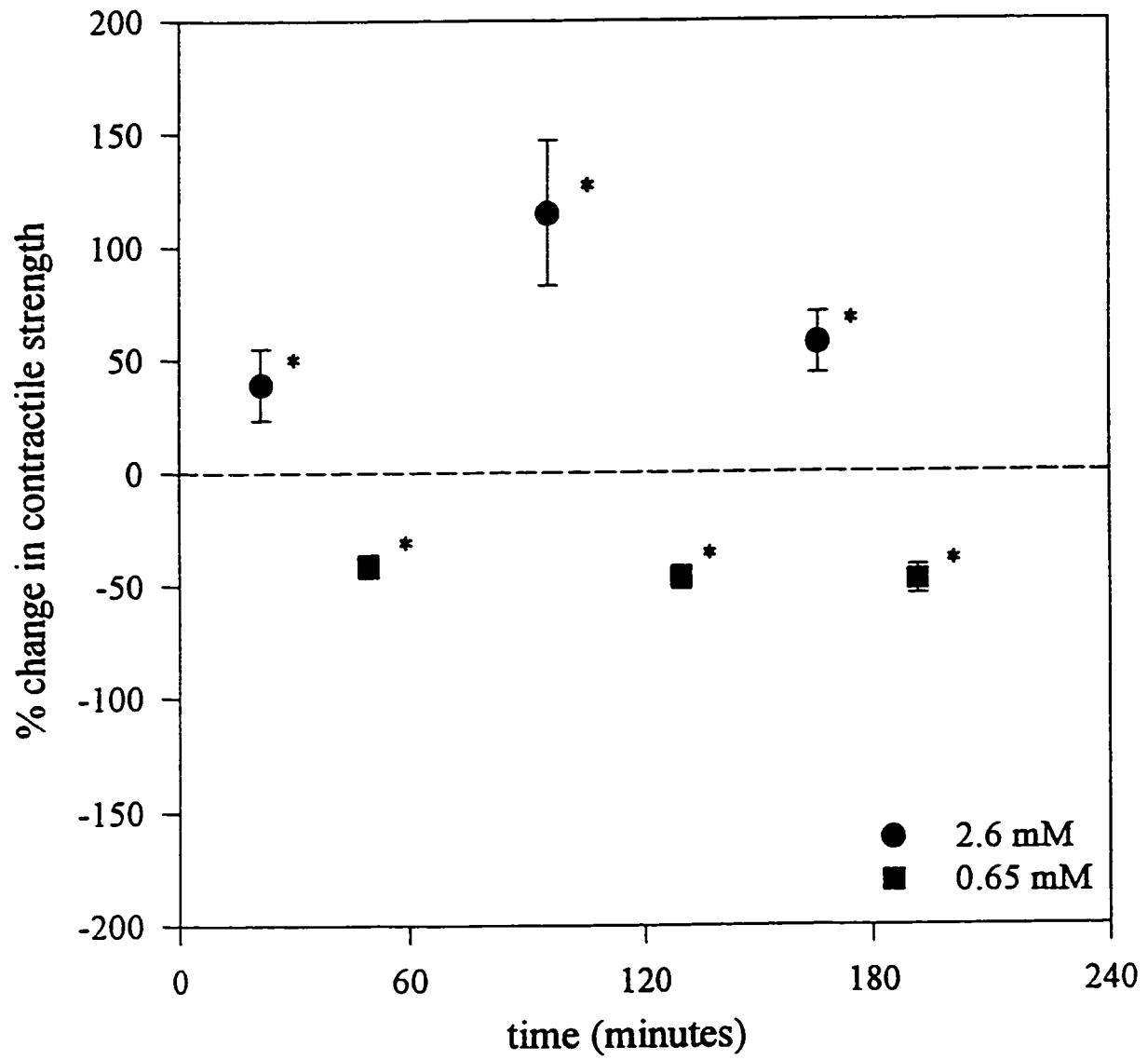
increase in the strength of contraction. Upon comparison to control values (1.3 mM), it is observed that the doubling of extracellular  $[Ca^{2+}]$  to 2.6 mM causes a dramatic increase in contractile strength (average of the three treatments results in a 121% stronger contraction of the muscle;  $P < 0.01$ ). Conversely, a 50% decrease in  $[Ca^{2+}]$  to 0.65 mM resulted in an average 48% reduction of atrial strength ( $P < 0.05$ ).

The exposure of ventricular muscle to various calcium concentrations resulted in changes in contractile strength which are shown in Figures 17 and 18. A comparison to control values reveals a 45% weaker contraction ( $P < 0.01$ ) when the  $[Ca^{2+}]$  is halved and a 70% stronger contraction ( $P < 0.01$ ) is observed with a doubling of extracellular calcium levels (Figure 17). When the  $Ca^{2+}$  levels were increased by 50% to 1.95 mM, the ventricular tissue displayed a 15% increase in contractile strength when compared to control values (Figure 18). Comparison of the cardiac tissues illustrates that the ventricular muscle is twice as strong ( $P < 0.05$ ) as the atrial muscle under control conditions of 1.3 mM  $Ca^{2+}$  (Table 1). Further comparison shows that a doubling of  $Ca^{2+}$  concentration from 1.3 mM to 2.6 mM causes a 121% increase in atrial strength (Figure 16) but only a 70% increase in ventricular strength (Figure 17). These data suggest that the atrium is more sensitive than the ventricle to increases in calcium levels.

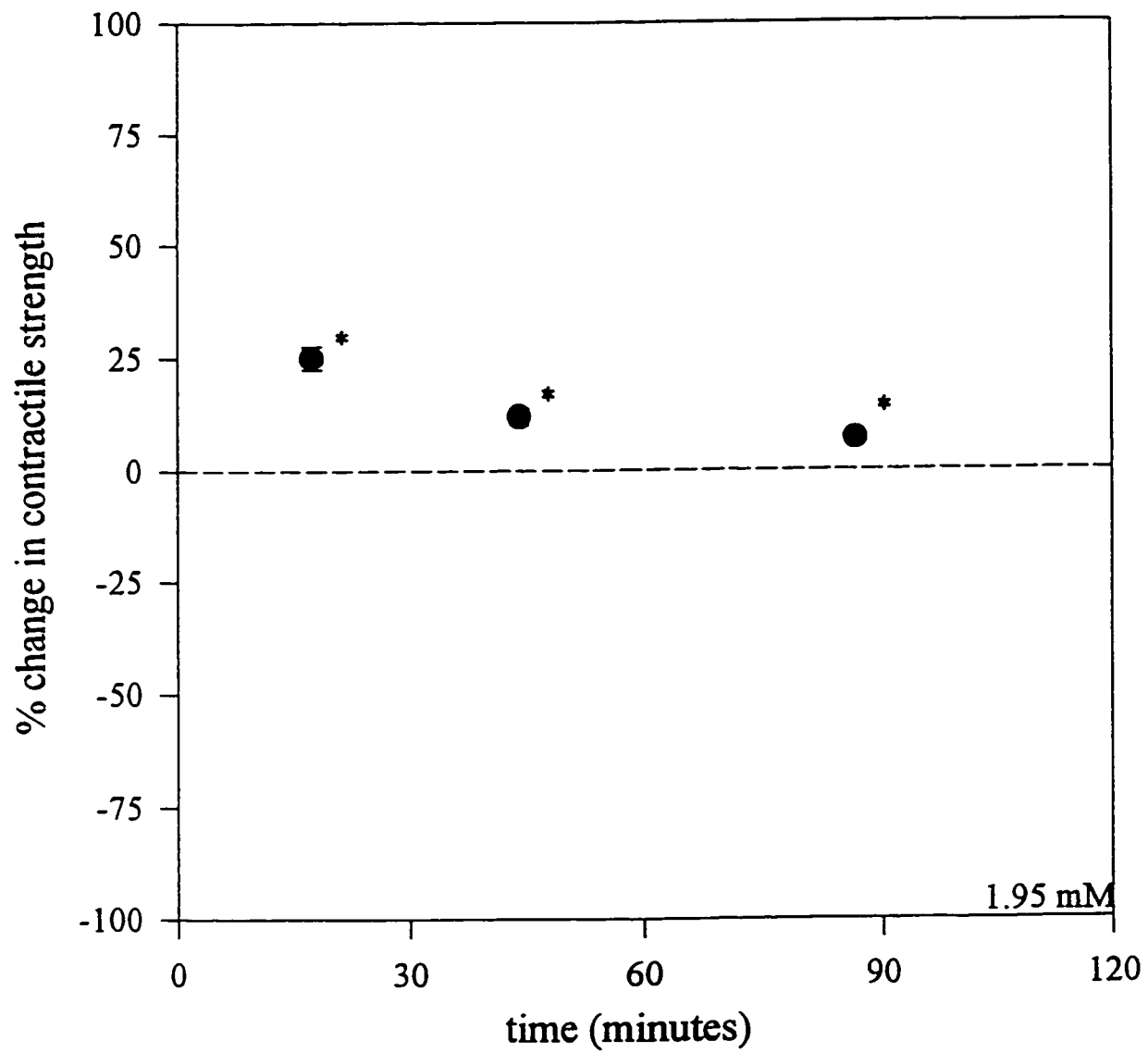
#### **v) Interspecies Comparisons**

Figure 19 depicts the average % change in contractile strength of atrial muscle following exposure to high and low  $[Ca^{2+}]$  solutions when compared to control values for all species tested. The eel had the greatest sensitivity to high calcium. Although the rat data are included, they are not fully comparable because the  $[Ca^{2+}]$  increase and decrease were only 20% as compared to the doubling or halving in the other species. When the rat ventricle was exposed to a calcium

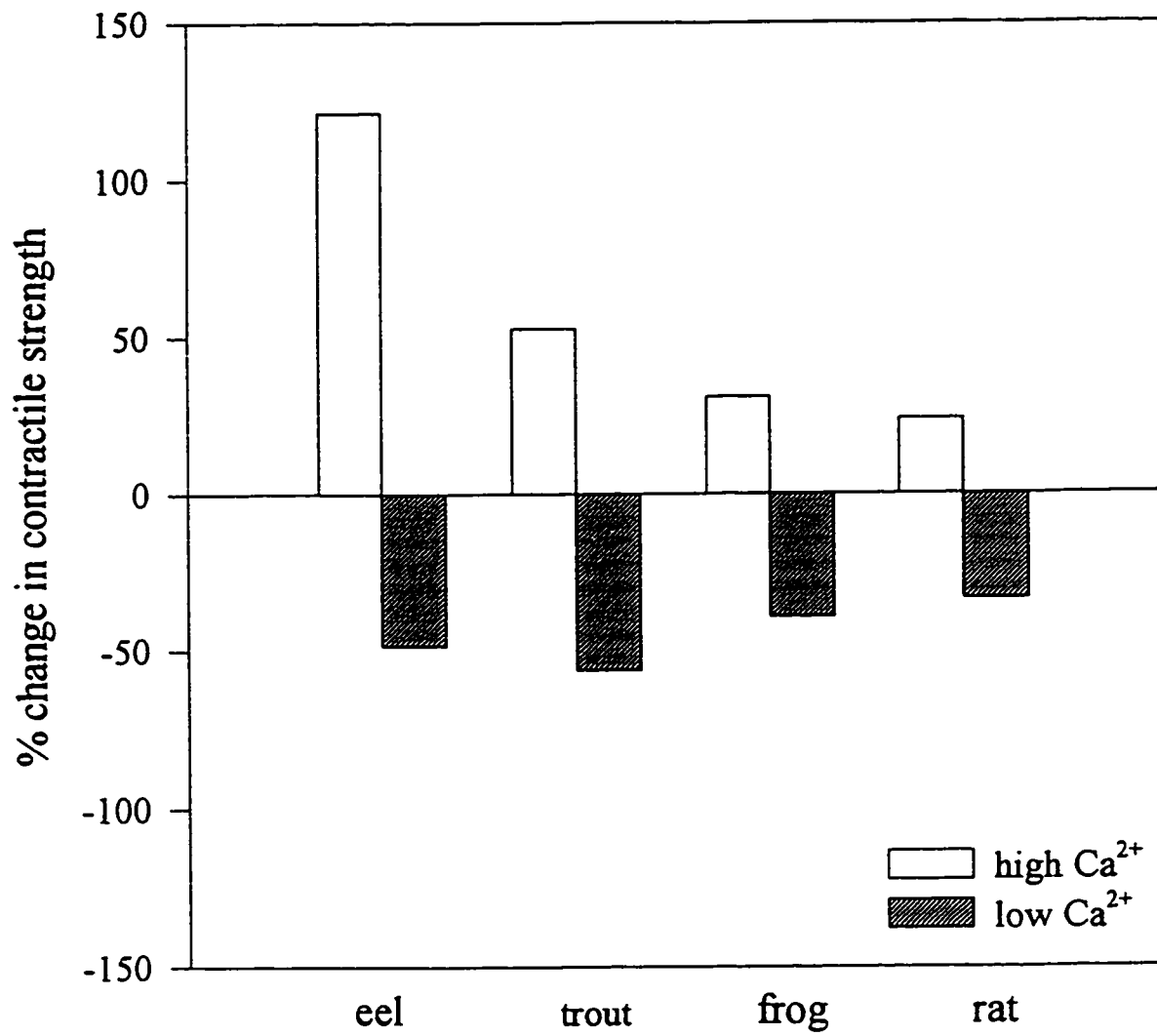
**Figure 17.** Effect of a doubling to 2.60 mM and a halving to 0.65 mM of extracellular  $[Ca^{2+}]$  on the strength of contraction in electrically paced freshwater eel (*Anguilla rostrata* LeSueur) ventricular strips. Results are expressed as percent change in contractile strength observed from the immediately preceding control calcium (1.30 mM) treatment (mean  $\pm$  SEM, N = 13). \* indicates a statistically significant difference from the control calcium treatments (P < 0.05) shown in Fig. 14.



**Figure 18.** Effect of a 50% increase to 1.95 mM of extracellular  $[Ca^{2+}]$  on the strength of contraction in freshwater eel (*Anguilla rostrata* LeSueur) ventricular strips. Results are expressed as percent change in contractile strength observed from the immediately preceding control calcium (1.3 mM) treatment (mean  $\pm$  SEM, N = 12). \* indicates a statistically significant difference from the normal calcium treatments (P < 0.05) shown in Fig. 15.



**Figure 19.** Effect of high and low  $[Ca^{2+}]$  on atrial muscle contractile strength from various vertebrates. The data are expressed as mean percent increase from control values. High  $[Ca^{2+}]$  = double the control  $[Ca^{2+}]$  for the frog, trout and eel and +20% for the rat. Low  $[Ca^{2+}]$  = 50% of the control  $[Ca^{2+}]$  for the frog, trout and eel and -20% for the rat.



solution double that of the control, it exhibited very irregular contractions that were not considered to be normal. It can be suggested then that the rat cardiac muscle can not withstand large fluctuations in extracellular calcium levels. These data also show that there is no detectable pattern in reactivity to low extracellular calcium levels between the species examined.

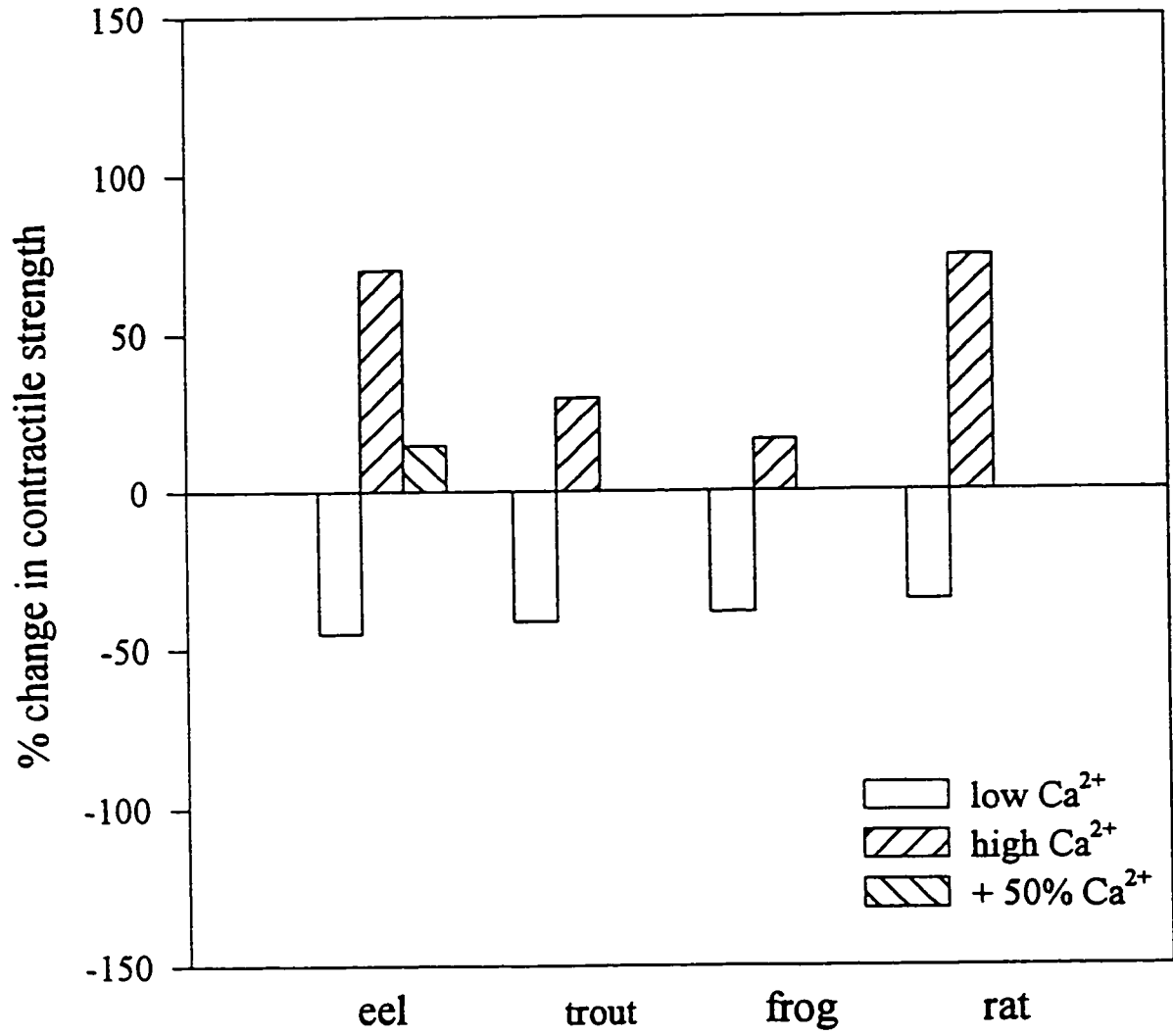
The effects of changes in extracellular  $\text{Ca}^{2+}$  on ventricular contractile strength in the different animals used are shown in Figure 20. A 50% increase in  $\text{Ca}^{2+}$  caused only a small increase in contractile strength for the eel. Considering that the rat was exposed to a much smaller increase in extracellular calcium levels (20%) and exhibited a larger increase in strength of contraction, and that it could not contract regularly when exposed to a doubling of extracellular  $[\text{Ca}^{2+}]$ , it is the most sensitive to high calcium among the vertebrates tested in this study.

It is also interesting to note that both the eel atrial (Figure 19) and ventricular (Figure 20) strips were more sensitive to a doubling of extracellular  $[\text{Ca}^{2+}]$  than trout cardiac tissues. The same increase in  $[\text{Ca}^{2+}]$  resulted in a % increase in strength of contraction in the eel that was double that observed in the trout.

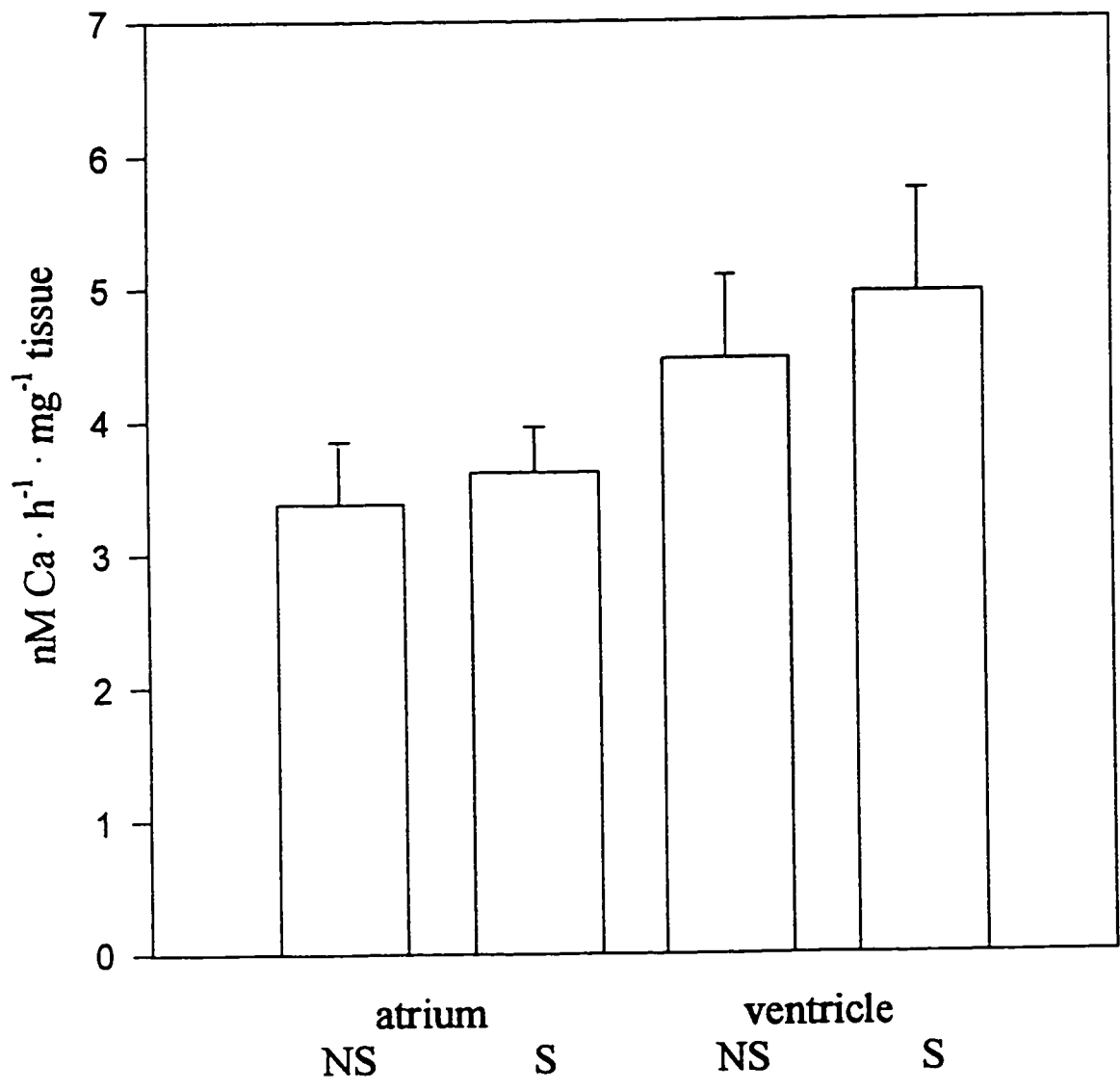
### **B. Calcium Uptake by Eel Cardiac Muscle**

Contracting and non-contracting atrial and ventricular muscle were exposed to  $^{45}\text{Ca}$  to determine the rate of calcium uptake. No difference was observed in the uptake rate either between contracting and non-contracting cardiac tissues or between atrial and ventricular  $^{45}\text{Ca}$  uptake (Figure 21).

**Figure 20.** Effect of high and low  $[Ca^{2+}]$  on ventricular muscle contractile strength from various vertebrates. The data are expressed as mean percent increase from control values. High  $[Ca^{2+}]$  = double the control  $[Ca^{2+}]$  for the frog, trout and eel and +20% for the rat. Low  $[Ca^{2+}]$  = 50% of the control  $[Ca^{2+}]$  for the frog, trout and eel and -20% for the rat. A +50%  $[Ca^{2+}]$  is also shown for the eel.



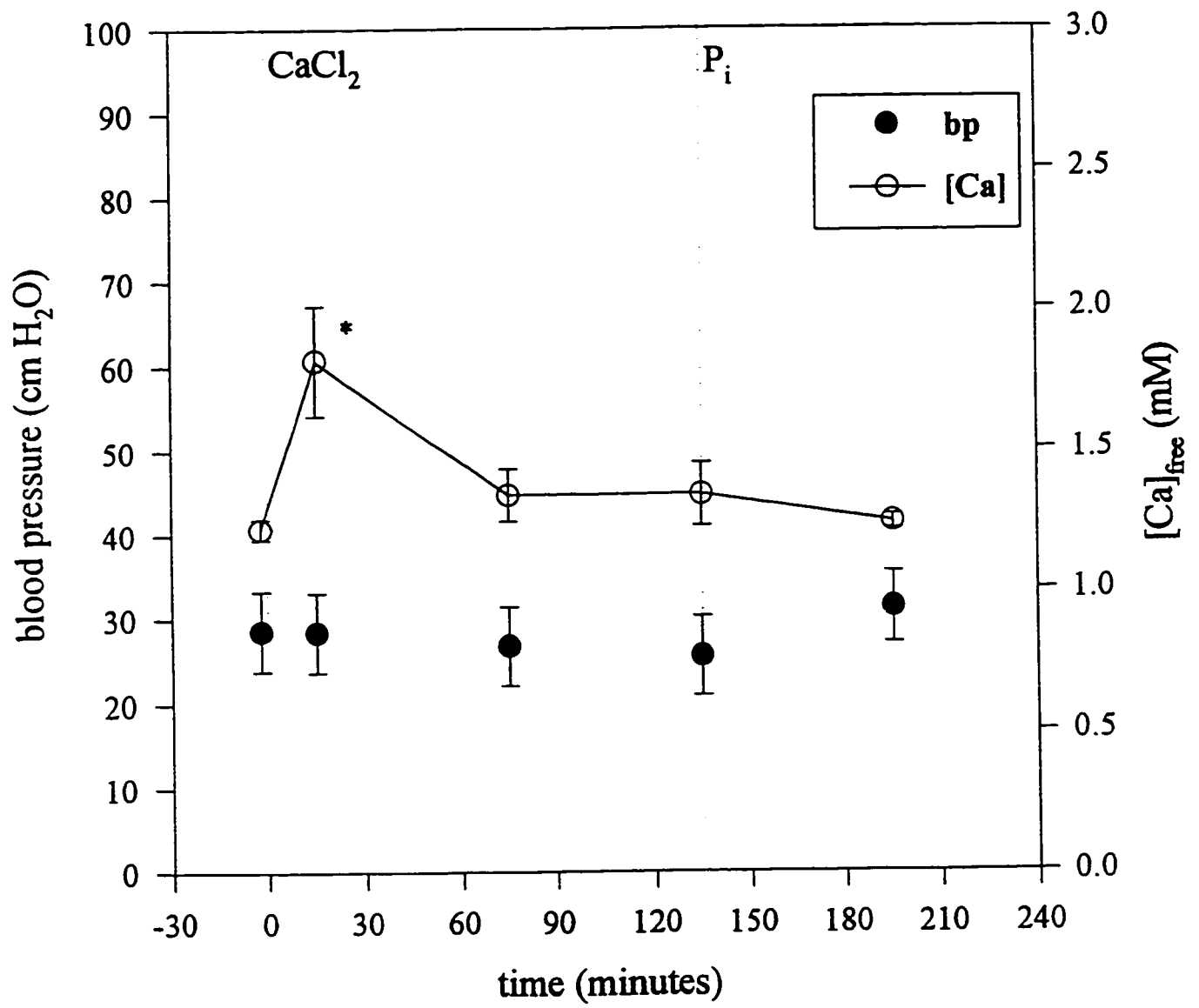
**Figure 21.** Calcium uptake in contracting and non-contracting freshwater eel (*Anguilla rostrata* LeSueur) atrial and ventricular muscle. Heart strips were either non-stimulated (NS) and thereby not contracting or stimulated (S) to contract. Each strip was exposed to  $^{45}\text{Ca}$  and the uptake was measured. Values are expressed as mean  $\pm$  SEM where N = 9 for the non-stimulated atrium and 10 for all other groups.



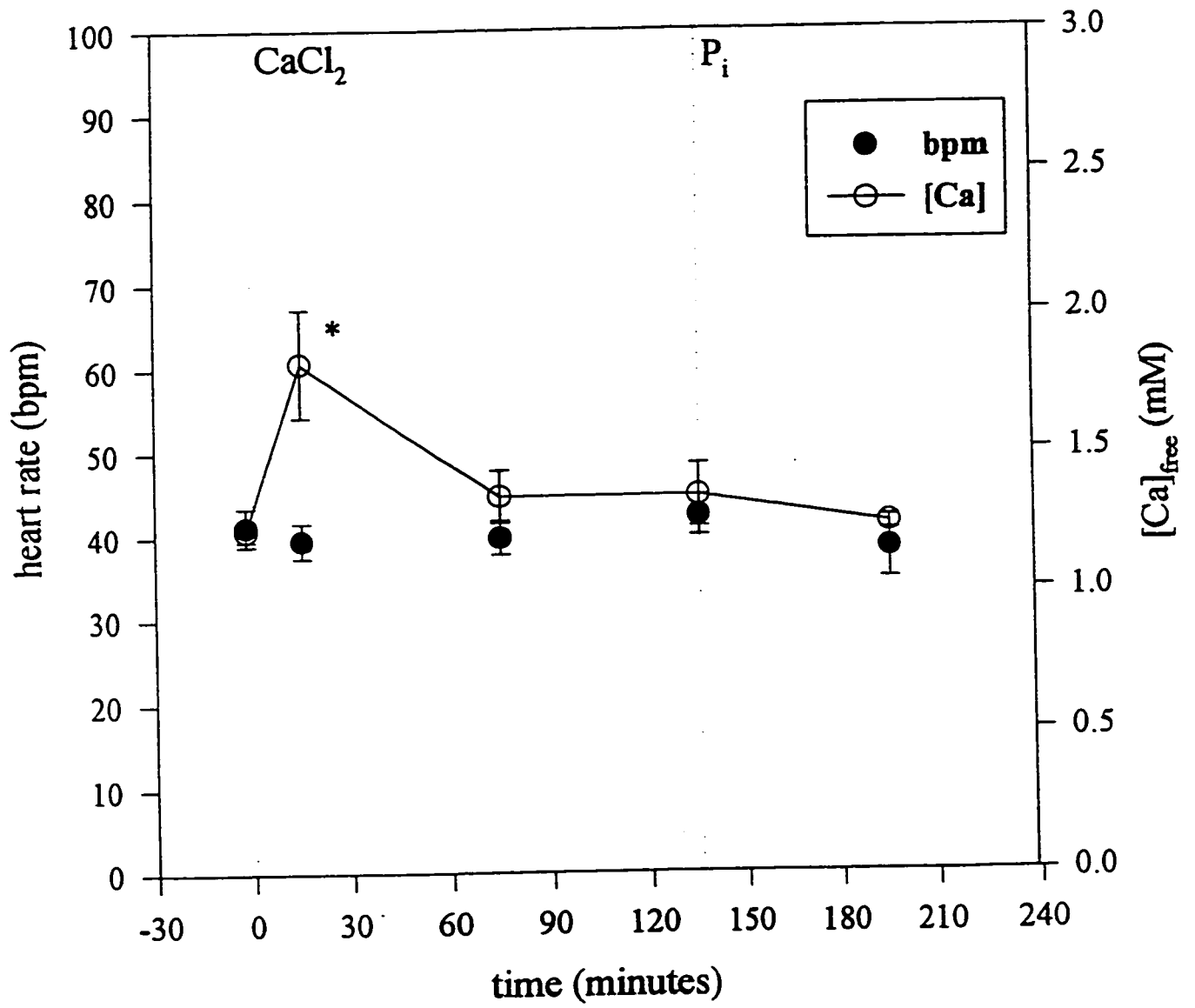
### **C. Plasma Calcium, Blood Pressure and Heart Rate**

Plasma calcium levels (ultrafiltrable fraction) were significantly increased ( $P < 0.01$ ) by 50% following an *in vivo* injection of  $\text{CaCl}_2$  at  $T=0$  (Figure 22). This increase in calcium had no effect on blood pressure (Figure 22) or heart rate (Figure 23). Attempts to decrease extracellular calcium levels by injecting  $\text{P}_i$  were ineffective and no decrease of blood calcium was observed.

**Figure 22.** Effect of varying calcium concentrations on blood pressure in the freshwater eel (*Anguilla rostrata* LeSueur). Calcium chloride was injected at T = 0 to increase extracellular calcium levels and sodium phosphate was injected at T = 135 minutes to decrease extracellular calcium levels. Values are expressed as mean  $\pm$  SEM, where N = 5. \* indicates a statistically significant difference from the pre-injection values (P < 0.01).



**Figure 23.** Effect of varying calcium concentrations on heart rate in the freshwater eel (*Anguilla rostrata* LeSueur). Calcium chloride was injected at T = 0 to increase extracellular calcium levels and sodium phosphate was injected at T = 135 minutes to decrease extracellular calcium levels. Values are expressed as mean  $\pm$  SEM, where N = 5. \* indicates a statistically significant difference from the pre-injection values (P < 0.01).



## Discussion

An investigation into the effects of altered calcium levels on some cardiovascular parameters was done in the American eel (*Anguilla rostrata* LeSueur) to determine the sensitivity of its cardiac muscle relative to the sensitivity seen in other representative vertebrates.

As expected (Driedzic and Gesser, 1988; Hansen and Gesser, 1987; Keen *et al.*, 1992; Meissner *et al.*, 1996; Nielsen and Gesser, 1983; Orchard *et al.*, 1991; Vornanen, 1996; Yee *et al.*, 1984), a positive inotropic effect on force generation was observed in all species studied for both types of cardiac tissue (Figures 19 and 20) with increasing extracellular calcium concentrations ( $[Ca^{2+}]_o$ ). These data agree with that already known about the heart. That is, extracellular calcium is ultimately responsible for the contraction of cardiac tissue as it is not possible to maintain contraction in cardiac cells in the absence of extracellular calcium (Gesser and Poupa, 1978).

In all the species examined, except for the rat, the atria was more sensitive to increases in  $Ca^{2+}$  than the ventricles where a larger increase in contractile strength was observed when compared to control values (Table 1). A possible explanation for this is that the wall of the atrium is much thinner than that of the ventricle, therefore decreasing the diffusion distance for calcium and rendering it more sensitive to high  $[Ca^{2+}]_o$ . The fact that the rat ventricle seems to be more sensitive to calcium is strange, but may have something to do with the action potential. Atrial action potentials are of short duration and have only a brief plateau during which calcium entry occurs whereas the ventricle has a much longer plateau. This increased level of calcium entering the ventricle may, at high extracellular calcium levels, cause an increase in contractile

strength greater than that occurring in the atrium. Another possibility is that the atria was energy-starved. The opening of the calcium release channel is stimulated by high concentrations of ATP, and a fall in the concentration of this nucleotide would have a negative inotropic effect whereby the strength of contraction would be smaller than expected (Katz, 1992).

Rat and trout exhibited stronger overall atrial than ventricular strength whereas the frog and eel ventricle were significantly stronger than the atrium under control conditions (Table 1). A stronger ventricular strength might be expected given that this portion of the heart ultimately is responsible for the development of pressure necessary to deliver the blood throughout the vascular system. In the experimental set up, the whole rat atrium was used since it was too small to cut. This may have played a role in the stronger contraction observed in the atria since the muscle fibers were potentially less damaged than those of the cut ventricles. The fact that the trout atria exhibited a stronger contraction than the ventricles is unexplainable.

A 50% decrease in  $[Ca^{2+}]_o$  from control values *in vitro* had a negative inotropic effect on both atrial (Figure 19) and ventricular (Figure 20) muscles in all examined species. The reduction of contractile strength was of similar magnitude for all species (approximately 50% for the atria and 40% for the ventricles). Rat ventricular strips exposed to a 50% decrease in  $[Ca^{2+}]$  exhibited an approximate 20% decrease in strength of contraction when compared to control values (Meissner *et al.*, 1996). There was not as much variation in sensitivity to low extracellular calcium as was observed during exposure to high  $[Ca^{2+}]_o$ , although a 50% decrease in contractile strength could cause serious effects on cardiac output and blood pressure *in vivo* lowering both of these and rendering the animal incapable of dealing with any increase in metabolic demands. It seems that vertebrate hearts as a whole do not cope well with a decrease

in extracellular calcium *in vitro*. In their natural habitat, a  $[Ca^{2+}]_o$  deficiency would be corrected either by mobilizing calcium from bone (frog and rat) or increasing calcium uptake at the gills (trout and eel). This suggests that although sensitive to a lowering of extracellular calcium levels, there have been no adaptive responses to low calcium.

Upon comparison of sensitivity to high  $[Ca^{2+}]_o$  in the cardiac tissues of all the vertebrates studied, the rat heart tissue showed the greatest response under the experimental conditions of this study. It is important to remember that the high  $[Ca^{2+}]_o$  to which the rat cardiac muscle (Figures 3 and 4) was exposed was only 20% higher than the control saline as the tissue exhibited irregular contractions and did not recover following exposure to an extracellular calcium concentration double that of the control. Previous studies where rat atrial (Nielsen and Gesser, 1983) and ventricular (Meissner *et al.*, 1996) strips were exposed to an extracellular calcium concentration up to four times that of normal saline showed a 20% increase in contractile strength occurring with a doubling of  $[Ca^{2+}]_o$  followed by a maximum 10% increase with higher  $[Ca^{2+}]_o$ . These data contradict the observations reported here and would lead to the conclusion that rat cardiac tissue is rather insensitive to  $[Ca^{2+}]_o$  increases. Unfortunately I have no explanation for this discrepancy.

Eel cardiac tissue proved to be extremely sensitive to high  $[Ca^{2+}]_o$  where both the atrial and ventricular strips had a stronger reaction to the increase than the trout or frog (Figures 19 and 20, respectively). These results would indicate that the eel relies on extracellular calcium for the generation of tension and is extremely responsive to changes in these levels. The differences observed between the species investigated upon exposure to changes in  $[Ca^{2+}]_o$  may have something to do with the source of activator calcium responsible for the generation of

tension in cardiac tissue.

The origin of the primary activator calcium responsible for the regulation of contractility differs among vertebrate species. In mammals, an influx of  $\text{Ca}^{2+}$  through the sarcolemmal (SL) L-type channels, otherwise known as dihydropyridine receptors (DHPRs), initiates cardiac contraction. This relatively small influx of extracellular calcium is thought to stimulate the release of larger  $\text{Ca}^{2+}$  stores through  $\text{Ca}^{2+}$ -release channels (ryanodine receptors, RyRs) from the sarcoplasmic reticulum (SR) (Fabiato, 1983), which is ultimately responsible for the generation of force (Weir, 1990).

A look at lower vertebrates reveals startling differences when compared to mammals, where calcium release from the SR is not responsible for delivering the calcium required for contraction. It seems that these animals have a greater reliance on extracellular calcium for direct activation of the myofilaments. Although the SR is well developed (Page and Niedergerke, 1972; Santer, 1974) it is sparse in comparison to mammals. The magnitude of extracellular calcium influx across the SL was shown in frog (Morad and Cleeman, 1987) to be sufficient to support myocardial contraction. In fish, it has been strongly suggested through a number of observations, that the transsarcolemmal  $\text{Ca}^{2+}$  influx is large relative to the cytosolic and myofilament requirement for  $\text{Ca}^{2+}$  (Tibbits and Moyes, 1992). These differences in calcium delivery to the myofilaments may also result in differences in calcium sensitivity of the contractile apparatus.

Ryanodine, a natural plant alkaloid, is known to block the release of  $\text{Ca}^{2+}$  from the SR (Sutko and Kenyon, 1983). Tissue sensitivity to ryanodine is considered to reflect the dependence of contractility on release from intracellular stores. Studies have shown that the

administration of ryanodine causes a decrease in strength of contraction in rat and mouse (Stemmer and Akera, 1986). A study done on skipjack tuna showed that force of contraction in atrial strips decreased following treatment with ryanodine (Keen *et al.*, 1992). These fish have heart rates resembling more those found in mammals than other fish where resting values may average 120 bpm in spinally blocked skipjack tuna (Bushnell *et al.*, 1990). These results would suggest that SR  $\text{Ca}^{2+}$  release is an important component of activator calcium in the maintenance of high heart rates. On the other hand, ryanodine had no effect on the force of contraction of sea raven, cod (Driedzic and Gesser, 1988) and crucian carp (Vornanen, 1996). A recent study in trout showed that the twitch force of atrial muscle was depressed by ryanodine at steady state whereas ventricular muscle showed no response (Gesser, 1996). When adrenaline was added with ryanodine, a decrease in force was observed for both types of muscle. *In vivo*, adrenaline causes a positive chronotropic effect on heart rate. Therefore, this suggests that SR in trout supports force development during adrenaline induced increases in heart rate. It has been suggested that in the amphibian myocardium SR  $\text{Ca}^{2+}$  release is of no significance in contraction where ventricular tissue is relatively insensitive to ryanodine treatment (Nayler, 1963).

All of my observations indicate that eel cardiac muscle *in vitro* is very sensitive to changes in extracellular calcium levels. These results coincide with the idea that eels, being lower vertebrates, do not rely on calcium release from the SR for the generation of force in cardiac muscle. These data are confounded by the results obtained in the calcium uptake experiment (Figure 21). No difference was observed between contracting and non-contracting strips for either type of tissue suggesting that the eel heart relies on intracellular calcium stores for the development of contraction. If extracellular  $\text{Ca}^{2+}$  is playing a major role in the generation

of force, then an accumulation of  $^{45}\text{Ca}$  should have been observed in the tissues of contracting muscles. This contradicts the observations made in the previous experiments where increases in  $[\text{Ca}^{2+}]_o$  resulted in large increases in force of contraction. Thomas *et al.* (1996) showed that trout have approximately the same density of RyRs and DHPRs which would suggest that there is a potential for the use of SR calcium release in these vertebrates. As mentioned earlier, contractility of atrial muscle in skipjack tuna at  $25^\circ\text{C}$  was substantially reduced following ryanodine treatment which suggests that SR  $\text{Ca}^{2+}$  release may be temperature dependent. Ventricular strips from trout at temperatures between  $15^\circ$  and  $25^\circ\text{C}$  are able to maintain force of contraction (Hove-Madsen, 1992) which implies that the SR may contribute activator calcium at higher temperatures.

No experiments have been done to describe the SR in eels as well as eliminate it as a possible source of calcium responsible for the generation of cardiac force. A blocking of SR  $\text{Ca}^{2+}$  release with the application of ryanodine would give some good insight as to the role of the SR in providing activator calcium.

The next step in determining sensitivity of the eel to changes in extracellular calcium levels was to artificially induce hypercalcaemia and hypocalcaemia by injection of  $\text{CaCl}_2$  or phosphate respectively. Injection of  $\text{CaCl}_2$  increased the extracellular plasma calcium concentration by 50% 15 minutes post-injection (Figures 22 and 23). One hour later the plasma ultrafiltrable  $[\text{Ca}^{2+}]$  returned to control values. This demonstrates that the calcium control mechanisms functioned rapidly to restore normocalcaemia. Unfortunately, phosphate-loading had no effect on plasma calcium levels. This method was taken from a study done by Wagner *et al.* (1997) in which they administered a one-dose injection of 76 mg of  $\text{P}_i$  per kg of fish diluted

in 10 ml per kg body weight of trout. This caused a significant decrease in total plasma calcium concentration 30 minutes after the injection which persisted up to two hours post-injection. The dose injected into the eels in this study was equal to 100 mg of  $P_i$  per kg of fish. This dose should have been sufficient to decrease plasma calcium concentrations, but no change was recorded. The only explanation for this result would be that a higher dose is required in eels, since this was the only difference between the two protocols.

High extracellular plasma calcium concentration had no effect on either blood pressure (Figure 22) or heart rate (Figure 23) in the American eel. Bailey and Fenwick (1975) found that experimentally induced hypercalcaemia resulting in a doubling of plasma calcium concentrations caused a significant 24% decrease in blood pressure two hours following the infusion. Comparison of these results, although seemingly contradictory, shows that blood pressure decreases with increasing plasma calcium and that the decrease does not occur rapidly. In the present study, hypercalcaemia was induced only for a short period of time as we were only interested in its immediate effects on the heart and the study period may not have been sufficient to cause a similar cardiovascular response. No previous studies have been done to determine the minimum time required for a cardiovascular response to occur due to an elevation in plasma calcium concentrations. The overall response of an increase in extracellular calcium concentration seems to be a decrease in the electrical activity of the heart although the contractile elements themselves appear to be unaffected (Ueba *et al.*, 1971). A decrease in electrical activity of the heart would lead to a decrease in heart rate with a consequent decrease in blood pressure. A high extracellular plasma calcium concentration did not have an effect on heart rate (Figure 23). Further studies on the electrical activity of the heart would be essential in

determining the effect of changes in  $[Ca^{2+}]_o$ , but an experimental obstacle occurs. There has not yet been any electrocardiogram (ECG) traces obtained from non-anaesthetized eels. I unsuccessfully attempted to implant electrodes in eels while simultaneously inducing experimental hypercalcaemia. The ECG traces obtained were not readable because the animals moved and this resulted in heart readings which were mixed with skeletal impulses. Taking ECG measurements in anaesthetized animals is much easier, but poses other problems.

Tricaine methanesulfonate (MS-222) has been shown by Iwama *et al.* (1989) to cause a decrease in plasma pH, among other physiological disturbances, at stage III of anesthesia, which coincides with the loss of body movements and the cessation of opercular movements in the rainbow trout. MS-222 is the most commonly used fish anesthetic and is routinely used to sedate eels in the laboratories at the University of Ottawa. Mammalian studies have shown that acidosis results in a decrease of  $Ca^{2+}$  loading and  $Ca^{2+}$ -induced  $Ca^{2+}$  release of the sarcoplasmic reticulum (Driedzic and Gesser, 1994). Acidosis has also been suggested to impair the cooperation of cardiac cells by its tendency to depress gap junctions. Therefore, an ECG from an anaesthetized animal should be analyzed carefully, and may not represent what is occurring in a non-sedated animal.

The experimental evidence reported from the *in vivo* experiments is consistent with the hypothesis that eel cardiac tissue is insensitive to increases in extracellular calcium levels, at least of short duration. In their natural habitat, eels would usually experience only short spans of hypercalcaemia as stanniocalcin is released in response to an elevation of extracellular calcium levels (Hanssen *et al.*, 1991, 1992). This antihypercalcaemic hormone inhibits  $Ca^{2+}$  influx from the water via the gills (Lafeber and Perry, 1988) in order to restore calcium homeostasis.

In summary, eel atrial and ventricular muscle is extremely sensitive to increases in extracellular plasma calcium concentrations *in vitro* reacting with a strong positive inotropic effect. Conversely, *in vivo* hypercalcaemia had no effect on either blood pressure or heart rate. It seems that something is present in the whole-animal preparation which alleviates the effects of high plasma calcium levels on the cardiovascular system which are evident in the *in vitro* preparation thus resulting in normal heart functioning. Further studies are required to determine how the eel accomplishes cardiovascular regulation in such a hypercalcaemic environment.

## References

- Bailey, R. E. (1957). The effect of estradiol on serum calcium, phosphorus, and protein of goldfish. *J. Exp. Biol.* **136**, 455-468.
- Bailey, J. R. and Fenwick, J. C. (1975). The effect of stanniectomy and plasma calcium levels on blood pressure in the eel, *Anguilla rostrata* LeSueur. *Comp. Biochem. Physiol.* **51A**, 693-697.
- Björnsson, B. T., Haux, C. Förlin, L. and Deftos, L. J. (1986). The involvement of calcitonin in the reproductive physiology of the rainbow trout. *J. Endocrinol.* **108**, 17-23.
- Bushnell, P. G., Brill, R. W. and Bourke, R. E. (1990). Cardiorespiratory responses of skipjack tuna (*Katsuwonus pelamis*), yellowfin tuna (*Thunnus obesus*) to acute reductions of ambient oxygen. *Can. J. Zool.* **68**, 1857-1865.
- Butler, D. G. (1969). Corpuscles of Stannius and renal physiology in the eel (*Anguilla rostrata*). *J. Fish Res. Bd Can.* **26**, 639-654.
- Butler, D. G. and Oudit, G. Y. (1994). Dorsal aortic and organ blood flow decrease following stanniectomy in freshwater North American eels (*Anguilla rostrata* LeSueur). *Comp. Biochem. Physiol.* **108A**, 229-238.
- Butler, D. G. and Oudit, G. Y. (1995). Corpuscles of Stannius and blood flow regulation in freshwater North American eels, *Anguilla rostrata* LeSueur. *J. Endocr.* **145**, 181-194.
- Cano, T. M., Perry, S. F. and Fenwick, J. C. (1994). Cholinergic control of stanniocalcin release in the rainbow trout, *Oncorhynchus mykiss*, and the American eel, *Anguilla rostrata*. *Gen. Comp. Endocrinol.* **94**, 1-10.

- Chan, D. K. O. and Chester Jones, I. (1968). Regulation and distribution of plasma calcium and inorganic phosphate in the European eel (*Anguilla anguilla* L.). *J. Endocr.* **42**, 109-117.
- Chan, D. K. O., Chester Jones, I., Henderson, I. W. and Rankin, J. C. (1967). Studies on the experimental alteration of water and electrolyte composition in the eel (*Anguilla anguilla* L.). *J. Endocr.* **37**, 297-317.
- Chang, A. C.- M., Janosi, J., Hulsbeek, M., de Jong, D., Jeffrey, K. J., Noble, J. R. and Reddel, R. R. (1995). A novel human cDNA highly homologous to the fish hormone stanniocalcin. *Mol. Cell. Endocrinol.* **112**, 241-247.
- Dacke, C. G. (1979). Calcium Regulation in the Osteichthyes. In "Calcium Regulation in Sub-Mammalian Vertebrates", pp. 99-122. Academic Press, New York.
- Driedzic, W. R. and Gesser, H. (1988). Differences in force-frequency relationships and calcium dependency between elasmobranch and teleost hearts. *J. Exp. Biol.* **140**, 227-241.
- Driedzic, W. R. and Gesser, H. (1994). Energy metabolism and contractility in ectothermic vertebrate hearts: Hypoxia, acidosis, and low temperature. *Physiol. Rev.* **74**, 221-258.
- Fabiato, A. (1983). Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. *Am. J. Physiol.* **245**, C1-C14.
- Fenwick, J. C. (1974). The corpuscles of Stannius and calcium regulation in the North American eel (*Anguilla rostrata* LeSueur). *Gen. Comp. Endocrinol.* **23**, 127-135.
- Fenwick, J. C. (1982). Some evidence concerning the nature of the hypocalcemic factor in the Stannius corpuscles. In "Comparative Endocrinology of Calcium Regulation" (C. Oguro and P. K. T. Pang, Eds.), pp. 167-172. Jpn. Sci. Press, Tokyo.

- Fenwick, J. C. (1985). The prevention of hypercalcemia is the primary role of the corpuscles of Stannius. In "Current Trends in Comparative Endocrinology" (B. Lofts and W. N. Holmes, Eds.), pp. 823-826. Hong Kong University Press, Hong Kong.
- Fenwick, J. C. and Brasseur, J. G. (1991). Effects of stanniectomy and experimental hypercalcemia on plasma calcium levels and calcium influx in American eels, *Anguilla rostrata*, LeSueur. *Gen. Comp. Endocrinol.* **82**, 459-465.
- Gesser, H. (1996). Cardiac force-interval relationship, adrenaline and sarcoplasmic reticulum in rainbow trout. *J. Comp. Physiol. B* **166**, 278-285.
- Gesser, H. and Poupa, O. (1978). The role of intracellular  $Ca^{2+}$  under hypercapnic acidosis of cardiac muscle: Comparative aspects. *J. Comp. Physiol.* **127**, 307-313.
- Guyton, A. C. (1991). Parathyroid hormone, calcitonin, calcium and phosphate metabolism, vitamin D, bone, and teeth. In "Textbook of Medical Physiology", 8th edition, pp. 868-884. Saunders, Montreal.
- Haddad, M., Roder, S., Olsen, H. S. and Wagner, G. F. (1996). Immunocytochemical localization of stanniocalcin cells in the rat kidney. *Endocrinology* **137**(5), 2113-2117.
- Hansen, S. P. and Gesser, H. (1987). Extracellular  $Ca^{2+}$ , force and energy state in cardiac tissue of rainbow trout. *Am. J. Physiol.* **253**, R838-R847.
- Hanssen, R. G. J. M., Lafeber, F. P. J. G., Flik, G. and Wendelaar Bonga, S. E. (1989). Ionic and total calcium levels in the blood of the European eel (*Anguilla anguilla*): Effects of stanniectomy and hypocalcin replacement therapy. *J. Exp. Biol.* **141**, 177-186.
- Hanssen, R. G. J. M., Aarden, E. M., Van der Venne, W. P. H. G., Pang, P. K. T. and Wendelaar Bonga, S. E. (1991). Regulation of secretion of the teleost fish hormone stanniocalcin:

- Effects of extracellular calcium. *Gen. Comp. Endocrinol.* **84**, 155-163.
- Hanssen, R. G. J. M., Mayer-Gostan, N., Flik, G. and Wendelaar Bonga, S. E. (1992). Influence of ambient calcium levels on stanniocalcin secretion in the European eel (*Anguilla anguilla*). *J. Exp. Biol.* **162**, 197-208.
- Hirano, T. (1989). The Corpuscles of Stannius. In "Vertebrate Endocrinology Fundamentals and Biomedical Implications", pp. 139-162. Academic Press, San Diego.
- Holmes, W. N. and Donaldson, E. M. (1969). The Body Compartments and the Distribution of Electrolytes. In "Fish Physiology" (W. S. Hoar and D. J. Randall, Eds.), Vol. I, pp. 1-89. Academic Press, New York.
- Hove-Madsen, L. (1992). The influence of temperature on ryanodine sensitivity and the force-frequency relationship in the myocardium of rainbow trout. *J. Exp. Biol.* **167**, 47-60.
- Hurwitz, S. (1996). Homeostatic control of plasma calcium concentration. *Crit. Rev. Biochem. Mol. Biol.* **31**, 41-100.
- Iwama, G. K., McGeer, J. C. and Pawluk, M. P. (1989). The effects of five fish anaesthetics on acid-base balance, haematocrit, blood gases, cortisol, and adrenaline in rainbow trout. *Can. J. Zool.* **67**, 2065-2073.
- Katz, A. M. (1992). Physiology of the Heart, 2<sup>nd</sup> edition, 687 pp. Raven Press, New York.
- Keen, J. E., Farrell, A. P., Tibbits, G. F. and Brill, R. W. (1992). Cardiac physiology in tunas. II. Effect of ryanodine, calcium, and adrenaline on force-frequency relationships in atrial strips from skipjack tuna, *Katsuwonus pelamis*. *Can. J. Zool.* **70**, 1211-1217.
- Lafeber, F. P. J. G., Flik, G., Wendelaar Bonga, S. E. and Perry, S. F. (1988). Hypocalcin

- from the Stannius corpuscles inhibits gill calcium uptake in trout. *Am. J. Physiol.* **254**, R891-R896.
- Lafeber, F. P. J. G., Hanssen, R. G. J. M., Choy, Y. M., Flik, G., Herrmann-Etlee, M. P. M., Pang, P. K. T. and Wendelaar Bonga, S. E. (1988a). Identification of hypocalcin (teleocalcin) isolated from trout Stannius corpuscles. *Gen. Comp. Endocrinol.* **69**, 19-30.
- Lafeber, F. P. J. G. and Perry, S. F. (1988). Experimental hypercalcemia induces hypocalcin release and inhibits branchial  $\text{Ca}^{2+}$  influx in freshwater trout. *Gen. Comp. Endocrinol.* **72**, 136-143.
- Lopez, E., Peignoux-Deville, J., Lallier, E., Martelly, E. and Millet, C. (1976). Effects of calcitonin and ultimobranchiaectomy (UBX) on calcium and bone metabolism in the eel, *Anguilla anguilla* L. *Calcif. Tiss. Res.* **20**, 173-186.
- McLean, F. C. and Hastings, A. B. (1935). Clinical estimation and significance of calcium-ion concentrations in the blood. *Am. J. Med. Sci.* **189**, 601-613.
- Meissner, A., Szymanska, G. and Morgan, J. (1996). Effects of dantrolene sodium on intracellular  $\text{Ca}^{2+}$  handling in normal and  $\text{Ca}^{2+}$  overloaded cardiac muscle. *European J. Pharmacol.* **316**, 333-342.
- Morad, M. and Cleeman, L. (1987). Role of  $\text{Ca}^{2+}$  channel in development of tension in heart muscle. *J. Mol. Cell. Cardiol.* **19**, 527-553.
- Nayler, W. G. (1963). Effects of ryanodine on cardiac muscle. *Am. J. Physiol.* **204**, 973-978.
- Nielsen, K. E. and Gesser, H. (1983). Effects of  $[\text{Ca}^{2+}]_o$  on contractility in the anoxic cardiac muscle of mammal and fish. *Life Sciences* **32**, 1437-1442.
- Norberg, B., Björnsson, B. T., Brown, C. L., Wichardt, U., Defetos, L. J. and Haux, C. (1989).

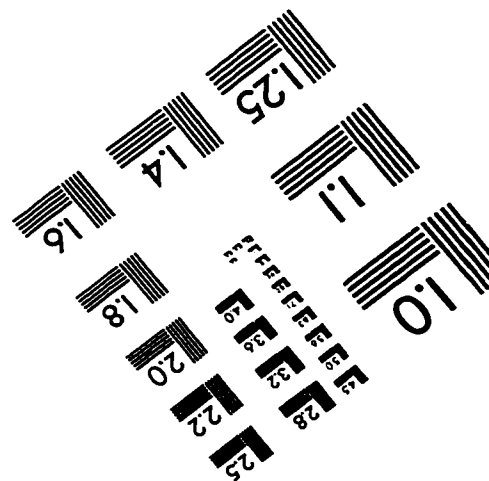
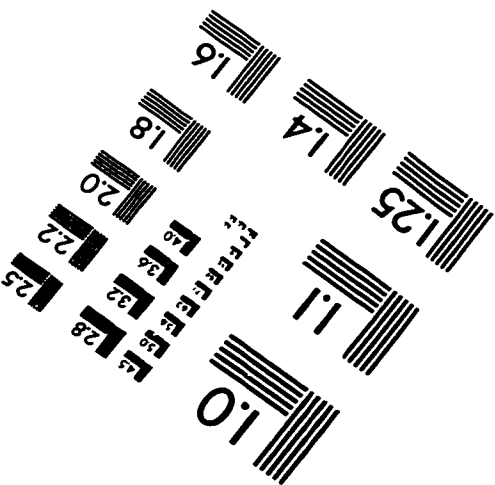
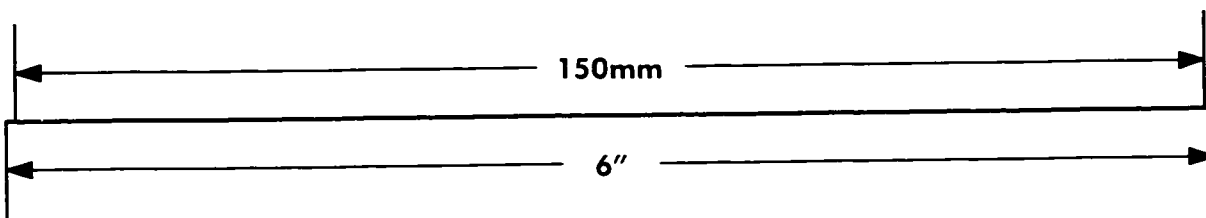
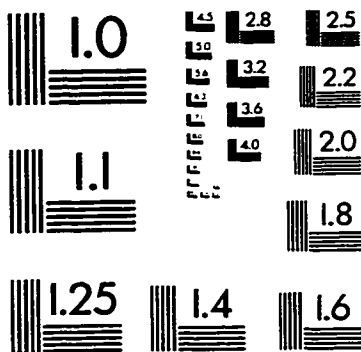
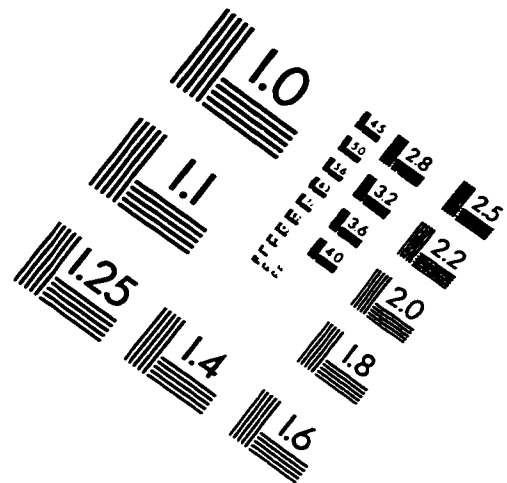
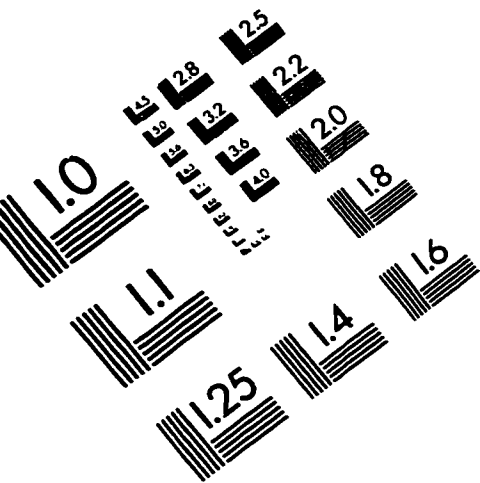
- Changes in plasma vitellogenin, sex steroids, calcitonin, and thyroid hormones related to sexual maturation in female brown trout (*Salmo trutta*). *Gen. Comp. Endocrinol.* **75**, 316-326.
- Orchard, C. H., Hamilton, D. L., Astles, P., McCall, E. and Jewell, B. R. (1991). The effect of acidosis on the relationship between  $\text{Ca}^{2+}$  and force in isolated ferret cardiac muscle. *J. Physiol.* **436**, 559-578.
- Page, S. G. and Niedegerke, R. (1972). Structures of physiological interest in the frog heart ventricle. *J. Cell Sci.* **11**, 179-203.
- Pang, P. K. T. (1973). Endocrine control of calcium metabolism in teleosts. *Amer. Zool.* **13**, 775-792.
- Pang, P. K. T., Griffith, R. W., Maetz, J. and Pic, P. (1980). Calcium uptake in fishes. In "Epithelial Transport in Lower Vertebrates" (B. Lahlou, Ed.), pp. 122-132. Cambridge University Press, Cambridge.
- Perry, S. F., Goss, G. G. and Fenwick, J. C. (1992). Interrelationships between gill chloride cell morphology and calcium uptake in freshwater teleosts. *Fish Physiol. Biochem.* **10**, 327-337.
- Perry, S. F., Seguin, D., Lafeber, F. P. J. G., Wendelaar Bonga, S. E. and Fenwick, J. C. (1989). Depression of whole-body calcium uptake during acute hypercalcaemia in American eel, *Anguilla rostrata*, is mediated exclusively by the corpuscles of Stannius. *J. Exp. Biol.* **147**, 249-261.
- Ringer, S. (1883). A further contribution regarding the influence of the different constituents of the blood on the contraction of the heart. *J. Physiol.* **4**, 29-42.

- Robertson, D. R. (1970). The ultimobranchial body in *Rana pipiens*. XI. Response to increased dietary calcium-evidence of possible physiological function. *Endocrinology* **87**, 1041-1050.
- Rosenberg, G. A. (1991). Calcium Channel Blockers in Neurological Disorders. In "Calcium Channels: Their Properties, Functions, Regulation, and Clinical Relevance" (L. Hurwitz, L. D. Partridge and J. K. Leach, Eds.), pp. 377-384. CRC Press, Boston.
- Santer, R. M. (1974). The organization of the sarcoplasmic reticulum in teleost ventricular myocardial cells. *Cell Tissue Res.* **151**, 395-402.
- So, Y. P. and Fenwick, J. C. (1979). *In vivo* and *in vitro* effects of Stannius corpuscle extracts on the branchial uptake of <sup>45</sup>Ca in stanniectomized North American eels (*Anguilla rostrata* L.). *Gen. Comp. Endocrinol.* **37**, 143-149.
- Stemmer, P. and Akera, T. (1986). Concealed positive force-frequency relationships in rat and mouse cardiac muscle revealed by ryanodine. *Am. J. Physiol.* **251**, H1106-H1110.
- Sundell, K., Björnsson, B. T., Itoh, H. and Kawauchi, H. (1992). Chum salmon (*Oncorhynchus keta*) stanniocalcin inhibits *in vitro* intestinal calcium uptake in Atlantic cod (*Gadus morhua*). *J. Comp. Physiol. B* **162**, 489-495.
- Sutko, J. L. and Kenyon, J. L. (1983). Ryanodine modification of cardiac muscle responses to potassium-free solutions. *J. Gen. Physiol.* **82**, 385-404.
- Takagi, Y., Nakamura, Y. and Yamada, J. (1985). Effects of the removal of corpuscles of Stannius on the transport of calcium across the intestine of rainbow trout. *Zool. Sci.* **2**, 523-530.
- Thomas, M. J., Hamman, B. N. and Tibbits, G. F. (1996). Dihydropyridine and ryanodine

- binding in ventricles from rat, trout, dogfish and hagfish. *J. Exp. Biol.* **199**, 1999-2009.
- Tibbits, G. F., Hove-Madsen, L. and Bers, D. M. (1991). Calcium transport and the regulation of cardiac contractility in teleosts: a comparison with higher vertebrates. *Can. J. Zool.* **69**, 2014-2019.
- Tibbits, G. F. and Moyes, C. D. (1992). Excitation-contraction coupling in the teleost heart. *In* "Fish Physiology" (W. S. Hoar, D. J. Randall and A. P. Farrell, Eds.), Vol. XIIA, pp. 267-304. Academic Press, San Diego.
- Ueba, Y., Ito, Y. and Chidsey C. A. (1971). Intracellular calcium and myocardial contractility - I. Influence of extracellular calcium. *Am. J. Physiol.* **220**, 1553-1557.
- Urasa, F. M. and Wendelaar Bonga, S. E. (1987). Effects of calcium and phosphate on the corpuscles of Stannius of the teleost fish, *Oreochromis mossambicus*. *Cell Tissue Res.* **249**, 681-690.
- Verbost, P. M., Butkus, A., Willems, P. and Wendelaar Bonga, S. E. (1993a). Indications for two bioactive principles in the corpuscles of Stannius. *J. Exp. Biol.* **177**, 243-252.
- Verbost, P. M., Flik, G., Fenwick, J. C., Greco, A. M., Pang, P. K. T. and Wendelaar Bonga, S. E. (1993b). Branchial calcium uptake: possible mechanisms of control by stanniocalcin. *Fish Physiol. Biochem.* **11**, 205-215.
- Vornanen, M. (1996). Effect of extracellular calcium on the contractility of warm- and cold-acclimated crucian carp heart. *J. Comp. Physiol. B* **165**, 507-517.
- Wagner, G. F. (1993). Stanniocalcin: Structure, function and regulation. *In* "Biochemistry and Molecular Biology of Fishes" (Hochachka and Mommsen, Eds.), Vol. 2, pp. 419-434. Elsevier Science Publishers.

- Wagner, G. F., Niu, P. D., Jaworski, E., Radman, D. and Chiarot, C. (1997). Development of a dose-response bioassay for stanniocalcin in fish. *Mol. Cell. Endocrinol.* **128**, 19-28.
- Weir, W. G. (1990). Cytoplasmic  $[Ca^{2+}]_i$  in mammalian ventricle: Dynamic control by cellular processes. *Annu. Rev. Physiol.* **52**, 467-485.
- Wendelaar Bonga, S. E., Greven, J. A. A. and Veenhuis, M. (1976). The relationship between the ionic composition of the environment and the secretory activity of the endocrine cell types of Stannius corpuscles in the teleost *Gasterosteus aculeatus*. *Cell Tiss. Res.* **175**, 297-312.
- Wendelaar Bonga, S. E. and Pang, P. K T. (1992). Control of calcium regulating hormones in the vertebrates: parathyroid hormone, calcitonin, prolactin, and stanniocalcin. *Int. Rev. Cytol.* **128**, 139-213.
- Yee, H. F. and Jackson, D. C. (1984). The effects of different types of acidosis and extracellular calcium on the mechanical activity of turtle atria. *J. Comp. Physiol. B* **154**, 385-391.
- Zarrow, M. X., Yochim, J. M. and McCarthy, J. L. (1964). Physiological fluids for isolated tissues. In: "Experimental Endocrinology: a sourcebook of basic techniques". p. 478. Academic Press, New York.

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