

Factors affecting the release of atrial natriuretic factor (ANF)  
from the heart of rainbow trout (*Oncorhynchus mykiss*)

by

Katherine Louise Cousins

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## APPROVAL

Name: **Katherine Louise Cousins**

Degree: **Master of Science**

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**FACTORS AFFECTING THE RELEASE OF ATRIAL NATRIURETIC FACTOR  
(ANF) FROM THE HEART OF RAINBOW TROUT (ONCORHYNCHUS  
MYKISS)**

Examining Committee:

Chair: Dr. L. Bendell-Young, Assistant Professor

Dr. A. P. Farrell, Professor, Senior Supervisor  
Department of Biological Sciences, SFU

Dr. B. A. McKeown, Professor  
Department of Biological Sciences, SFU

Dr. K. R. Delaney, Assistant Professor  
Department of Biological Sciences, SFU

Dr. T. Williams, Assistant Professor  
Department of Biological Sciences, SFU  
Public Examiner

Date Approved 27<sup>th</sup> Jun 1995

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Factors Affecting the Release of Atrial Natriuretic Factor (ANF)

From the Heart of Rainbow Trout (Oncorhynchus mykiss)

Author:

(signature)

Katherine Louise Cousins

(name)

January 27th, 1995

(date)

## ABSTRACT

Atrial natriuretic factor (ANF), released from the heart of most vertebrates, has hypotensive, natriuretic and diuretic effects. In mammals, the primary stimulus for ANF release is atrial detension, but in fish the mechanisms mediating ANF release from the heart are unknown. Furthermore, it is unclear how much the atrium and the ventricle contribute to total ANF release. Therefore, the objectives of this thesis were to: 1) quantitatively describe the location of ANF in the rainbow trout (*Oncorhynchus mykiss*) heart; and 2) investigate the mechanistic factors influencing its release from the *in situ* trout heart. In addition, as ionic regulation differs between freshwater (FW) and seawater (SW) fish, and ANF is involved in sodium regulation, the factors mediating ANF release from the trout heart were investigated in both FW and SW fish.

Using immunogold labeling, dense ANF secretory granules were identified in both the atrial and ventricular myocardium. However, these secretory granules were considerably more numerous in the atrium than in the ventricle. To quantify the absolute amount of ANF in the trout atrium and ventricle, ANF was assayed using a radioimmunoassay (RIA) specific for human ANF-(99-126). Preliminary studies using Western blot analysis had confirmed that human ANF effectively cross-reacts with trout ANF. Immunoreactive ANF (ir-ANF) content in the atrium and ventricle was  $3.10 \pm 0.24$  ng·g<sup>-1</sup> wet tissue weight and  $0.04 \pm 0.01$  ng·g<sup>-1</sup> wet tissue weight, respectively ( $N = 7$ ). Thus, the atrium contained 92% of the total ir-ANF stored in the heart. The results from the immunogold labeling, when combined with those from the direct measurement of ir-ANF in atrial and ventricular extracts, strongly suggest that the atrium is the primary source of ANF in rainbow trout.

In the FW perfused trout hearts, ANF secretion at basal filling pressures was unaffected by increases in afterload. This suggests that stretch of the atrium and not the

ventricle is the main contributor to total ANF release. In addition, hypertension on the arterial side of the heart does not appear to have a negative feedback mechanism on ANF release. In both FW and SW perfused trout hearts, increases in venous filling pressure ( $P_i$ ) resulted in proportional increases in both cardiac output ( $\dot{Q}$ ) and rate of release of ANF. For example, at maximum  $\dot{Q}$  ( $P_i = 0.36 \pm 0.01$  kPa) rate of ANF ( $189.43 \pm 29.74$   $\text{pg}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  heart) was 6-fold greater than at basal levels ( $P_i = -0.09 \pm 0.04$  kPa and ANF Release =  $29.50 \pm 1.85$   $\text{pg}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  heart). As in mammals, these data are consistent with atrial stretch mediating ANF release.

SW perfused hearts were capable of higher cardiac outputs than FW hearts, and the maximum rate of ANF release was also greater, but more variable. This suggests that sea water-acclimation of rainbow trout at least temporarily increases the capacity for ANF release. The mechanisms underlying these differences between FW and SW trout hearts are unknown.

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## *Chapter I*

### **GENERAL INTRODUCTION**

Atrial natriuretic factor (ANF) is a hormone that is synthesized by and secreted from the hearts of both invertebrates (Nehls *et al.*, 1985; Agnisolo *et al.*, 1989; Vesely *et al.*, 1991) and vertebrates (Chapeau *et al.*, 1985; Ryu *et al.*, 1992; Baranowski and Westenfelder, 1989). It acts at various receptor sites to cause natriuretic (i.e. increased sodium excretion), diuretic (i.e. increased urine production) and hypotensive (i.e. low blood pressure) effects. Thus, the cardiac tissue not only serves to pump body fluids, but it also is capable of modulating its own work load by affecting blood volume and composition (Ackermann, 1986).

ANF was not discovered until the early 1960's. It was discovered that atrial distension in dogs could cause diuresis (Henry *et al.*, 1956), but the factor that caused this effect remained a mystery for eight years. During this period, investigators working with dogs found that an infusion of saline would increase urine flow independent of the animal's circulating aldosterone concentration (DeWardener *et al.*, 1961). This finding prompted other investigators to search for an elusive "third factor" thought to be released into the blood when venous blood volume was increased (Levinsky and Lalone, 1963). Meanwhile, other studies utilizing a relatively new technique, electron microscopy, found dense granules in the cytoplasm of atrial tissues in guinea pigs and rats (Kisch, 1955, 1956). Finally, Jamieson and Palade (1964) suggested that the atrial granules looked similar to other hormone storage granules and could be the source of the mysterious third factor. Many recent studies have now identified ANF as a cardiac hormone.

Thus, at present, an extensive literature exists regarding ANF, but the majority of the literature is based on mammalian systems. For instance, it is now known where ANF

is located and what physiological effects this hormone exerts on a variety of mammalian tissues. Also, mammalian ANF has been isolated and sequenced for several species and the complementary deoxyribonucleic acid (cDNA) for the hormone has been cloned.

In fish, however, very little is known about ANF structure and biosynthesis. Also, the factors that induce ANF release from the fish heart have never been studied. Mammalian ANF injected into fish can elicit responses similar to those found in mammals (Duff and Olson, 1986), and although it has not been determined, this suggests that there may be a high sequence homology between fish and mammalian ANF. The purpose of this thesis was to quantitatively describe the location of ANF in a fish heart and to investigate the factors influencing its release.

### **Terminology**

As the interest in cardiac hormones grew, so did the literature and subsequent nomenclature. For example, the mammalian prohormone was called atrial natriuretic factor (DeBold *et al.*, 1981), gamma-atrial natriuretic peptide (Kangawa *et al.*, 1984), cardiodilatin-126 (Forssmann, 1986) and cardionatrin IV (Flynn *et al.*, 1985). Not surprisingly, the 28 amino acid peptide was also given a cascade of names such as atrial natriuretic polypeptide (Kangawa and Matsuo, 1984), cardiodilatin-28 (Flynn *et al.*, 1983), cardionatrin (Currie *et al.*, 1984a), atriopeptin (Atlas *et al.*, 1984) and auriculin (Marin *et al.*, 1985). In addition, shortened forms of mammalian ANF were discovered and named auriculum B and auriculin A (Atlas *et al.*, 1984) or atriopeptin III (Currie *et al.*, 1984b). Consequently, to reduce confusion and standardize the nomenclature, an international committee decided that the 126 amino acid prohormone would be called proANF or ANF-(1-126) and the circulating 28 carboxyl-terminal amino acid would be called ANF or ANF-(99-126) (Dzau *et al.*, 1987).

## Molecular Structure and Biosynthesis of Mammalian ANF

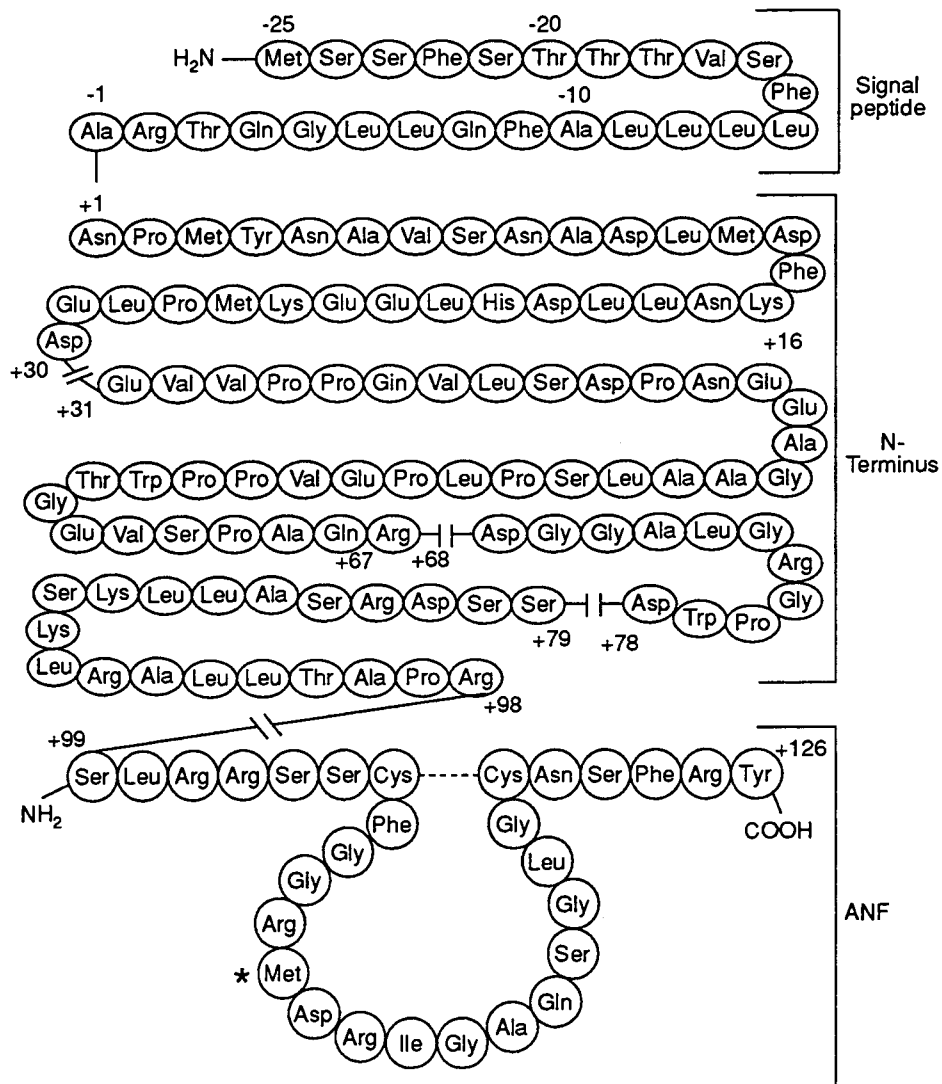
Generally, mammalian cardiac hormones are derived from a common preprohormone that consists of either 151 (human) or 152 (rat) amino acids (Kangawa and Matsuo, 1984; Kangawa *et al.*, 1984; Greenwald *et al.*, 1984; Oikawa *et al.*, 1985; Seidman *et al.*, 1984). From the preprohormone, a signal peptide consisting of 25 (human) or 24 (rat) amino acids is co-translationally cleaved resulting in the generation of a 126 (human) or 128 (rat) amino acid prohormone (Flynn *et al.*, 1985; Seidman *et al.*, 1985).

When released into the circulating blood, the mammalian prohormone is cleaved at four positions (Fig 1.1). The cleavage at position Arginine (98)-Serine (99) results in the creation of a 28 amino acid carboxyl-terminal peptide (Fig. 1.1). This is the active and circulating hormone and it has a molecular weight of approximately 3,000 to 5,000 Da (Napier *et al.*, 1984) and a half-life in the venous blood of about 3 minutes at 37 °C (Espiner and Nicholls, 1987). Also, three other peptides are derived from the amino-terminus of the mammalian prohormone and have diuretic properties (Vesely *et al.*, 1987), but are not as potent as the active hormone (Martin *et al.*, 1990).

In rats, the amino acid sequence of the active hormone, ANF-(99-126), is similar to the human ANF except for the substitution of isoleucine for methionine at position +110 and the addition of two arginine residues at the carboxyl-terminal (Kangawa and Matsuo, 1984; Oikawa *et al.*, 1985). Within the ringed structure of ANF-(99-126) (Fig 1.1), the sequence phenylalanine (+106) through to glycine (+114) is critical for the hormone's biological actions because amino acid substitutions in this region result in a loss of activity (Haasman *et al.*, 1988). Furthermore, the disulfide bridge between the two cysteine residues and the three carboxyl-terminal amino acids, namely phenylalanine, arginine and tyrosine, are also essential for the hormone's biological activity (Misono *et al.*, 1984; 1985).

Figure 1.1. A schematic diagram showing the amino acid sequence of the preprohormone for human atrial natriuretic factor. The signal peptide is cleaved between the two amino acids alanine (-1) and asparagine (+1) to produce the prohormone. The prohormone is cleaved at four positions to produce four biologically active peptides. ProANF-(1-30) is called the long-acting sodium stimulator (Martin *et al.*, 1990) while ProANF-(31-67) is called the vessel dilator (Martin *et al.*, 1989). The last of the active amino-terminal peptide chains is ProANF-(79-98) which is called the kaliuretic hormone (Martin *et al.*, 1990). The amino acids arginine (+98) and serine (+99) are cleaved producing the most potent of the natriuretic hormones, ANF-(99-126) (Martin *et al.*, 1990). Note that in rats at position +110, which is indicated with an asterisk (\*), the amino acid methionine is replaced by isoleucine. Also in rats, two arginine residues are added between the tyrosine residue and the carboxyl-terminal (modified after Vesely, 1992).





Commercial radioimmunoassays, such as the one used in this thesis (Peninsula Laboratories, Inc., Belmont, CA), bind specifically to the epitope of the tyrosine-carboxyl-terminal. Radioimmunoassays using rat ANF antisera have failed to show a cross-reaction with rainbow trout antigens (C. Redekopp and J.R. Ledsome, pers. commun.). This suggests that in rat ANF, the single amino acid substitution and/or the two arginine residue additions may have somehow changed its binding specificity with antisera from other animal species. Immunoblotting techniques, however, such as Western blotting, can be used to establish species specificity. Therefore, in Chapter 3 of this thesis I assessed the species specificity between human ANF antiserum and trout ANF using a Western blot.

Although small changes in amino acid sequencing occur between species, the metabolic controls of the ANF biosynthetic pathway and secretion are presumably the same (Burgess and Kelly, 1987). For instance, the mammalian ANF messenger-RNA is translated on ribosomes of the rough endoplasmic reticulum (RER) which results in the production of the prohormone. The prohormone is then co-translationally transported into the RER lumen (Flynn *et al.*, 1985; Seidman *et al.*, 1985). At the RER membrane the signal peptide is cleaved to produce proANF and then the peptide is likely packaged in the Golgi cisternae and stored in secretory granules (Thibault *et al.*, 1989). An enzyme called atrioactivase, which cleaves ANF-99-126 from the prohormone, has been found in mammalian atrial tissues, but the cleavage site still remains undetermined (Imada *et al.*, 1988).

To date, the only fish ANF that has been isolated and sequenced is from the atria of freshwater eels (Takei *et al.*, 1989). Takei and Balment (1993) reported that the sequence homology of eel ANF is approximately 62% to that of mammalian ANF. Thus, ANF appears to be a highly conserved peptide (see Vesely, 1992 for review). Table 1.1 presents a summary of immunoreactive-ANF (ir-ANF) content found in the heart tissues

**Table 1.1.** The ir-ANF content in the heart ( $\text{ng}\cdot\text{mg}^{-1}$  wet weight) and plasma ( $\text{pg}\cdot\text{mL}^{-1}$ ) of several vertebrate species. References indicated in parentheses are as follows: (a) Gutkowska *et al.*, 1985; (b) Wilson *et al.*, 1986; (c) Kim *et al.*, 1989; (d) Takei *et al.*, 1990; (e) Uva *et al.*, 1993; (f) Westenfelder *et al.*, 1988; (g) Smith *et al.*, 1991; (h) Baeyens *et al.*, 1989; (i) Kim *et al.*, 1991; (j) Ryu *et al.*, 1992; (k) Brandt *et al.*, 1994; (l) Evans *et al.*, 1989; (m) Uemura *et al.*, 1990. Where available, all values are means  $\pm$  S.E.M. R = right, L = left and ND = not detectable. See text regarding values indicated by an asterisk (\*).

		Atrium (ng·mg <sup>-1</sup> )	Ventricle (ng·mg <sup>-1</sup> )	Plasma (pg·mL <sup>-1</sup> )
<b>Mammals</b>				
Human (a)		160 (R)		65.3 ± 2.5
Dog (b)		4.78 ± 0.03 (L)	0.015	77 ± 4
	(k)	1.93 ± 0.02 (R)		34 ± 10
Mouse (c)		46.3 ± 6.1 (L)	0.630 ± 0.125	
		28.4 ± 2.0 (R)		
Rat (c)		237.0 ± 28.0 (L)	ND	135.2 ± 12.2
		344.5 ± 38.5 (R)		
Rabbit (c)		12.9 ± 1.1 (L)	ND	72.5 ± 4.5
		11.5 ± 0.9 (R)		
	(b)	9.86 ± 0.05 (R+L)		53 ± 4
<b>Birds</b>				
Chicken (c)		0.71 ± 0.10 (L)	0.051 ± 0.010	
		0.62 ± 0.08 (R)		
<b>Reptiles</b>				
Freshwater Turtle	<i>Amyda japonica</i> (c)	0.80 ± 0.09 (L)	0.076 ± 0.012	ND
	(h)	0.87 ± 0.09 (R)		47.0 ± 4.0
<b>Amphibians</b>				
Frog	<i>Rana nigro maculata</i> (c)	27.50 ± 2.71	1.02 ± 0.13	
	<i>Rana dybowskii</i> (j)	5.23 ± 1.21*	0.53 ± 0.12*	
<b>Fish</b>				
Hagfish	<i>Myxine glutinosa</i> (l)			83 ± 22
	(m)	0.36 ± 0.02	0.01 ± 0.00	
Sting Ray	<i>Dasyatis sabina</i> (l)			209 ± 50
Dogfish	<i>Squalus acanthias</i> (l)			129
Freshwater Eel	<i>Anguilla japonica</i> (d)	0.203 ± 0.013	0.025 ± 0.004	116.7 ± 18.6
	(c)	0.080 ± 0.010	ND	
	(m)	5.21 ± 0.48	0.04 ± 0.00	45.7 ± 9.9
Rice Eel	<i>Manopterus albus</i> (i)	0.01261*	0.00316*	
Catfish	<i>Pelteobagrus fulvidraco</i> (c)	0.157 ± 0.026	0.214 ± 0.009	
	<i>Silurus asotus</i> (i)	0.00942*	0.00346*	
Silver carp	<i>Carassius auratus</i> (i)	0.00863*	0.00470*	
Snakehead	<i>Channa argus</i> (i)	0.00570*	0.00186*	
	<i>Channa maculata</i> (m)	0.29 ± 0.02	0.03 ± 0.00	70.3 ± 12.6
Antarctic Teleosts	<i>Chionodraco hamatus</i> (e)			181 ± 27
	<i>Pagothenia bernacchii</i> (e)			97 ± 3
Gleenling	<i>Hexagrammos otakii</i> (m)	0.08 ± 0.01	0.01 ± 0.00	
Flounder	<i>Pseudopleuronectes americanus</i> (l)			30
Sculpin	<i>Myoxocephalus octodecimspinosus</i> (l)			100 ± 10
Dacefish	<i>Zauo platypus</i> (c)	0.213 ± 0.020	0.470 ± 0.042	
Utah Trout	<i>Gila atraria</i> Freshwater (f)			146 ± 27
	Saltwater (f)			347 ± 21
Rainbow Trout	<i>Oncorhynchus mykiss</i> (m)	0.35 ± 0.02	0.01 ± 0.00	8.9 ± 0.9
	Freshwater (g)			21.87 ± 2.0
	Saltwater (g)			75.16 ± 6.8

and plasma of several vertebrate species. Whereas, data for mammalian species are representative, data for non-mammalian species are comprehensive to the best of my knowledge. Table 1.1 shows that, generally, ANF content is greater in the atrium than in the ventricle and that plasma ANF concentrations are considerably lower than those found in cardiac tissue. In earlier mammalian studies, ANF was not found in the ventricles. This oversight may have been due to the exceptionally low concentrations normally present in ventricular tissues. Interestingly, human ventricular ANF is usually found in extremely low concentrations, but during periods of circulatory distress, such as congestive heart failure, it becomes a major contributor to the total ANF release (Ding *et al.*, 1987; and see Genest and Cantin, 1988 for review).

As electron microscopic studies have revealed a large number of secretory granules in the ventricles of non-mammalian species, several investigators suggested that there may be a phylogenetic trend to concentrate ANF in the atria (Reinecke *et al.*, 1987). The values presented in Table 1.1, however, do not seem to support this hypothesis. With the exception of the fish values, the ventricular ANF contents found in amphibians, reptiles, birds and mammals are much lower than those for the atrial tissues.

Several studies report ANF cardiac tissue content for fish species in which the values ( $\text{ng}\cdot\text{mg}^{-1}$  wet weight) are in the same range as those found in turtles and birds, but are considerably lower than those found for mammals (Table 1.1). One study by Kim *et al.*, (1991) reported ANF cardiac tissue values for four fish species that are two orders of magnitude lower than those found by Kim *et al.*, (1989). Moreover, a second paper by the same authors (Ryu *et al.*, 1992) reported ANF values in the cardiac tissues of a frog (*Rana dybowskii*) that are also lower than those found for a related species (*Rana nigromaculata*). Consequently, the ANF values reported by these two papers should be viewed with some circumspect. Furthermore, the fish ANF plasma values range widely from 347 to  $8.9 \text{ pg}\cdot\text{mL}^{-1}$ . With the exception of the freshwater eel, seawater fish tend to have

higher ANF plasma values than those reported for freshwater fish. Overall, very little is known about non-mammalian ANF biosynthesis and there appears to be some discrepancy regarding ANF concentrations found in non-mammalian cardiac tissues and plasma. Therefore, in Chapter 3, I investigated the myocardial ANF contents and the plasma ANF concentrations for rainbow trout.

### **Release of ANF**

In mammalian cardiac studies, the most effective and best documented stimulus for ANF release is atrial distension (Dietz, 1984; Lang *et al.*, 1985; Ledsome *et al.*, 1985; Anderson *et al.*, 1986; Edwards *et al.*, 1988; Agnoletti *et al.*, 1989); however, it is unknown if this stimulus is unique to the mammalian system or universal amongst the other vertebrate groups. In diving turtles (*Pseudemys scripta*), an increase in their central blood volume, resulting from the constriction of their pulmonary and systemic systems, is a primary stimulus for ANF release (Baeyens *et al.*, 1989). In birds, amphibians and fish, the stimuli resulting in the release of ANF are still unknown, although they are presumed to be the same as those known for mammals. Nevertheless, fish cardiac myocytes do produce and secrete ANF (Baranowski and Westenfelder, 1989), but the mechanisms that induce ANF release from a working fish heart remain to be investigated.

In mammals, it is unclear if an ionic mediator, such as  $\text{Na}^+$ , can stimulate or hamper ANF production and release. For example, a decrease in dietary sodium results in a decrease in atrial ANF (DeBold, 1979), but when the dietary  $\text{Na}^+$  intake is increased there is no change in the atrial ANF content (Takayanagi *et al.*, 1985; Dietz, 1987). Many other mammalian studies, however, show that increases in dietary salts will increase circulating plasma ANF levels (Tanaka *et al.*, 1984; Sagnella *et al.*, 1985; Weidmann *et al.*, 1986). When rainbow trout are fed high salt diets, their blood and pulse pressures increase (Eddy *et al.*, 1990), but it is unknown if the plasma ANF levels also changed.

What is known, however, is that an infusion of human ANF ( $10 \mu\text{g}\cdot\text{kg}^{-1}$  for 10 minutes) into these fish did not show an effect on the blood or pulse pressures (Eddy *et al.*, 1990).

In addition, it is also unclear to what degree the neurotransmitters, such as adrenaline, noradrenaline and acetylcholine, can affect ANF secretion. For instance, several mammalian studies report that neurotransmitters can cause the release of ANF (Schiebinger *et al.*, 1987; Wong *et al.*, 1988; Agnoletti *et al.*, 1992), yet other studies report they have no effect on ANF release (Haass *et al.*, 1987; LaChance and Garcia, 1988; Onwochei and Rapp, 1988). It has also been shown that mammalian ANF release increases with increases in heart rate (Schiebinger and Linden; 1986; Yamamoto *et al.*, 1988a; Ngo *et al.*, 1989) and that this ANF release is independent of cardiac innervation (Rankin *et al.*, 1987; Deray *et al.*, 1990). As previously mentioned, nothing is known about the factors that influence ANF release fish hearts; therefore, Chapter 3 of this thesis investigates the factors that induce ANF release from the hearts of freshwater-acclimated and seawater-acclimated trout.

### **Biological Actions of ANF**

Once ANF is released into the circulation, it has a key role in the regulation of blood pressure through a variety of actions. However, all hormones, including ANF, must first bind to a specific membrane receptor before they can mediate their actions. In mammals, specific binding sites for ANF have been found in many tissues, such as the aorta (Hirata *et al.*, 1984; Vandlen *et al.*, 1985), kidney (Murphy *et al.*, 1985), brain (Quirion *et al.*, 1984), pituitary (Kurihara *et al.*, 1987), lung (Kudo *et al.*, 1986), and retina (Fernandez-Durango *et al.*, 1989). There are three basic types of ANF receptors found in mammalian tissues: namely, ANF-A and ANF-B receptors which are linked to increased guanylate cyclase activity (Kudo *et al.*, 1986; Schulz *et al.*, 1989); and ANF-C receptor, or the clearance receptor, which is not associated with a change in guanylate

cyclase activity (Fuller *et al.*, 1988). Nothing is known about ANF receptors in amphibians, reptiles or birds, to the best of my knowledge. In fish, however, ANF receptors have been found on teleost intestines (O'Grady *et al.*, 1985), gills and kidneys (Duff and Olson, 1992b; Kloas, 1993), but their molecular structures are unknown.

In mammals, when ANF binds to a receptor it forms an ANF-receptor complex (Hirata *et al.*, 1985) that initiates an intracellular response. The ANF-receptor complex is then internalized, ANF is degraded and the receptor reappears on the cytoplasmic membrane (Hirata *et al.*, 1985). In mammals, after ANF binds to its receptor, there is an increase in the intracellular level of cyclic guanosine monophosphate (cGMP) (Hamet *et al.*, 1984). The enzyme guanylate cyclase catalyzes the conversion of guanosine triphosphate to cGMP, which in turn initiates the final physiological effect (Stryer, 1988). As ANF-A and -B receptors are associated with an increase in guanylate cyclase activity, this suggests that in mammals cGMP could be the secondary messenger for ANF (Waldman *et al.*, 1984).

The biological actions of ANF in mammalian systems are well documented. For instance, in rats and dogs, ANF inhibits aldosterone release from the adrenal glomerulosa (Kudo and Baird, 1984) and renin secretion from the kidneys (Maack *et al.*, 1984). In the plasma, renin is the enzyme that catalyzes the splitting of angiotensin I from angiotensinogen (Vander *et al.*, 1990). The inhibition of aldosterone and renin release by ANF are mediated through cGMP (Kurtz *et al.*, 1986; Henrich *et al.*, 1988). Thus, in mammals, ANF produces hypotension primarily by opposing their renin-angiotensin-aldosterone system (Ackermann, 1986; Atlas *et al.*, 1986).

In mammals, ANF also inhibits the oral uptake of water and salt (Antunes-Rodrigues *et al.*, 1985) and stimulates their excretion by the kidney (Sonnenberg, 1986; Sonnenberg *et al.*, 1986). Once again, the diuretic and natriuretic effects of ANF are mediated by cGMP (Huang *et al.*, 1986). In rats, ANF binding sites are present in the



jejunum (Bianchi *et al.*, 1989), but here the actions of ANF are unclear. For instance, in one study ANF reduced water, sodium and chloride absorption in the rat intestine (Seeber *et al.*, 1986) while in another study ANF increased their absorption (Kanai *et al.*, 1987).

In other vertebrates, such as turtles and frogs, all that is known about ANF activity is that it lowers blood pressure by acting as a vasorelaxant (Cho *et al.*, 1988; Chiu *et al.*, 1990). In birds, such as the white leghorn chicken (*Gallus g. domesticus*), atrial extracts have been shown to cause a mild vasodilation of their aorta (Reinecke *et al.*, 1985) and a weak natriuresis and diuresis in their kidney (Greg and Wideman, 1986). The mediating systems for these responses have not been established, but they are assumed to be similar to the mammalian system (i.e. mediated by cGMP).

In fish, ANF-C gill receptors have been shown to remove at least 60% of an inter-arterial injection of synthetic ANF (Duff and Olson, 1992b; Olson and Duff, 1993). In the seawater killifish, ANF binding to specific receptors in their gill epithelium stimulates  $\text{Na}^+\text{-K}^+\text{-Cl}^-$  co-transporter proteins and this results in the retention of water (Scheide and Zadunaisky, 1988). In the intestine of the seawater flounder water uptake occurs when ANF binds to specific receptors that inhibit the  $\text{Na}^+\text{-K}^+\text{-Cl}^-$  co-transporters (O'Grady *et al.*, 1985). ANF also inhibits the rhythmic and tonic contractions in the rainbow trout gut (Jensen and Olson, 1994) and when synthetic rat ANF is injected into rainbow trout, it relaxes the arterial vessels by inhibiting intracellular calcium release (Olson and Meisner, 1989). Again, the mediating systems for the above responses have not been established, but they are assumed to be mediated by cGMP.

## **Objectives**

The objectives of this thesis are as follows:

1. To establish that ANF is present in the trout myocardium and specifically in the atrial tissue.

2. To establish that trout ANF can be detected and quantified using standard biochemical techniques.

These two objectives formed the focus of the first part of my research (Chapter 2) which centered on the location of ANF granules in the trout myocardium using both light and electron microscopy coupled with immunogold and immunofluorescence techniques. This was accomplished by using a mammalian antibody to immunoreactively label trout ANF in standard histochemical and biochemical techniques.

The second part of this thesis (Chapter 3) investigated factors influencing the release of ANF from the rainbow trout heart. The physiological studies of ANF release utilized a working perfused *in situ* heart preparation that is known to be capable of performing at work levels up to the maximum levels observed *in vivo* (Farrell *et al.*, 1988). The research objectives in Chapter 3 were as follows:

3. To determine the constancy of cardiac ANF release to a sustained increase in atrial volume/pressure.

4. To determine if cardiac ANF release was a graded or "all-or-none" response to variations in atrial volume/pressure.

and,

5. To determine if cardiac ANF release differs between freshwater-acclimated and seawater-acclimated trout.

## *Chapter 2*

# **IMMUNOHISTOCHEMICAL AND ULTRASTRUCTURAL STUDY OF ATRIAL NATRIURETIC FACTOR IN RAINBOW TROUT MYOCARDIUM**

## **INTRODUCTION**

Early anatomical studies, using electron microscopy, were an essential component in the discovery of ANF. Kisch (1955; 1956) was the first to describe dense granules in the atria of guinea pigs and rats. Later, Jamieson and Palade (1964) were the first to suggest that these granules contained a hormone; namely, ANF. As the development of the electron microscope improved, so did various techniques, such as immunocytochemistry and quantitative microscopy, that are associated with electron microscopy. The immunogold technique has been used to quantify an ir-ANF increase in the ventricular tissues of hamsters in severe congestive heart failure (Genest and Cantin, 1988). Another study used both immunofluorescence and immunogold to label ANF in heart and salivary glands of water-deprived and sodium-deficient rats (Cantin *et al.*, 1984). When the two methods gave identical results the ultrastructural results were confirmed (Cantin *et al.*, 1984). Thus, with the assistance of these new microscopic techniques, it is now generally accepted that the dense granules found in vertebrate cardiac tissues contain the membrane bound prohormone ANF-(1-126) (Chapeau *et al.*, 1985; Kawata *et al.*, 1985; Reinecke *et al.*, 1985).

Therefore, the objective of this chapter was to locate, describe and quantify ANF in the rainbow trout myocardium. To accomplish this objective, trout myocardial tissues were subjected to both light and electron microscopic techniques to determine the immunohistochemical distribution of ANF. In rainbow trout, previous studies using the

peroxidase-antiperoxidase (PAP) technique have shown more ir-ANF in the atrium than in the ventricle (Reinecke *et al.*, 1985). The PAP technique involves the incubation of tissues with an enzyme-antibody complex and is diffusion limited yielding micrographs with poor resolution. This study, however, used a light microscopic technique involving immunofluorescent staining to grossly identify the location of ANF in the tissues. Then, transmission electron microscopy (TEM) was used to yield detailed ultrastructural morphology of the cardiac myocytes. Tissues were also prepared for transmission immunoelectron microscopy (TIEM). This technique involved the competitive binding of a colloid-gold labeled antibody to a specific antigen (Faulk and Taylor, 1971). TIEM allows the precise identification and location of specific macromolecules, such as ANF, in the myocardial cells.

## MATERIALS AND METHODS

### Animals

Rainbow trout (*Oncorhynchus mykiss* [Walbaum]) were obtained from a local supplier (West Creek Trout Farms, Aldergrove, B.C.) and held indoors in a 2,000 L fiberglass tank supplied with flowing, dechlorinated water (12:12 hour photoperiod, pH 6.5). Before sampling, all animals were acclimated to 13 °C for at least two weeks. At sampling time, seven control animals were quickly and fully anesthetized (MS222; 1:5,000 w/v) before receiving a blow to the head. The hearts were then immediately removed from the body of the animals and quickly rinsed in saline and blotted dry to remove any adhering blood cells. In addition, five trout hearts were obtained from the *in situ* heart preparation (see Chapter 3) and prepared for light and electron microscopy.

## Light Microscopy

The atrium and ventricle from four animals, two control and two *in situ* hearts, were dissected apart and then quickly frozen and stored at -80 °C. The frozen tissues were immersed in an OCT compound (Tissue Tek II, Miles Inc.) and cryosectioned at -28 °C. Fourteen- $\mu$ m thick sections were mounted on slides and stored in a sealed container at -20 °C.

The sections were prepared for fluorescence microscopy by first preincubating them in a phosphate-buffered saline (PBS; pH 7.3; see Table 2.1) for 5 minutes. The primary antibody was prepared by diluting human anti-alpha ANF-99-126 (Peninsula Laboratories, Inc., Belmont, CA) 1:200 v/v in the PBS buffer. The primary antibody was introduced to the sections at one end of the slide while the PBS buffer was removed with a pipette at the other end. The sections were then transferred to a moist chamber and allowed to incubate in the primary antibody overnight (at 4 °C). The controls were incubated in normal rabbit serum. After the primary incubation, the sections were once again washed with PBS (5 X 5 minutes) and then incubated for one hour at room temperature with an anti-rabbit immunoglobulin-G fluorescein isothiocyanate (FITC) conjugate (Sigma Chemical, St. Louis, MO) diluted 1:160 v/v in PBS. This was followed with 5 X 5 minute washes in PBS. The tissues were immediately photographed using an Olympus-AHBS3 fluorescence microscope.

## Electron Microscopy

While immersed in a 2.5% glutaraldehyde primary fixative, atrial and ventricular tissues from eight animals (5 control and 3 *in situ* hearts) were dissected apart and cut into approximately 1 mm<sup>3</sup> blocks with a clean razor blade. After 10 minutes, the tissue blocks destined for immunogold staining (TIEM) were transferred with a fine brush into a 0.05 M sodium cacodylate buffer (pH 7.2; Table 2.1). After three 10 minute rinses in the

cacodylate buffer, the TIEM tissues were dehydrated in a series of ethanol rinses (each 10 minutes in 30%, 50% 70%, 85%, 95%, and twice in absolute ethanol). Next, the TIEM tissues were rapidly embedded by decreasing temperature with each graded exchange of ethanol with Lowicryl K4M (Altman *et al.*, 1984). The TIEM samples were placed in pre-cooled gelatin capsules filled with 100% Lowicryl K4M and allowed to equilibrate and polymerize for 24 hours at 4 °C under a 360 nm light source.

The chopped tissue blocks destined for TEM were transferred to a fresh primary 2.5% glutaraldehyde solution, where they remained for three hours. After the primary fixation, the tissue blocks were washed three times for 5 minutes each in a cacodylate buffer. Subsequently, the TEM tissues were immersed for one hour at room temperature in 1.0% buffered osmium tetroxide (Table 2.1). After the osmium post-fixation, the samples were washed twice for 10 minutes each in cacodylate buffer and then dehydrated in a series of ethanol rinses for 10 minutes (each in 30%, 50% 70%, 85%, 95%, and twice in 100% ethanol). Next, the TEM samples were subjected to a series of graded changes from absolute ethanol to absolute propylene oxide and then embedded in a vinylcyclohexene dioxide resin (Spurr Low Viscosity; J.B. EM Services, Point Claire, Dorval, Quebec).

After embedding, all tissue blocks were uniformly sectioned (< 70 nm) using a ultramicrotome and glass knife. The sections were then carefully mounted on 200-mesh per inch copper grids. The TIEM sections were immersed for 5 minutes in an incubation buffer (pH 7.3; Table 2.1) consisting of 0.05 M phosphate, 0.154 M NaCl and 1.0% bovine serum albumin (modified from Wang and Haunerland, 1991) and then the sections were incubated with human anti-alpha ANF-99-126 diluted 1:10 in incubation buffer for 24 hours (4 °C). After the primary incubation, the sections were transferred to a washing buffer (pH 7.3; Table 2.1) which consisted of 0.05 M phosphate, 0.308 M NaCl, 2.0% bovine serum albumin and 0.1% Triton X100. After five 10 minute rinses in the washing

**Table 2.1 Immunohistochemical Buffers and Reagents**Phosphate-Buffered Saline *10X stock solution* (pH 7.3)

80 g NaCl  
 2 g KCl  
 11.5 g Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O  
 2 g KH<sub>2</sub>PO<sub>4</sub>  
 1000 mL distilled water

## 0.05 M Sodium Cacodylate Buffer (Hayat, 1981)

*Stock A:* 42.8 g Na<sub>2</sub>(CH<sub>3</sub>)AsO<sub>2</sub>·3H<sub>2</sub>O      *Stock B:* 2 mL HCl  
 1000 mL distilled water                              8 mL distilled water  
 Mix 50 mL stock *A* with 4.2 mL stock *B* (pH 7.2)

## 2.5% Glutaraldehyde Primary Fixative

25.0 mL 50% glutaraldehyde  
 187.5 mL sodium cacodylate buffer (pH 7.2)  
 287.5 mL distilled water

## 1.0% Osmium Tetroxide

10 mL 4% OsO<sub>4</sub>  
 30 mL sodium cacodylate buffer (pH 7.2)

## Incubation Buffer

*Stock A:* 3.44 g KH<sub>2</sub>PO<sub>4</sub>                              *Stock B:* 6.75 g Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O  
 500 mL distilled water                              500 mL distilled water  
 Mix 19.6 mL stock *A* and 130.7 mL stock *B* (pH 7.3)  
 In 50 mL of the above mixture add: 0.50 g bovine serum albumin  
 0.45 g NaCl

## Washing Buffer

19.6 mL of the incubation buffer stock *A* was mixed with  
 130.7 mL of incubation buffer stock *B* (pH 7.3)  
 In 50 mL of the above mixture add: 1.0 g bovine serum albumin  
 0.9 g NaCl  
 50 μL Triton X100

## 5.0% Uranyl Acetate

0.99 g UO<sub>2</sub>(C<sub>2</sub>H<sub>3</sub>O<sub>2</sub>)<sub>2</sub>·2H<sub>2</sub>O and 20 mL distilled water

## 2.6% Lead Citrate (Reynolds, 1963)

1.33 g lead citrate  
 8.0 mL 1N NaOH (pH 12) and 50 mL distilled water

buffer, the sections were incubated for one hour in 1.0% goat anti-rabbit immunoglobulin-G labeled with 10 nm colloidal gold particles (Sigma Chemical, St. Louis, MO) at room temperature. Once again the sections were rinsed in the washing buffer (3 X 10 minutes) and then in distilled water (2 X 10 minutes). Both the TEM and TIEM ultrathin sections were post-stained with 5.0% uranyl acetate and 2.6% lead citrate (Reynolds, 1963). In the final step, the sections were placed in a specimen holder and viewed using a Philips 300 electron microscope at accelerating voltages of 80 kV. (Buffer conditions and all reagents are presented in Table 2.1.)

### Quantitative Analysis

Equation (1) was used to calculate the fraction of bound gold particles ( $F_p$ ) within a fixed area ( $A_t$ ; Eq. 2) for an electron micrograph. The total area of gold particles ( $A_p$ ; Eq.3) was based on the assumption that the gold particles were uniform in size and circular in shape. Therefore, the area of gold was given by equation (4). In addition, the gold particles were small and sparsely distributed, such that they could be counted ( $N$ ) as separate. The study was able to meet the above assumptions because the diameter of a colloidal gold complex is  $10.37 \pm 0.58$  nm (mean  $\pm$  S.D.;  $N = 100$ ; Sigma Chemical, St. Louis, MO).

Equation 1. Fraction of marked area ( $F_p$ ) =  $A_p/A_t$

Equation 2. Total Area (units) of micrograph ( $A_t$ ) = Length x Width

Equation 3.  $A_p$  = number of particles ( $N$ ) x  $\alpha$

Equation 4. Area (units) of each gold particle ( $\alpha$ ) =  $\pi r^2$



## Statistical Procedures

Nonparametric statistical comparisons were made using the Mann-Whitney test. The significant differences were at the 95% confidence level.

## RESULTS

The fine structure of the rainbow trout ventricle was composed of tightly packed myofibril bundles that were irregularly distributed in the cytoplasm and surrounded by numerous mitochondria and lipid droplets (Figs. 2.1 to 2.3). The longitudinal section of the ventricular myofibrils displayed the familiar banding pattern found in mammalian cardiac muscle. Each Z-band occurred at regular intervals of approximately 1.9  $\mu\text{m}$  (Fig. 2.1).

A cross-section of the atrium shows the myofibrils in discrete bundles and sparsely distributed in the cytoplasm (Fig. 2.4). The myofibril organization in the trout atrium does not have the compact appearance of the mammalian atrium, such that the fibres appeared to be arranged in a loose mesh. The atrium contained numerous mitochondria that lie adjacent to the muscle bundles, but unlike the ventricle, it lacked the large lipid droplets.

Electron-dense homogeneously granulated material was found in both the atrial and ventricular myocardium, but the material was considerably more abundant in the atrium than in the ventricle (Figs. 2.2 to 2.5). The material was found in double membraned vesicles, presumed to be secretory vesicles (Figs. 2.3 and 2.5). The granules were usually found near the nucleus and were best seen in cross-sections (Figs. 2.2 and 2.5). The granules were oval or round in appearance and were  $0.17 \pm 0.01 \mu\text{m}$  in diameter (mean  $\pm$  S.E.M.,  $N = 16$ ).

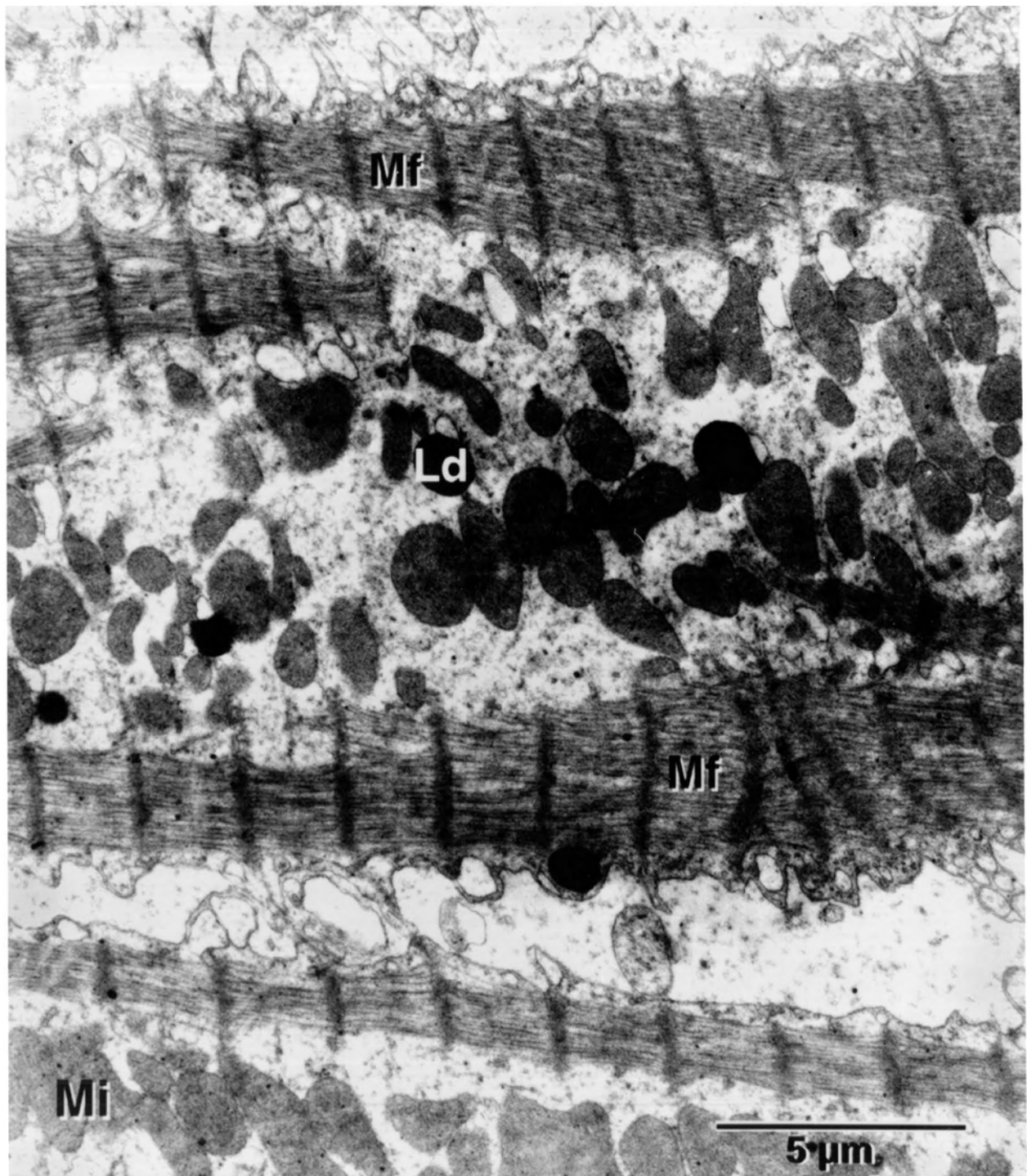
With both the immunofluorescent and immunogold procedures, ANF immunoreactivity was observed in the trout atrium and the trout ventricle (Figs. 2.7 and 2.9). No immunolabeling was noted in the positive controls (Figs. 2.6 and 2.8). Overall, immunolabeling was greater in the atrium than in the ventricle. The immunofluorescent labeling of ir-ANF was abundant in the atrium, but was sparsely distributed in the ventricle (Fig. 2.7).

In the atrial TIEM sections, immunoreactive colloid gold particles were also present in large clusters and were readily found, but in the ventricular sections the gold particles were sparse and often difficult to find (Fig 2.9). No immunoreactive gold binding was noted in the controls (Fig. 2.8). At all magnifications, the number of gold particles bound in a given area were found to be significantly higher ( $p < 0.05$ ) in the atrium than in the ventricle. Subsequently, the calculated fraction of bound gold particles ( $F_p$ ) observed at an original magnification of 42,000 X in the atrium ( $15.6 \pm 3.3 \times 10^{-3}$ ) was approximately 7 times greater than that found in the ventricle ( $2.2 \pm 0.7 \times 10^{-3}$ ). At the ultrastructural level, no descriptive or quantitative differences were noted between the control hearts and the *in situ* hearts (Table 2.2).

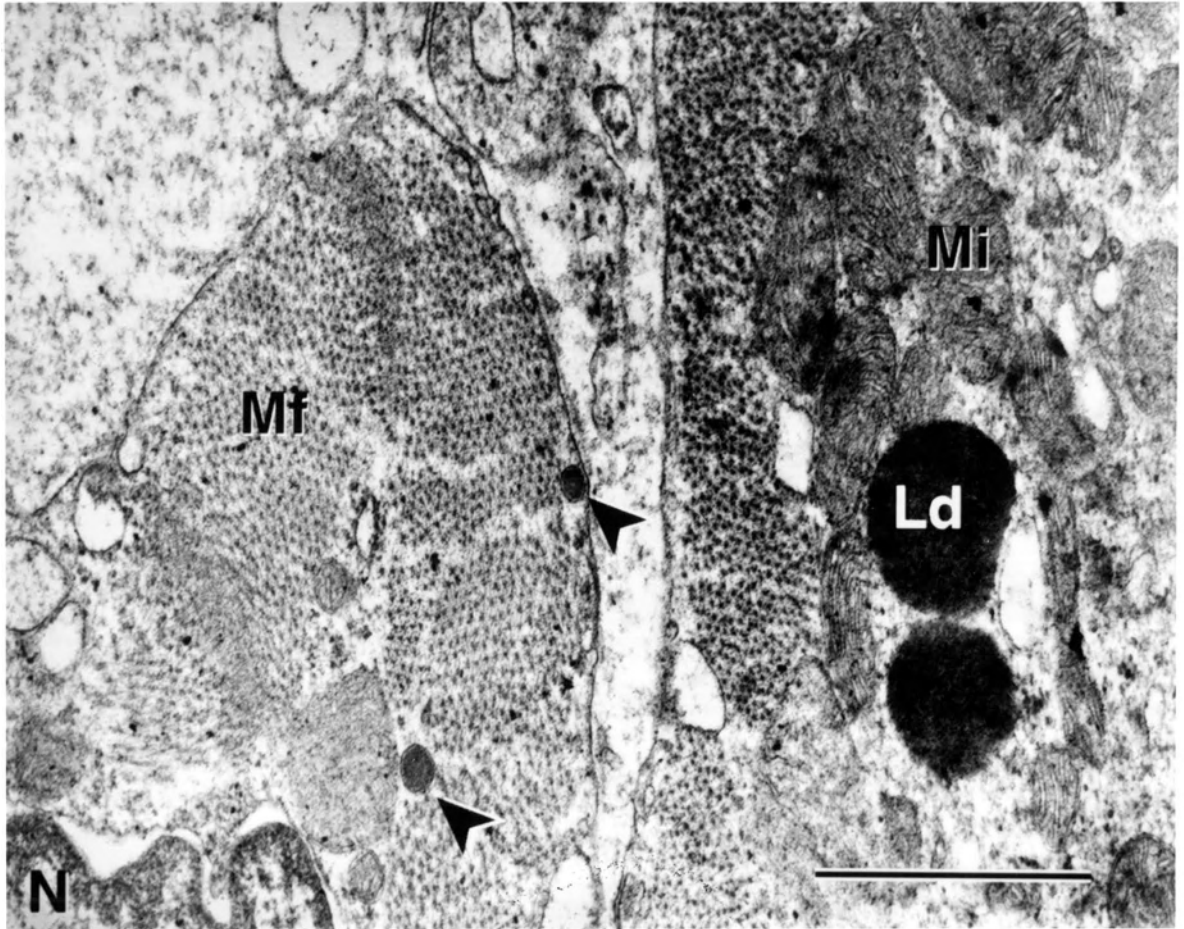
**Table 2.2.** The number of bound particles (N), total area of gold particles ( $A_p$ ) and the fraction of bound gold particles within a fixed area ( $F_p$ ) for rainbow trout atrial and ventricular tissues at an original magnification of 42,000 X. For all calculations:  $A_t = 3126.2 \text{ mm}^2$ ;  $\alpha = 0.785 \text{ mm}^2$ . No significant differences were noted between the mean values for the control ( $N = 5$ ) and *in situ* ( $N = 3$ ) groups (mean values  $\pm$  S.E.M.).

		ATRIUM	VENTRICLE
<b>N</b>	Control	$62.2 \pm 13.7$	$8.6 \pm 2.9$
	<i>In situ</i> Preparation	$56.3 \pm 19.6$	$5.3 \pm 1.2$
<b><math>A_p</math> (<math>\text{mm}^2</math>)</b>	Control	$48.9 \pm 10.2$	$6.8 \pm 2.3$
	<i>In situ</i> Preparation	$49.0 \pm 15.4$	$4.2 \pm 0.9$
<b><math>F_p</math> (<math>10^{-3}</math>)</b>	Control	$15.6 \pm 3.3$	$2.2 \pm 0.7$
	<i>In situ</i> Preparation	$15.7 \pm 4.9$	$1.3 \pm .03$

**Figure 2.1.** A low magnification transmission electron micrograph showing a longitudinal section of rainbow trout ventricle. The myofibrils (Mf) are seen with their distinct Z-bands. Several lipid droplets (Ld) are seen adjacent to mitochondria (Mi). Note that the fine granular material seen dispersed throughout the cytoplasm is a mixture of free ribosomes and glycogen particles. Bar represents 5  $\mu\text{m}$ . Original magnification 8,200 X.

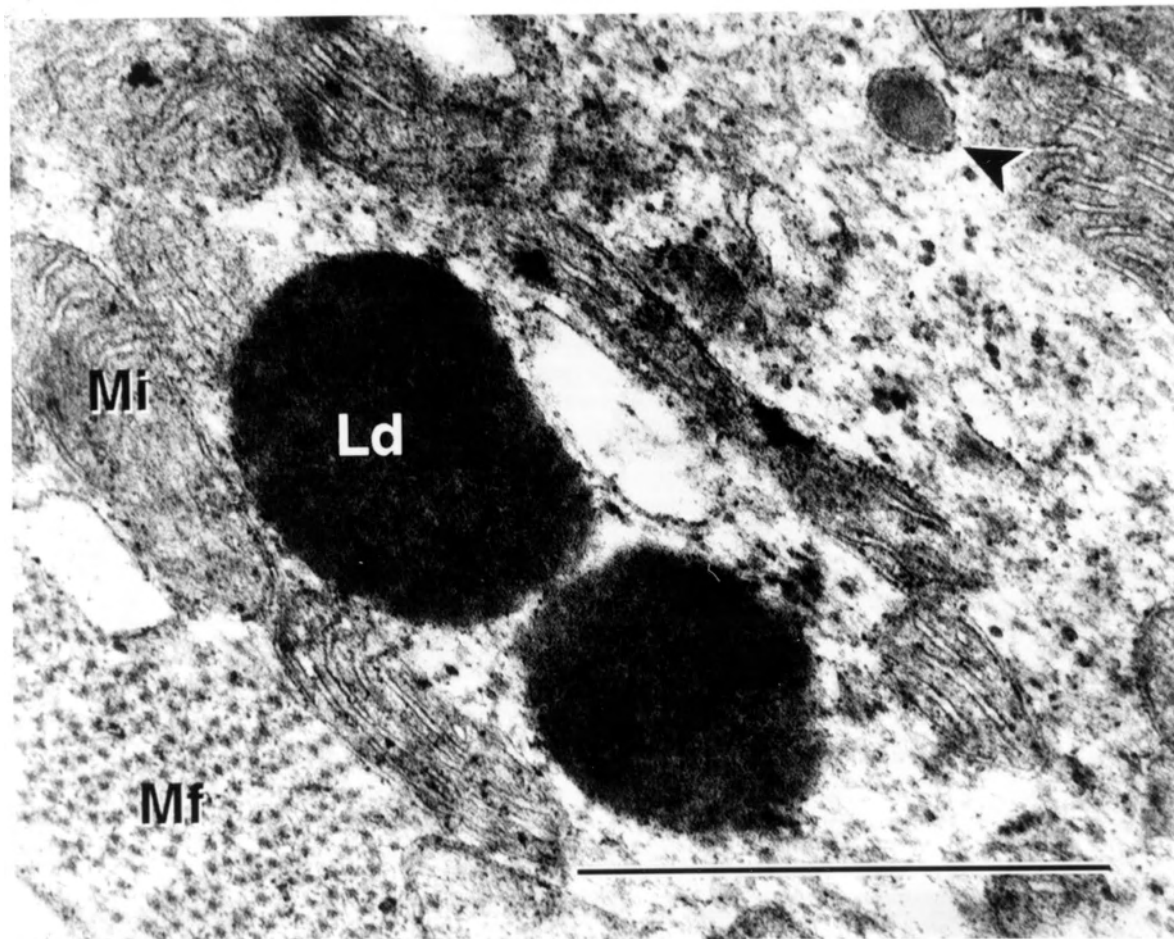


**Figure 2.2.** A transmission electron micrograph showing a cross-section of rainbow trout ventricle. The myofibrils (Mf) are seen arranged in discrete bundles. A close association between lipid droplets (Ld) and mitochondria (Mi) is seen in the cardiac muscle and fine granular material is dispersed throughout the cytoplasm. Two electron-dense granules with double membranes (arrows) are seen in close proximity to the nucleus (N). Bar represents 1  $\mu\text{m}$ . Original magnification 20,000 X.

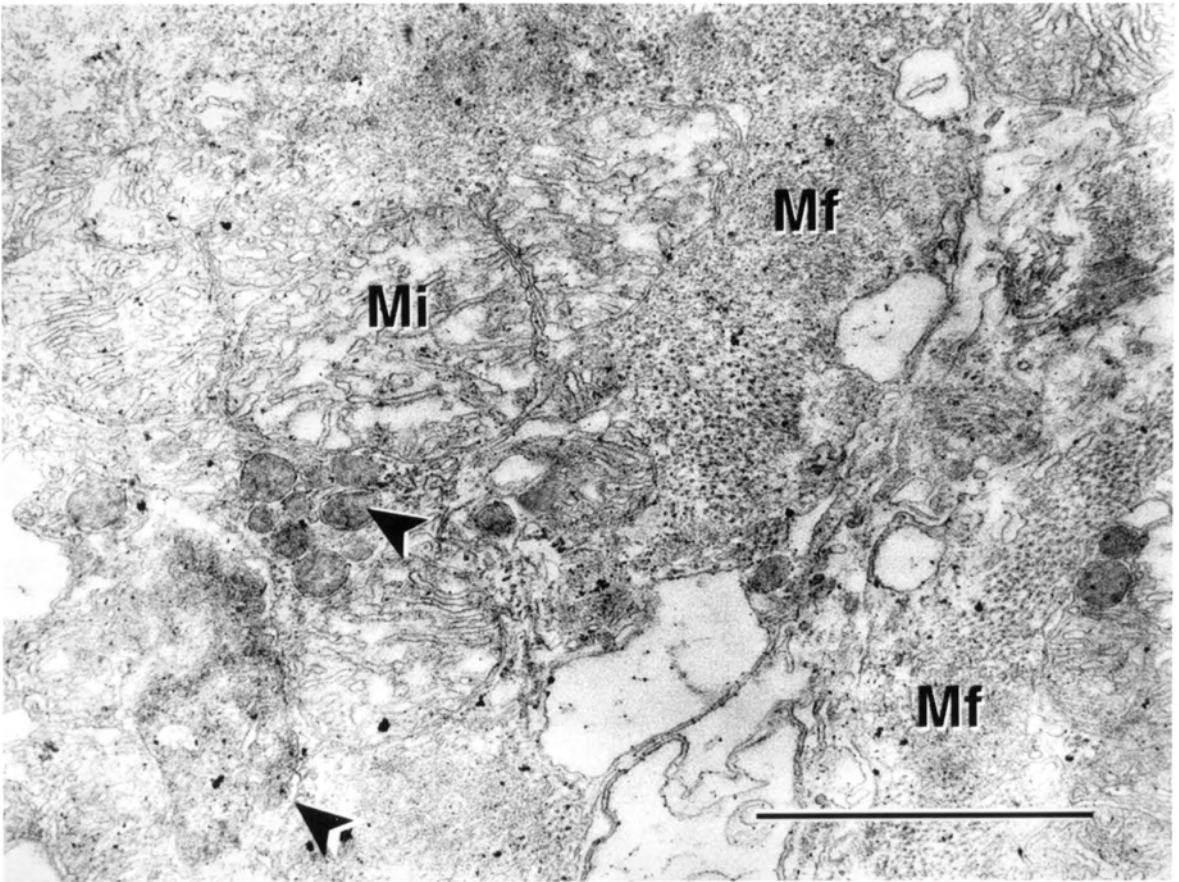


**Figure 2.3.** A transmission electron micrograph showing a cross-section of rainbow trout ventricle. The myofibrils (Mf) are seen in a discrete bundle and are adjacent to mitochondria (Mi) and lipid droplets (Ld). Granular material, such as free ribosomes and glycogen particles, are dispersed throughout the cytoplasm. Note that a single electron-dense granule is seen adjacent to a mitochondria (arrow). Also note that a double membrane is seen surrounding the electron-dense homogeneously granulated material. Bar represents 1  $\mu\text{m}$ . Original magnification 42,000 X.

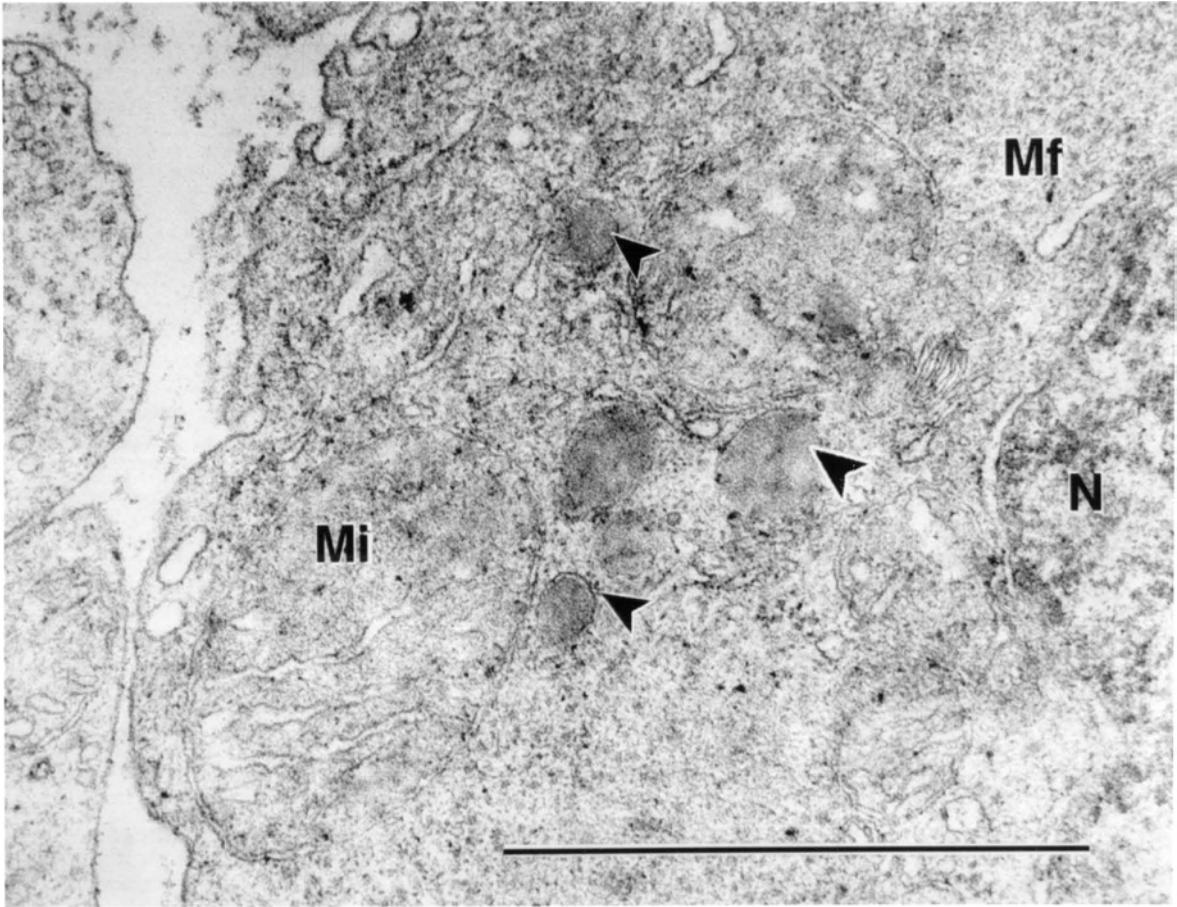




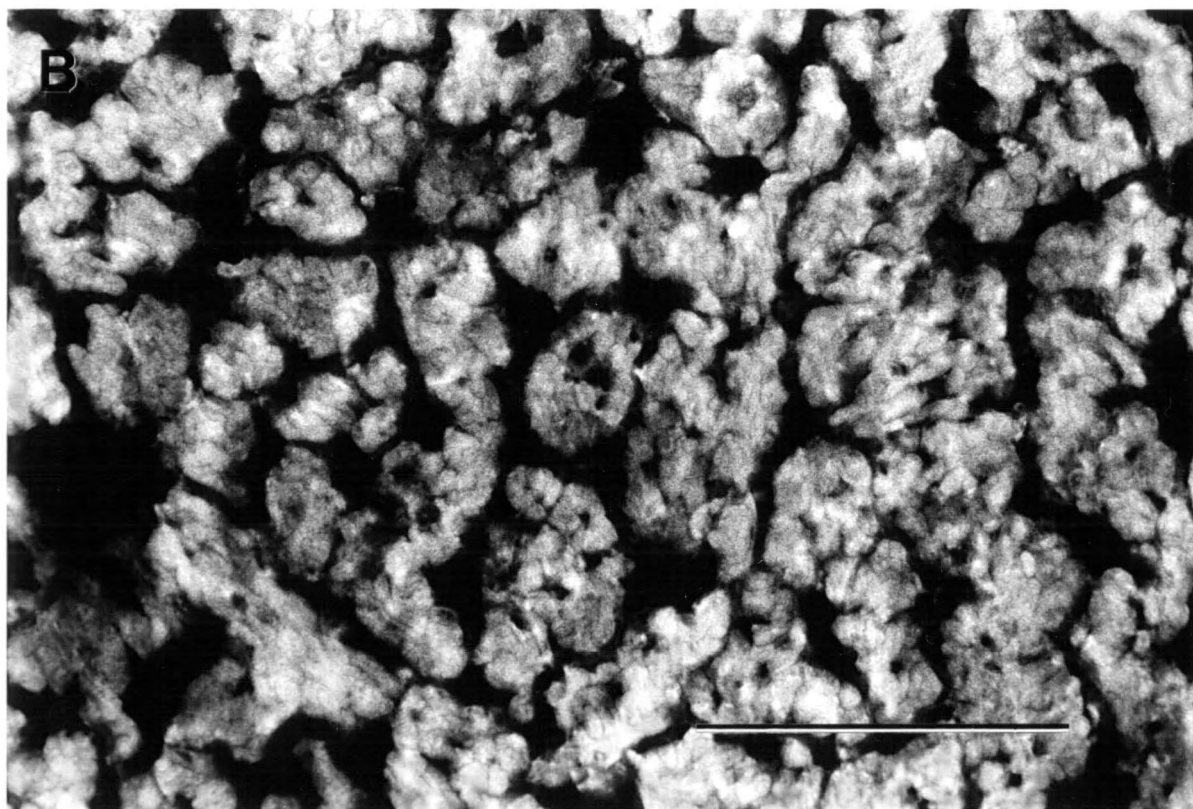
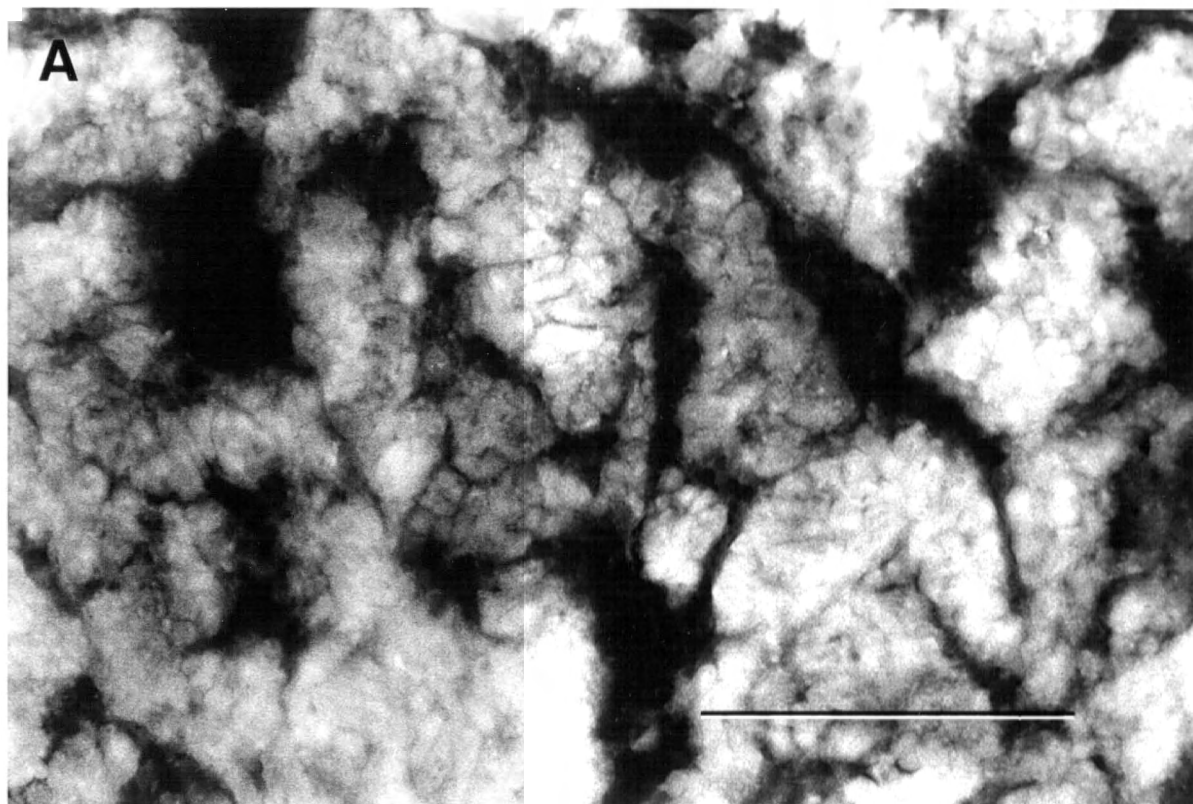
**Figure 2.4.** A transmission electron micrograph showing a cross-section of rainbow trout atrium. The myofibrils (Mf) are seen in bundles, but their orientation varies. Note that the overall granularity appears finer than that found in the ventricle (Fig. 2.2). Mitochondria (Mi) are seen adjacent to the myofibrils. Double membraned electron-dense granular vesicles are numerous and dispersed throughout the tissue (arrows). These vesicles are similar in shape and size to those found in the ventricle (Fig. 2.2). Note the large cluster of granulated material near the bottom left portion of the micrograph (arrow). Bar represents 1  $\mu\text{m}$ . Original magnification 20,000 X.



**Figure 2.5.** A transmission electron micrograph showing a cross-section of rainbow trout atrium. The myofibrils (Mf) are seen adjacent to the nucleus (N) and mitochondria (Mi). Several double membraned granular vesicles (arrows) are seen in close proximity to the nucleus. Note that these vesicles are similar in shape and size to the single vesicle shown in the ventricular tissue (Fig. 2.3). Bar represents 1  $\mu\text{m}$ . Original magnification 42,000 X.

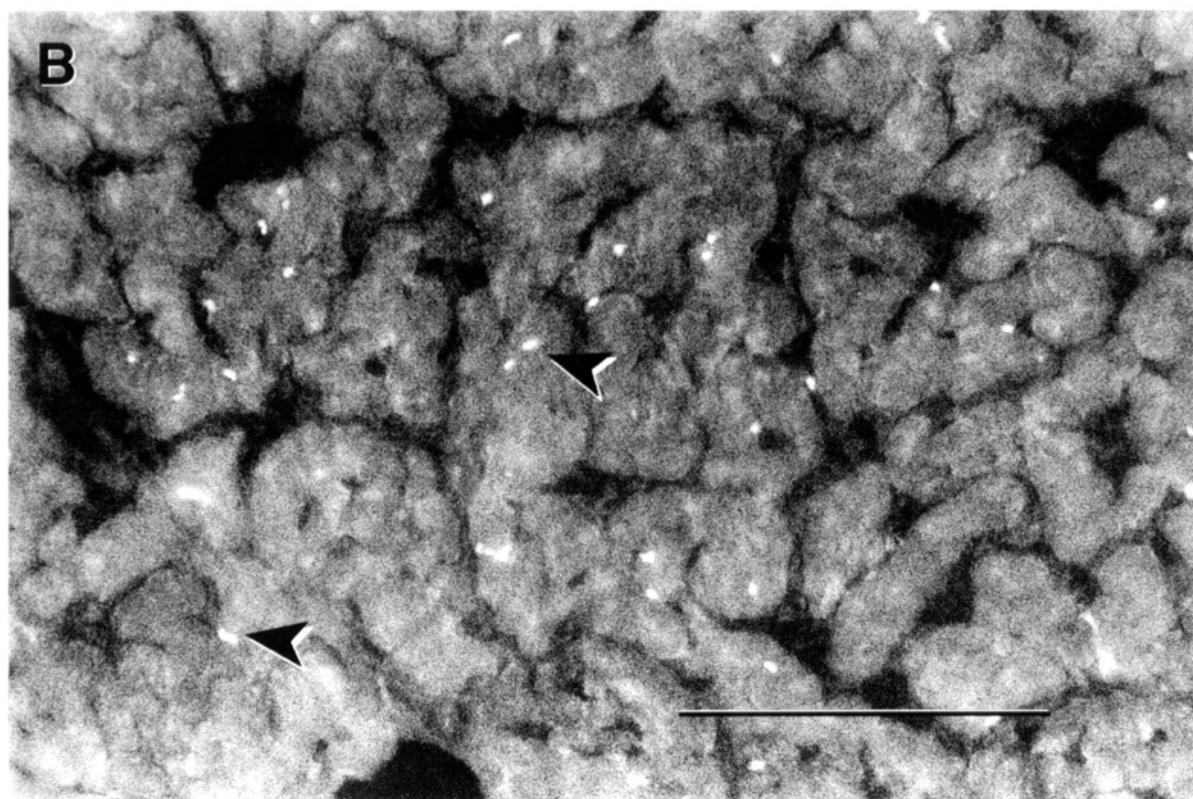
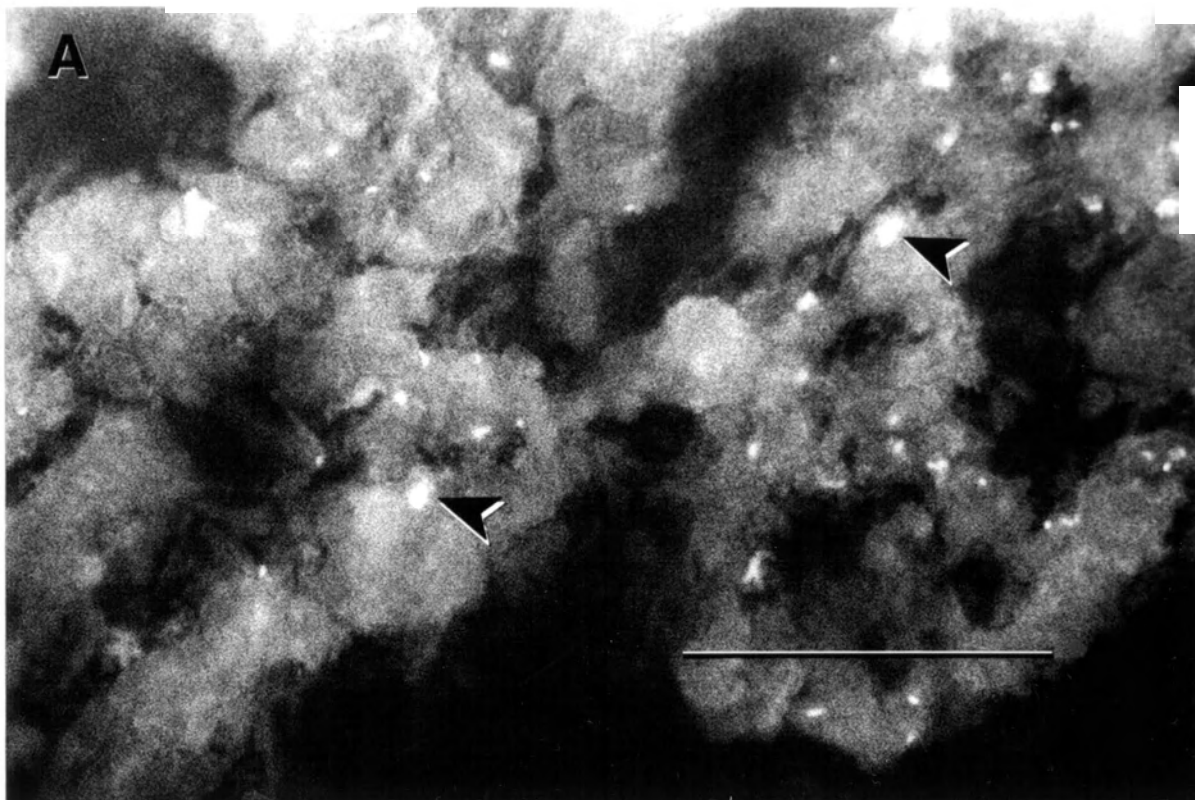


**Figure 2.6.** Immunofluorescence photomicrographs of sectioned control rainbow trout atrial (a) and ventricular (b) tissues. Note the absence of fluorescent labeling. Bar represents 100  $\mu\text{m}$ . Original magnification 100 X.



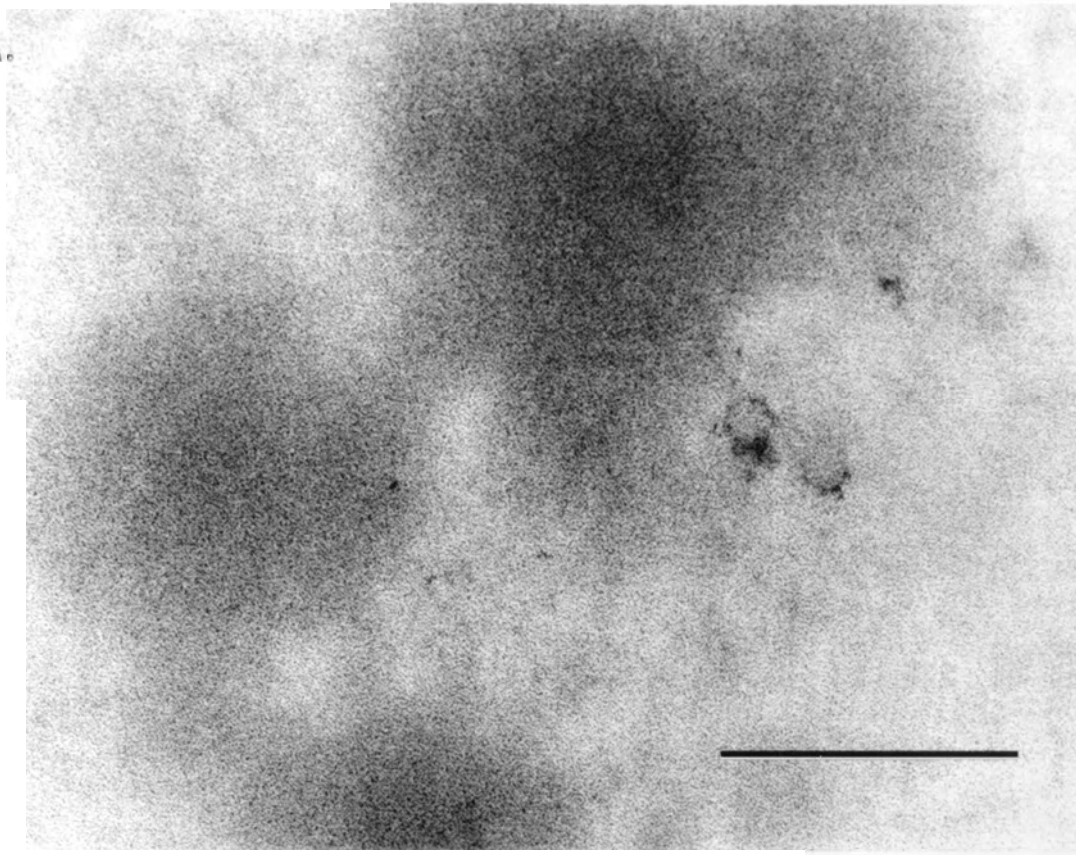
**Figure 2.7.** Immunofluorescence photomicrographs of sectioned rainbow trout myocardium. Note that the heterogeneous occurrence of ir-ANF (arrows) is greater in the atrial tissue (A) than in the ventricular tissue (B). Bar represents 100  $\mu\text{m}$ . Original magnification 100 X.



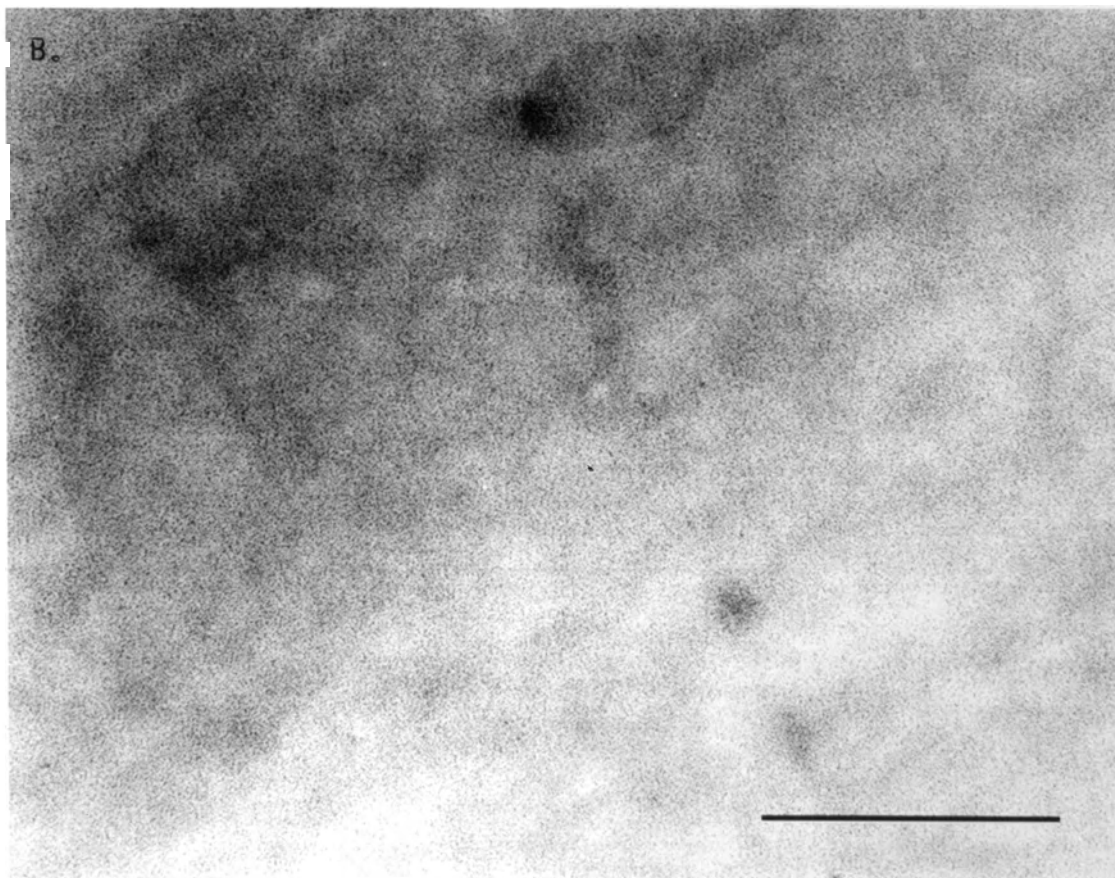


**Figure 2.8.** Immunohistochemical transmission electron micrographs of control rainbow trout myocardium. Unosmicated atrial (A) and ventricular (B) tissues were embedded in Lowicryl K4M and treated with a normal rabbit serum followed by staining with colloidal gold labeled secondary antibodies. The tissues were then post-stained with uranyl acetate and lead citrate. Note the absence of gold particles. Also note the lack of ultrastructural detail due to the lack of osmium tetroxide in the fixation process. Osmium tetroxide was not used because it can destroy or interfere with antigen binding sites. Bar represents 1  $\mu\text{m}$ . Original magnification 42,000 X.

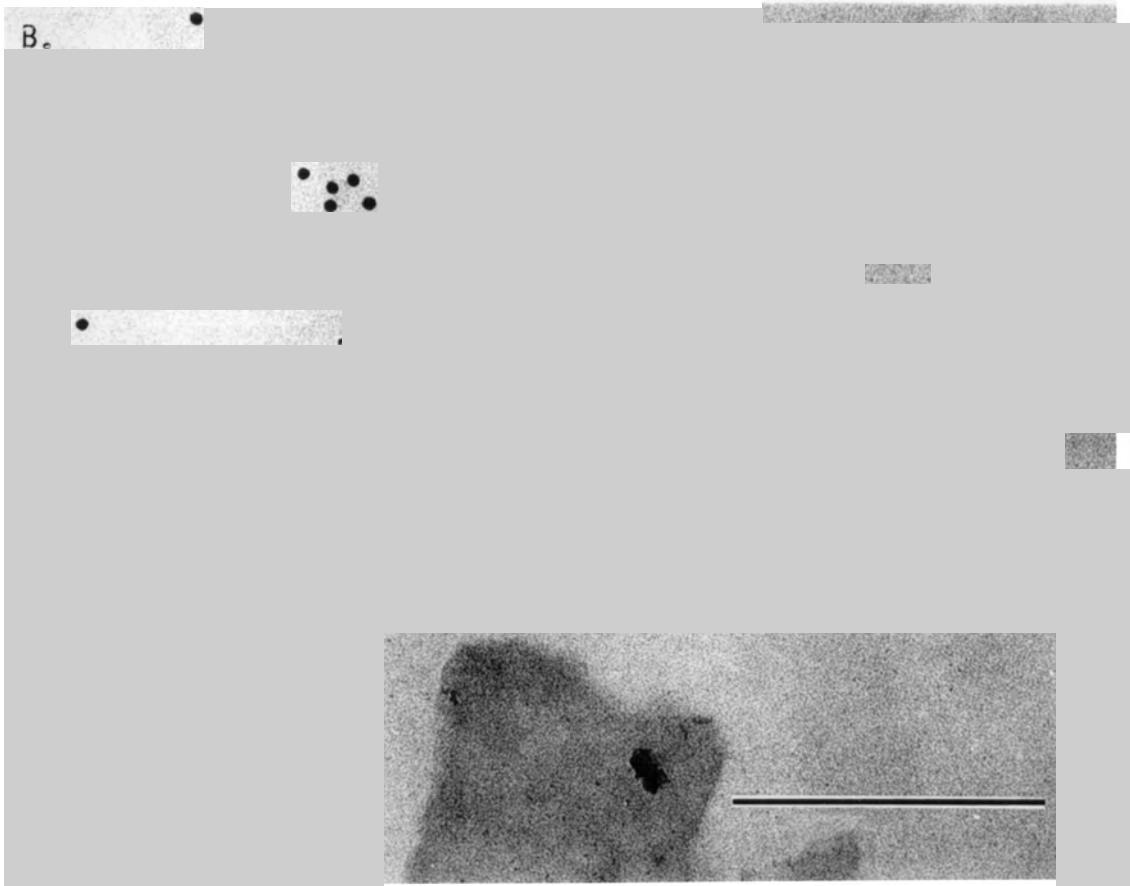
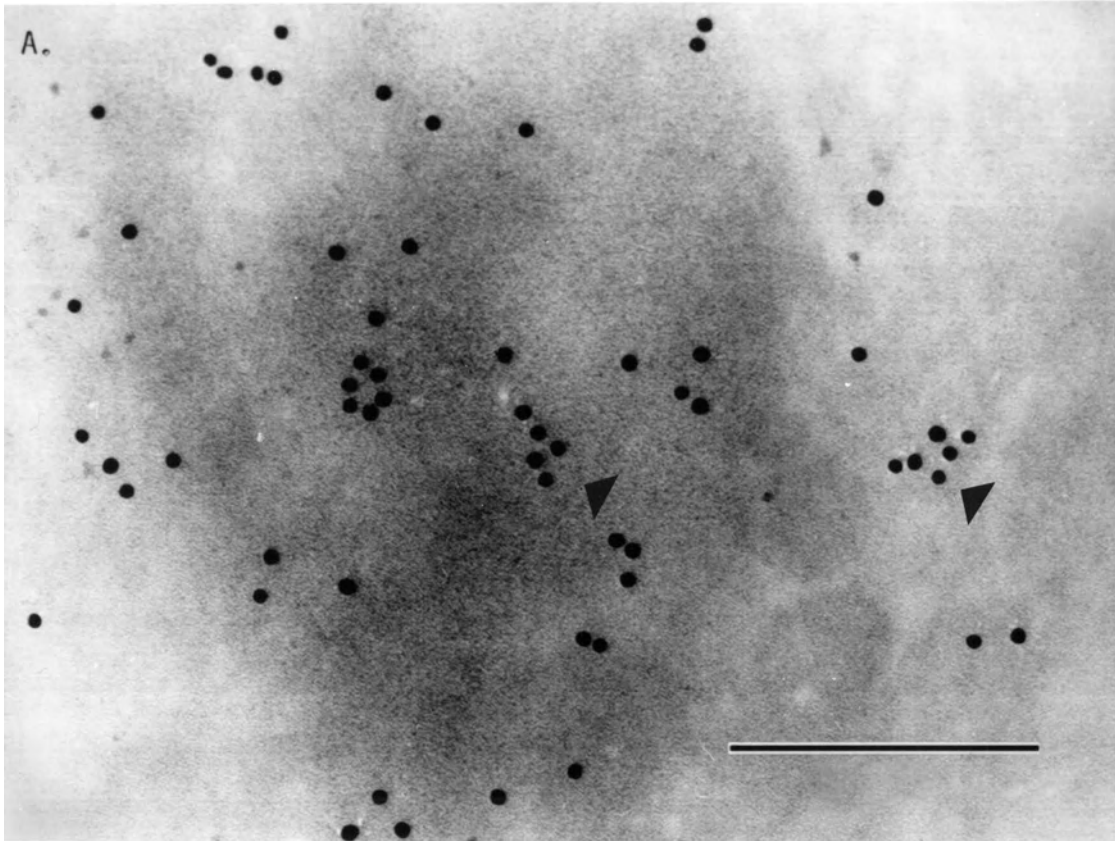
A.



B.



**Figure 2.9.** Transmission electron micrographs showing the immunohistochemical detection of ANF in rainbow trout myocardium. The atrial (A) and ventricular (B) tissues were embedded in Lowicryl K4M and treated with human anti-alpha ANF-(99-126) followed by staining with colloidal gold-labeled secondary antibodies. As in the controls (Fig. 2.8), the tissues were post-stained with uranyl acetate and lead citrate. Although there is very little ultrastructural detail due to the lack of an osmium fixative, faint membranes are seen surrounding two gold clusters (arrows). Note that gold particles are found in both the atrium and ventricle and that the particles are more numerous in the atrium. Also, note that the gold particles are found in discrete clusters. Bar represents 1  $\mu\text{m}$ . Original magnification 42,000 X.



## DISCUSSION

In a phylogenetic study by Reinecke *et al.* (1985), ANF was found in the hearts of mammals, reptiles, amphibians, birds, and bony fish. This phylogenetic study was also the first to locate electron-dense granules in the rainbow trout heart. Recent studies, utilizing immunofluorescence and immunogold labeling, report ir-ANF in the hearts of eels (Takei *et al.*, 1990; Cerra *et al.*, 1992), sharks (Reinecke *et al.*, 1987) and Antarctic teleosts (Uva *et al.*, 1993). All of these studies used a rat ANF antibody to label the fish ANF. In addition, all of the studies reported finding more ir-ANF in the atrium than in the ventricle. Moreover, a recent study quantitatively described the location of ir-ANF in the atria and ventricles of African lungfish (*Protopterus aethiopicus*) and also concluded that the atrium contained the majority of the total myocardial storage of ir-ANF (Larsen *et al.*, 1994). This is the first study to quantitatively describe the location of ir-ANF in the heart of rainbow trout using a human ANF antibody.

The fixation of tissues with glutaraldehyde and osmium tetroxide result in the stabilization of their fine ultrastructure so that they can be processed for viewing under an electron beam. Although osmium tetroxide is an excellent membrane stabilizer (Hayat, 1981), it is also a strong oxidizer and can destroy or interfere with antigen binding sites (Bendayan, and Zollinger, 1983; Bettica and Johnson, 1990). Consequently, in most immunogold studies, osmium tetroxide is not used and the ultrastructural detail is sacrificed to ensure immunoreactivity between the antigen and antibody. In this study, therefore, the TEM micrographs provided detailed ultrastructural information regarding the size and placement of secretory vesicles in the myocardial tissues while the immunolabeling procedures (TIEM) validated the presence and quantitative distribution of ir-ANF.

The TEM micrographs revealed that electron-dense homogeneously granulated material was contained in double membraned secretory vesicles and that these vesicles were present in both the atrial and ventricular sections (Figs. 2.2 to 2.5). These secretory vesicles were often found near the nucleus and in higher concentration in the atrium than in the ventricle. As Golgi bodies were commonly seen near the nucleus and as these structures are part of the cellular constitutive secretory pathway (Burgess and Kelly, 1987), it is not surprising that the granular vesicles were in this region of the cell. These granular vesicles were spherical in shape and had a mean diameter of  $0.17 \pm 0.01 \mu\text{m}$ . This value is similar for those found in mammalian studies ( $0.35 \mu\text{m}$ ; DeBold, 1986) and other fish species. For instance, the mean diameters of atrial granules for freshwater eels, African lungfish and Antarctic teleosts are  $0.158 \pm 0.001$  (Takei *et al.*, 1990),  $0.213 \pm 0.049$  (Larsen *et al.*, 1994), and  $0.21 \pm 0.04 \mu\text{m}$  (Uva *et al.*, 1993), respectively.

The immunogold labeling revealed that the human ANF-(99-126) antibody effectively cross-reacted with the rainbow trout ANF antigen. Every section treated with human ANF displayed gold labeling while the controls, which were treated with normal rabbit serum, displayed no labeling (Figs. 2.8 and 2.9). In addition, the immunogold labeling revealed that the atrium contained at least seven times more ir-ANF than the ventricle (Table 2.2). This finding is in agreement with that reported by Reinecke (1989). Bound colloidal gold particles were found in small clusters, consisting of 4 to 11 particles. In the atrium, gold clusters were abundant and usually found with other similar clusters. In the ventricle, small clusters of bound gold particles were also found, but these clusters were isolated and at great distances from other clusters.

The diameters of gold clusters ranged in size and shape. There are several explanations for this finding. First, a bound gold particle can fall a measurable distance from the epitope of an antigen. In this study, two antibodies were used to label the gold to the ANF epitope. The size of a single rabbit antibody is approximately 10 nm

(Kellenberger and Hayat, 1991). As previously mentioned in the methods, the diameter of the gold particle used in this study was 10 nm. Consequently, the distance between a gold particle and an antigen epitope could vary between 18 to 28 nm (Kellenberger and Hayat, 1991). Taking the distance between a gold particle and the antigen epitope into account, the diameters of the gold clusters were found to agree with the diameters of the secretory vesicles.

The second explanation for a scattered distribution of gold particles involves the three-dimensional surface of the ultrathin section. An ultrathin section is formed from the cleavage of a tissue block face. Consequently, as the section breaks away from the block face large macromolecules can either remain in or are removed from the section (Stierhof and Schwarz, 1991). If macromolecules, such as ANF, are only partially submerged into the section, then only a portion of the ANF will be labeled. In addition, a cleavage through the middle of a spherical granule, rather than through an end, could result in more ANF antigens being exposed to labeled antibodies.

Furthermore, the immunofluorescent micrographs demonstrated that ir-ANF in the trout myocardium was randomly distributed in large heterogeneously shaped clusters (Fig. 2.7). Consequently, during the ultrathin sectioning entire clusters could be missed. As a particular structure was not selected for, nor avoided, the likelihood of encountering an ir-ANF cluster was then assumed to be proportional to its occurrence in the tissue section. Thus, the final determination of the TIEM gold particle distribution was not only affected by the sectioning process, such as choosing the section orientation and thickness, but it was also affected by the random selection of the sample from the larger tissue pieces.

There was no significant difference noted between the means for the number of bound gold particles between the control F<sub>p</sub> ( $15.6 \pm 3.3 \times 10^{-3}$ ) and the *in situ* hearts ( $15.7 \pm 4.9 \times 10^{-3}$ ) (Table 2.2). There are several reasons for this finding, such as the quality



and sample position, but foremost is that this technique was labour intensive and required many more sections and a higher sample number than was performed.

This study is the first to demonstrate that human ir-ANF cross-reacted with rainbow trout ANF. This study was also the first to quantify the amount of ir-ANF present in the trout myocardium using immunofluorescence and immunogold labeling. Based on the gold labeling, approximately seven times more ir-ANF was found in the rainbow trout atrium than in the ventricle. This finding parallels the findings from the TEM micrographs, such that more secretory vesicles were found in the atrium than in the ventricle. As such the present study also is consistent with the general findings for mammalian and other fish species that the atrium contains more ANF than ventricle.

### *Chapter 3*

## **THE RELEASE OF ATRIAL NATRIURETIC FACTOR FROM AN *IN SITU* PERFUSED RAINBOW TROUT HEART**

### **INTRODUCTION**

In rainbow trout, as in mammals, atrial natriuretic factor (ANF) is a cardiac hormone with natriuretic and diuretic activity (Duff and Olson, 1986; Olson and Meisheri, 1989; Olson *et al.*, 1991; Olson and Duff, 1992a). In mammals, atrial distension results in the release of ANF (Dietz, 1984; Lang *et al.*, 1985; Ledsome *et al.*, 1985; Anderson *et al.*, 1986; Edwards *et al.*, 1988; Agnoletti *et al.*, 1989). Generally, very little is known regarding ANF release in non-mammalian animals, although ANF release has been shown to increase when turtles dive (Baeyens *et al.*, 1989). To date, the mechanism of ANF release in a fish heart is unknown. Therefore, the objective of this chapter was to determine and describe factors that induce ANF release from a rainbow trout heart.

The structure and function of a mammalian heart differs from a fish heart. In mammals, the heart consists of two atria and two ventricles which serve to pump blood through the lungs and then to the systemic circulation. The deoxygenated venous blood returns to the right side of the heart and then passes to the lungs. From the lungs the freshly oxygenated blood returns to the left side of the heart and is then recirculated. This permits maintenance of a high blood pressure to the systemic circulation while maintaining a relatively lower blood pressure to the lungs. In contrast, a fish heart is composed of a single atrium that receives and delivers deoxygenated venous blood to a single ventricle and then to the gills. Thus, in fish, the blood makes a single circuit during

which it is pumped through the respiratory or capillary circuit, oxygenated, distributed through the systemic capillary circuit and then returned to the heart.

In mammals, cardiac output is primarily regulated by regulation of heart rate, whereas in fish; cardiac output is regulated by regulation of stroke volume (Farrell, 1991). In both mammals and fish, the stroke volume is the volume of blood that is pumped in one beat of the heart and cardiac output is the product of stroke volume and heart rate. In mammals, ventricular filling is largely determined by the venous filling pressure, but it is also influenced by the time available for ventricular filling and the pressures generated during atrial and ventricular contraction (Eckert *et al.*, 1988). In addition, atrial filling pressure in mammals can vary considerably even when the stroke volume remains relatively constant (for review see Lakatta, 1986; Berne and Levy, 1992). In contrast, the teleost heart is encased in a semi-rigid pericardium resulting in *vis-a-fronte* filling (Farrell *et al.*, 1988). In this type of filling, a ventricular contraction produces a subambient pressure in the pericardial cavity that aids in atrial filling and venous return (Farrell, 1990). In comparison to mammalian hearts, ventricular filling in a teleost heart is fully dependent upon atrial filling which in turn is extremely sensitive to venous filling pressure (Farrell, 1991). Even though the mechanism of ventricular filling differs between mammalian and teleost hearts, I predict that the volume-loading of a teleost heart will induce ANF secretion, at least in part, because the changes in stroke volume resulting from changes in venous filling pressure are greater in the teleost heart than in the mammalian heart. I further predict that ANF release will be a graded response, such that step increases in filling pressure will result in proportional increases in ANF release.

Fish can face osmotic challenges very much different to that in mammals. Terrestrial mammals are in constant danger of dehydration, whereas fish face dehydration in marine environments and overhydration in freshwater. Marine fish are in hyperosmotic environments and are in danger of hypovolemia (i.e. low blood volume). In contrast, fish

residing in freshwater have serum sodium and chloride contents above that of their environment and so they are in danger of hypervolemia (i.e. high blood volume).

In aquatic animals, the internal cellular composition is greatly dependent upon the ionic composition of the extracellular environment (Kirschner, 1991), so maintenance of a controlled extracellular milieu is critical to cellular function. Osmoregulation involves the exchange of salts and water between the extracellular environment and the external environment. Some fish can osmoregulate and live in both freshwater and marine environments. Members of the *Oncorhynchus* genus, for example, reproduce and develop as juveniles in freshwater environments, but spend the majority of their life span growing in the ocean. Euryhaline fish, such as adult rainbow trout, can inhabit freshwater and seawater environments while maintaining relatively constant blood volumes (Olson, 1992). This homeostatic ability is also reflected in their blood pressures. The ventral aortic blood pressures from freshwater rainbow trout are reported to be only slightly higher (5.33 kPa; Stevens and Randall, 1967) than those reported from seawater-acclimated rainbow trout (4.55 kPa; Thorarensen, 1994).

Fish, therefore, are faced with greater osmotic challenges, and hence greater blood volume challenges, than most mammals. Furthermore, depending on the efficiency of their regulatory mechanisms and on the environment, the nature of this challenge shows substantial qualitative differences. If my primary prediction that atrial stretch, which is associated with hypervolemia, is the main stimulus for atrial release of ANF in fish, then I further predict that there could be differences in the regulation of cardiac ANF secretion between freshwater-acclimated and seawater-acclimated rainbow trout.

The primary objective of this chapter was to investigate factors that induce ANF release from a rainbow trout heart. To accomplish this objective, I first had to determine that trout ANF antigens could be detected using a human antibody. To test the species specificity, I performed a Western blot using preparations of trout perfusate, plasma and

myocardial extracts tested against a human ANF-(99-126) standard. In addition, I serially diluted these same samples and assayed them using a radioimmunoassay specific for human ANF-(99-126). The resulting curves from these serial dilutions were then compared with that given by the standard. Once the species specificity was established, I tested my above predictions by collecting samples from an *in situ* perfused trout heart (Farrell *et al.*, 1986; 1988) and assaying them using the radioimmunoassay.

The *in situ* preparation was used for several reasons. First, in this preparation, the integrity of the pericardium was kept intact which allowed the fish heart to perform at work levels similar to those observed *in vivo*. This means that the results I obtain will have relevance to intact fish *in vivo*. The *in situ* preparation was also excellent for controlling the filling pressure to the trout heart. This was essential as my first prediction asserts that volume-loading a teleost heart will induce ANF secretion. In addition, this *in situ* preparation offered the advantage of studying the effect of mechanical stretch on ANF release without the interferences from other circulating hormones or central neuronal pathways.

Therefore, this chapter asked four primary questions:

- (1) Will human antibodies cross-react with rainbow trout antigens?
- (2) Which stimulus, increased venous filling pressure (i.e. volume-loaded) or increased diastolic output pressure (i.e. pressure-loaded) to the perfused trout heart, produces ANF secretion?
- (3) Is ANF release a graded or an "all-or-none" response?
- (4) Will ANF secretion differ between freshwater and seawater trout?

Additional questions included:

- (5) How much ANF is present in trout plasma and myocardial tissues?
- (6) What is the time course of ANF release?
- (7) How much ANF will be secreted at resting and maximum cardiac outputs and how will these rates of release affect plasma ANF concentrations?

## MATERIALS AND METHODS

### Animals

Rainbow trout ( $N = 35$ ) weighing between 300 to 600 grams were held in freshwater as described in Chapter 2. An additional group of 12 fish were transferred to an indoor 500 L fiberglass tank supplied with recirculating seawater (13 °C; salinity 28 ppt, pH 7.9). These trout were allowed to acclimate to the seawater for a period of at least 6 weeks and showed no signs of distress during this time period. The acclimation of rainbow trout to seawater has been shown to occur in approximately two weeks, such that plasma  $[Na^+]$  and  $[Cl^-]$  decline after 7 days and then stabilize to new levels (Johnston and Cheverie, 1985). Both the fresh and salt water trout were fed a maintenance diet (1% body weight) of commercial trout chow (Silver Cup Fish Food, Murray, UT) on a daily basis. At the time of sampling, both the freshwater and the salt water trout were vigorously feeding and appeared to be in good health.

### Radioimmunoassay

The measurement of ir-ANF content in both the collected perfusate samples and the heart extracts was accomplished using a commercially available radioimmunoassay (RIA) kit specific for human or canine  $\alpha$ -ANF-99-126 (Peninsula Laboratories, Belmont, CA). The human ANF antibody was selected for the RIA because other investigators failed to show cross-reactivity using rat ANF antibodies with trout antigens (C. Redekopp and J.R. Ledsome, pers. commun.), and more importantly, in Chapter 2 the human ANF antibody successfully immunolabeled the trout myocardium.

Triplicate 100  $\mu$ L volumes of either standard or sample were mixed with 100  $\mu$ L of primary antibody (rabbit anti-peptide serum) and incubated for 24 hours at 4 °C. After the first incubation, 100  $\mu$ L of  $^{125}I$ -ANF was added to the tubes and the mixture allowed

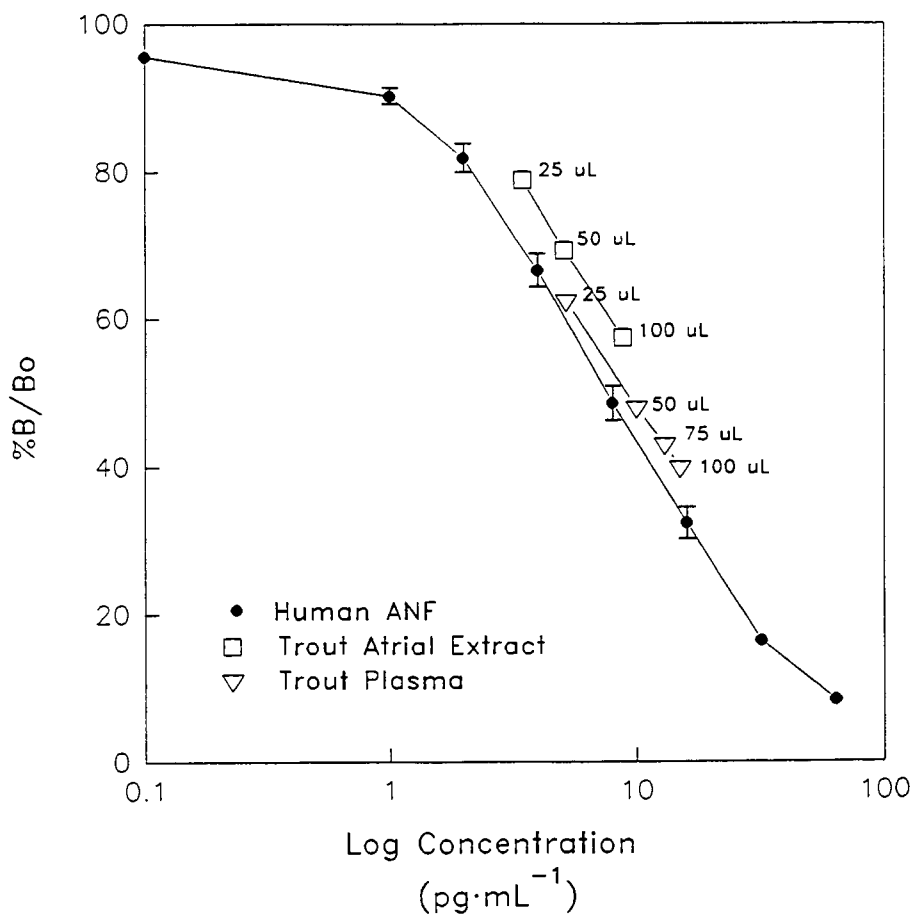
to incubate again for 24 hours at 4 °C. Next, 100 µL of goat anti-rabbit anti-immunoglobulin-G serum was added to each sample before a 100 µL aliquot of normal rabbit serum. The samples were then allowed to incubate for 90 minutes before the addition of 500 µL of RIA buffer (0.1 M sodium phosphate buffer, pH 7.4). Each sample was centrifuged (4 °C at 1,700 g for 20 minutes) and the supernatant carefully aspirated off before being counted in a gamma counter (Beckman, Mississauga, Ontario).

A pooled trout plasma sample served as the between-assay standard. The RIA between-assay coefficient of variation (CV = standard deviation/mean X 100%) was 12.33%. The minimum amount of ANF that could be detected was 0.1 pg·tube<sup>-1</sup> or 1 pg·mL<sup>-1</sup>. Figure 3.1 shows the RIA standard curve (mean ± S.D.; *N* = 13) and dilution curves for the trout plasma and atrial extracts. Analysis of the standard curves showed a binding of <sup>125</sup>I-ANF to antibodies of 28.0 ± 1.2 % and a nonspecific binding of 4.7 ± 0.3 %. The RIA ED<sub>50</sub> (i.e., the standard dose at which the percentage bound divided by the percentage bound in the zero standard is equal to 0.5) was 10.7 ± 0.7 pg·mL<sup>-1</sup>. Both the plasma and atrial extract dilution curves were parallel to the human ANF standard curve. In an analytical recovery experiment with 2 pg of human ANF standard added to a reconstituted volume-loaded trout perfusate sample, recovery was 85.3 ± 4.5 % (*N* = 3).

### **Biochemical Analysis**

Trout atrial and ventricular extracts, plus circulated perfusate samples, were subjected to a Western blot to confirm that the human α-ANF-(99-126) serum cross-reacted with the trout ANF. Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed on slab gels by the method of Laemmli (1970). Gels were prepared from a 30% acrylamide and methylene bis-acrylamide solution and polymerized using a combination of ammonium persulphate (APS) and tetramethylene diamine (TEMED) as catalysts. Eighteen percent separating gels and 4.0% stacking gels

**Figure 3.1.** A standard curve derived from 13 consecutive assays (mean  $\pm$  S.E.M.) for human  $\alpha$ -ANF-(99-126) with dilution curves for rainbow trout plasma ( $\nabla$ ) and atrial extract ( $\square$ ). Binding is given as the proportion of the tracer bound (B) expressed as a percentage of that in the zero standard ( $B_0$ ).





were utilized for all runs. (Gel conditions and all reagents used are presented in Table 3.1).

To concentrate the protein from the tissue extracts and perfusate samples, 100  $\mu\text{L}$  of each sample was mixed with 400  $\mu\text{L}$  of cold acetone. The mixtures were quickly vortexed and placed on ice for 10 minutes prior to being centrifuged (3,000 g for 10 minutes). Forty microlitres were taken and assayed for protein content (Bradford, 1976). Next, the prepared samples were resuspended 1:4 v/v with the loading buffer and then dissolved by placing them in a 95 °C water bath for 4 minutes. Twenty microlitres of each prepared sample, plus a low weight molecular standard (Biorad Laboratories, Hercules, CA), were loaded onto the gels and run at a constant current of 13 mA for one hour and then 15 mA for approximately 4 hours (Protein II slab gel, Model 1000/500 power supply, Biorad Laboratories, Hercules, CA).

After electrophoresis, the gels were allowed to equilibrate in a transfer buffer for at least 30 minutes. The separated proteins were then electrophoretically transferred, or electroblotted, out of the gels onto nitrocellulose (NC) membranes. First, each gel was carefully placed onto a transfer buffer soaked NC membrane and then subjected to a constant current of 454 mA for 30 minutes (Trans-Blot SD, Semi-dry Transfer cell, Biorad Laboratories). The gels were then removed and stained for 8 hours in a 0.1% Coomassie Blue R-250 fixative. Destaining was accomplished in approximately 6 hours using a 25% ethanol and 8.0% acetic acid solution. Afterward, the gels were stored in a 5.0% methanol and 1.0% acetic acid solution.

Concurrently, the blotted NC membranes were placed into a blocking solution for two hours. Next, the NC membranes were rinsed 3 times (each for 5 minutes) in TBS-T and then immersed in human anti-alpha ANF-99-126 serum (Peninsula Laboratories, Belmont, CA) for 24 hours. Once again, the NC membranes were rinsed in TBS-T for 5

**Table 3.1 Gradient SDS-PAGE Reagents and Gel Conditions****Stacking Gel**

1.3 mL 30% methylene bis-acrylamide  
6.25 mL 1.5 M Tris[hydroxymethyl]-aminomethane (pH = 6.8)  
0.25 mL 10% SDS  
3.4 mL distilled water  
125  $\mu$ L 10% APS  
12.5  $\mu$ L TEMED

**Separating Gel**

15 mL 30% methylene bis-acrylamide  
2.5 mL 0.5 M Tris[hydroxymethyl]-aminomethane (pH = 8.8)  
100  $\mu$ L 10% SDS  
6.1 mL distilled water  
50  $\mu$ L 10% APS  
10  $\mu$ L TEMED

**Running Buffer**

15 grams Tris[hydroxymethyl]-aminomethane (pH = 8.3)  
72 grams glycine  
5 grams SDS  
1000 mL distilled water

**Loading Buffer**

4.0 mL distilled water  
1.0 mL 0.5 M Tris[hydroxymethyl]-aminomethane (pH = 6.8)  
0.8 mL glycerol  
1.6 mL 10% SDS  
0.4 mL 2 $\beta$ -mercaptoethanol  
0.2 mL 0.05% bromophenol blue

**Transfer Buffer**

5.8 grams Tris[hydroxymethyl]-aminomethane  
2.92 grams glycine  
200 mL methanol  
3.8 mL 10% SDS

**Table 3.2 Nitrocellulose Membrane Conditions and Developers****Blocking Solution**

10 mL TBS 10X  
90 mL distilled water  
10 grams carnation skim milk

**TBS (10X)**

12.11 grams Tris[hydroxymethyl]-aminomethane (pH 8.0)  
87.66 grams NaCl  
1000 mL distilled water

**TBS-T (10X)**

12.11 grams Tris[hydroxymethyl]-aminomethane (pH 8.0)  
87.66 grams NaCl  
5.0 mL Tween 20  
1000 mL distilled water

**Carbonate Buffer**

3.36 grams NaHCO<sub>3</sub>  
0.813 grams MgCl<sub>2</sub>  
pH to 9.8 with NaOH  
400 mL distilled water

**BCIP Stock Solution**

15 mg BCIP  
1 mL distilled water

**NBT Stock Solution**

30 mg NBT  
1 mL distilled water

minutes. After the three rinses, the NC membranes were incubated in a TBS-T and an anti-immunoglobulin-G mixture for 3 hours. In the final steps, the NC filter papers were subjected to washes in TBS-T (3 X 5 minutes), TBS (1 X 5 minutes) and a carbonate buffer (1 X 10 minutes) and then developed in 5-bromo-4-chloro-3 indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) for 2 hours. After two 5 minute washes in distilled water, the NC membranes were allowed to air dry. (NC membrane conditions and developers used are presented in Table 3.2).

### ***In Situ* Heart Preparation**

The perfused trout heart preparation utilized in this study was similar to the method described by Farrell *et al.* (1986, 1988). Once fully anesthetized (MS222, 1:5000 w/v), the trout were quickly measured, weighed and then transferred to an operating table. During the surgery, the gills were irrigated with a chilled aerated MS222 solution (1:10,000 w/v). To prevent the formation of blood clots, the fish were injected with 75 USP of sodium heparin *via* the caudal vessels. A 1 mL sample of plasma was then taken from the dorsal aorta and stored on ice for subsequent analysis of ir-ANF. Next, an incision was made from the cloaca to a point just posterior to the pericardium. The gut was removed after tying off the vasculature to gain a better access to the hepatic vessels. The hepatic vessels were identified and all but one tied off with silk ligatures. A small incision was made in the remaining hepatic vein. An input cannula was inserted into the sinus venosus *via* the incised hepatic vein and then the incision was closed around the cannula with a silk suture. The saline perfusion was started immediately after the insertion of the input cannula and maintained during the entire experiment. As the saline level in the Marriot bottle relative to the fish determined the filling pressure of the saline into the heart, this was carefully monitored. This was important because the primary focus of the study was to determine if increases in filling pressure induced ANF release,

thus, it was critical to not stress the heart during the surgery. Once the gills were removed, the severed ventral aorta was exposed. The output cannula was then inserted into the bulbus arteriosus *via* the ventral aorta. In the final steps of the surgery, the ductus Cuvier were ligated to prevent backflow and to insure that the heart received input only from the cannula. Overall, the surgery took between 20 to 30 minutes to complete (Fig. 3.2).

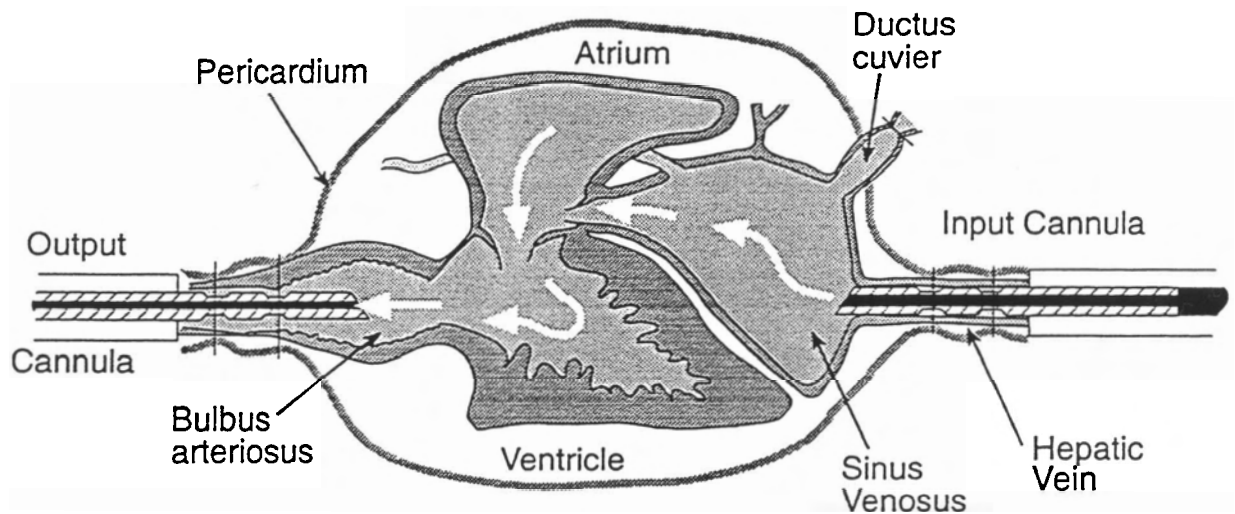
Immediately following the surgery, the cannulated fish was carefully transferred to a saline-filled bath maintained at 13 °C (Fig. 3.3). During the experiment, the filling pressure was varied to attain a cardiac output equaling that in resting rainbow trout *in vivo* (17 mL·min<sup>-1</sup>·kg<sup>-1</sup> body mass; Kiceniuk and Jones, 1977). Similarly, the diastolic output pressure was varied by adjusting the height of the output pressure head to 4.91 kPa (Graham and Farrell, 1989), again equaling the resting value for rainbow trout *in vivo*. As can be seen in Figures 3.2 and 3.3, the perfusate made a single pass through the interior of the heart chambers before collection at the end of the output tubing.

The filling ( $P_i$ ) and afterload ( $P_o$ ) pressures were referenced to the *in situ* bath saline level and measured using Micron pressure transducers (Narco Life Sciences, Houston, Texas). The cardiac output was continuously measured with an electromagnetic flow probe (Zepeda Instruments, Seattle, Washington). Both the pressure and flow signals were amplified and monitored on a strip chart recorder (Gould 2400, Cleveland, Ohio). In addition, the pressure and flow signals were converted to digital signals and instantaneously analyzed by a computer data acquisition program (Labtec Notebook Version 5.0, Laboratory Technologies Corporation, Wilmington, MA).

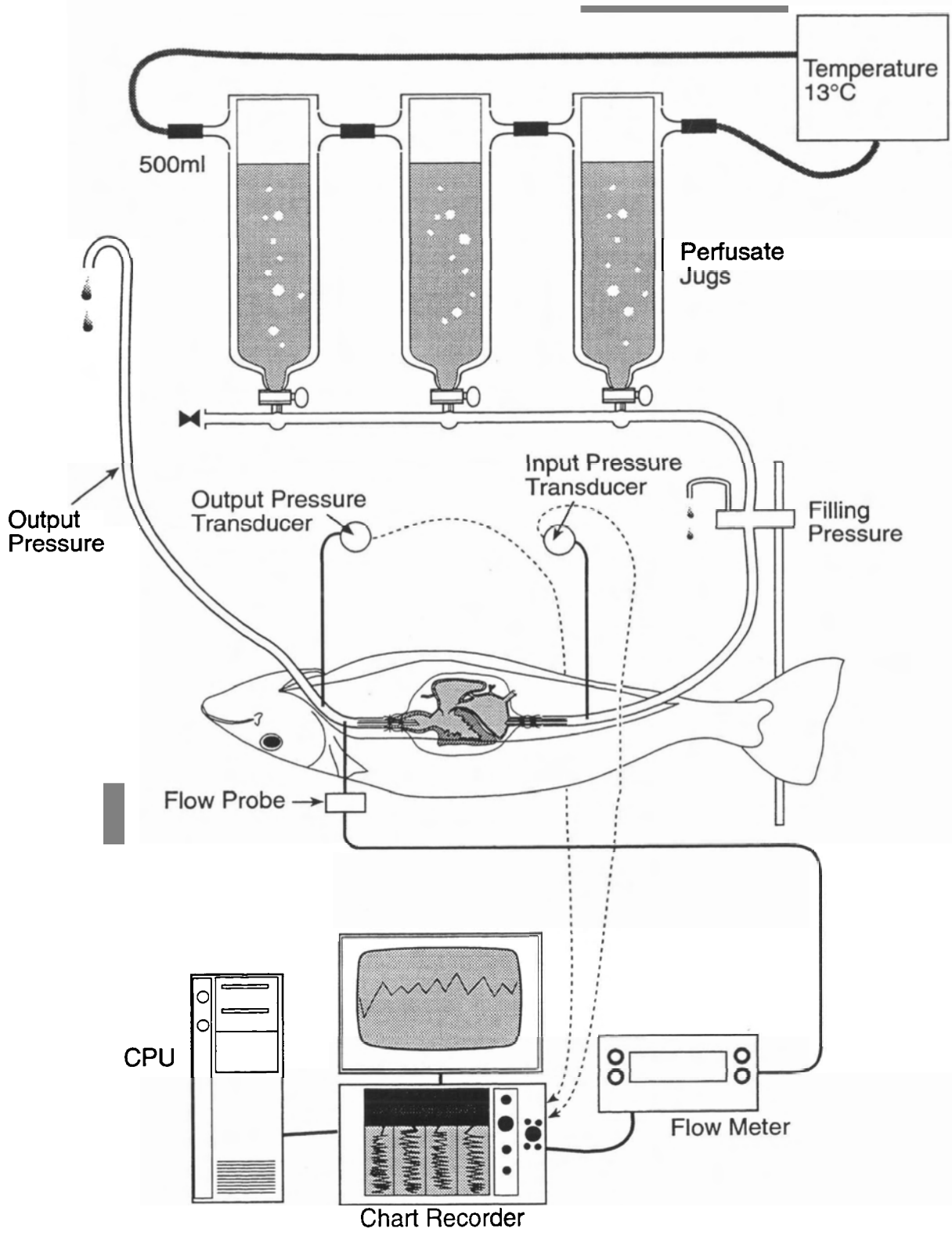
### **Perfusate**

The perfusion saline for the freshwater trout was composed of the following (mM): NaCl, 124.1; KCl, 3.1; CaCl<sub>2</sub>, 2.5; MgSO<sub>4</sub>, 0.9; dextrose, 0.5 (Keen *et al.*, 1993).

**Figure 3.2.** Schematic diagram showing a cannulated rainbow trout heart. The input cannula is inserted into the entrance of the sinus venosus *via* a hepatic vein. The output cannula is inserted into the bulbus arteriosus. Note that the integrity of the pericardium is maintained. Also notice that all entrances and exits to the heart are sealed. Arrows indicate the flow of aerated perfusate through the cannulated trout heart.



**Figure 3.3.** Schematic diagram showing the *in situ* perfused heart preparation. The cannulated animal is placed into an *in situ* bath maintained at 13 °C. The flow of perfusate into the heart is controlled by either raising or lowering the filling pressure head. The arterial resistance is simulated by either raising or lowering the output pressure head. Two pressure transducers and a flow probe are interfaced with a computer data acquisition system and a chart recorder.





The seawater perfusion saline consisted of the following (mM): NaCl, 150; KCl, 5; CaCl<sub>2</sub>, 2.3; MgSO<sub>4</sub>, 2.0; dextrose, 0.5 (modified from Farrell *et al.*, 1985). Both perfusate recipes were buffered with 10 mM N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES) with a pH of 7.85 at 13 °C. The osmolality of the freshwater and seawater perfusion solutions were 281 mOsm·kg<sup>-1</sup> and 328 mOsm·kg<sup>-1</sup>, respectively. Adrenaline (5 nM; bitartrate salt, Sigma Chemical, St. Louis, MO) was added at a constant rate (50 μ L·min<sup>-1</sup>) to the perfusate solution to provide a resting tonus to heart rate and reduced deterioration of the preparation (Farrell and Milligan, 1986).

During the experiment, the circulated perfusate was collected from the output tubing into one minute fractions and then immersed into an ethanol and dry ice mixture. Once completely frozen, the samples were subjected to a freeze drying process for approximately 24 hours. The samples were stored at room temperature in a powdered form until reconstituted with 1 mL of the radioimmunoassay buffer and assayed for ir-ANF.

### **Experimental Protocols**

Rainbow trout hearts were used *in situ* to investigate factors that induce ANF release. Three groups of fish were used in two experimental protocols which are presented in sections (A) and (B). In section (A), a group of freshwater trout was used to determine if volume-loading or pressure-loading a perfused heart resulted in ANF release. In section (B), freshwater-acclimated trout were used to determine if ANF release was a graded or "all-or-none" response. Seawater-acclimated trout were also subjected to the identical protocol as the freshwater-acclimated trout and a comparison was made between the two groups. A summary of the two experimental protocols is given below.

<b>Section A</b>	<b>Freshwater-Acclimated Trout</b> Control (CP) $N = 4$ Volume-Loaded(VL) $N = 7$ Pressure-Loaded (PL) $N = 7$
<b>Section B</b>	<b>Freshwater-Acclimated Trout</b> Graded or "All-or-None" ANF Release $N = 6$  <i>versus</i>  <b>Seawater-Acclimated Trout</b> Graded or "All-or-None" ANF Release $N = 6$

Control conditions for all preparations were a cardiac output of  $17 \text{ mL} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$  body mass and an output pressure at 4.91 kPa (Graham and Farrell, 1989; Kiceniuk and Jones, 1977). Then for 30 minutes the perfusate was collected in one minute fractions every 10 minutes and quickly frozen in an ethanol and dry ice mixture. These control conditions were followed for all preparations to allow the perfused *in situ* hearts to recover from the effects of the surgery.

#### **(A) Effect of Volume-Loading and Pressure-Loading on ANF Release**

The first objective was to establish if either an increase in atrial stretch/pressure or afterload would increase ANF release in freshwater trout hearts. In these experiments, the total duration of each *in situ* preparation was 60 minutes. Control fish (CP;  $N = 4$ ) remained at the control level for the entire time period while the perfusate was collected in one minute fractions every 10 minutes. It was predicted that the CP group would show no change in ANF release after the initial 30 minutes equilibration. The experimental groups, volume-loaded (VL;  $N = 7$ ) and pressure-loaded (PL;  $N = 7$ ), remained at a control level for 30 minutes before the experimental changes were introduced.

With the VL group, the filling pressure or input pressure ( $P_i$ ) was increased until the stroke volume and cardiac output did not increase further (Fig. 3.4). Note that heart rate remains essentially unchanged with this change in stroke volume and cardiac output (Farrell, 1984). At this point the heart was considered maximally volume-loaded (i.e., at the maximum stroke volume and cardiac output), such that it was at the peak of the Frank-Starling curve (Bennion, 1968; Farrell *et al.*, 1986). To determine if ANF release diminished after a certain time period, one VL perfused trout heart was set and maintained to a maximum stroke volume and samples collected every 30 minutes for two and half hours. It was predicted that the VL group would show an increase in ANF release and that this release would be sustained with a constant volume-loading stimulus.

In the PL group, the experimental change involved an increase in diastolic afterload or output pressure ( $P_o$ ), such that  $P_o$  was raised to a maximum value between 7.16 to 7.85 kPa while the filling pressure remained unchanged (Fig. 3.5). As the ventricle contained only 8% of the total ANF store, it was predicted that the PL ANF release would be similar to that in the CP group.

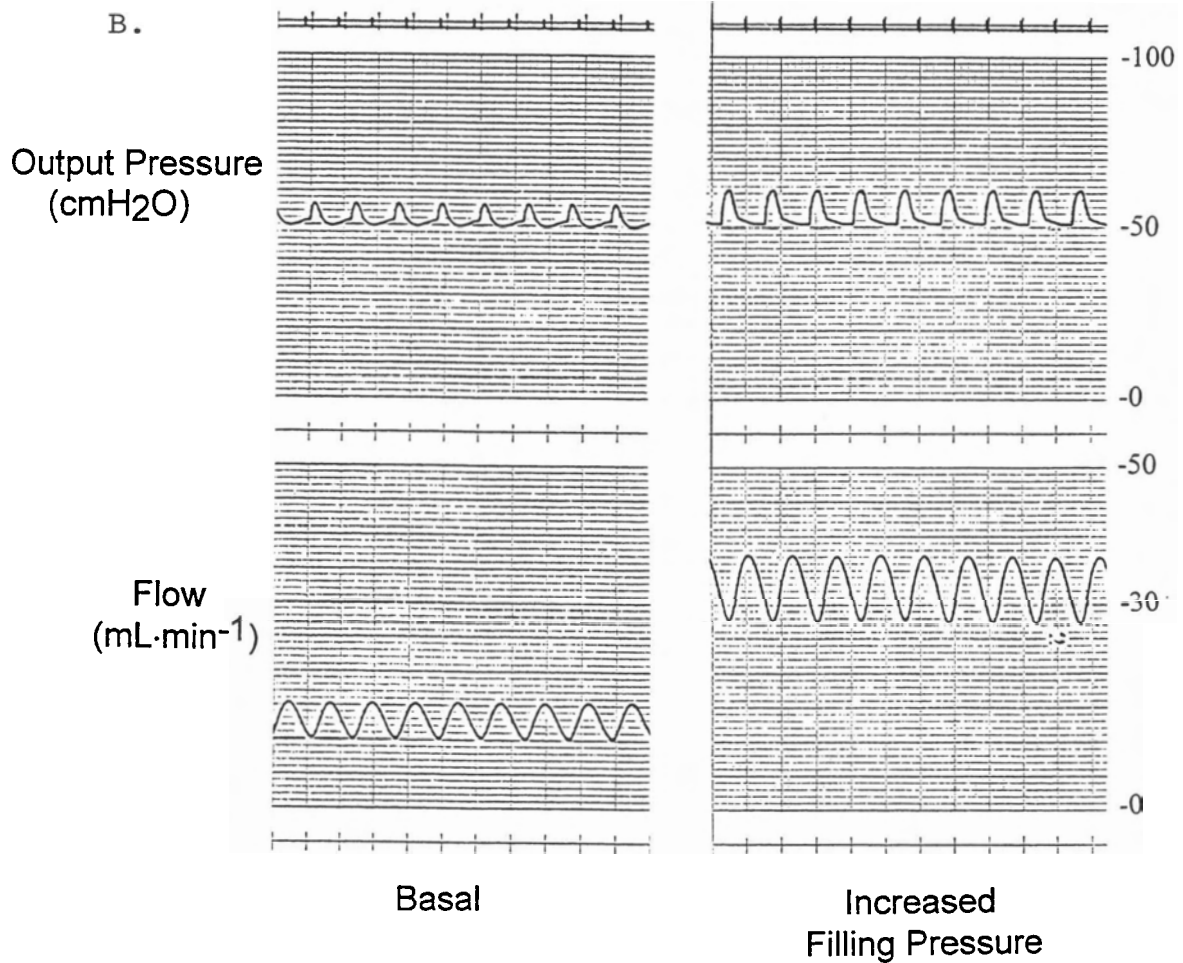
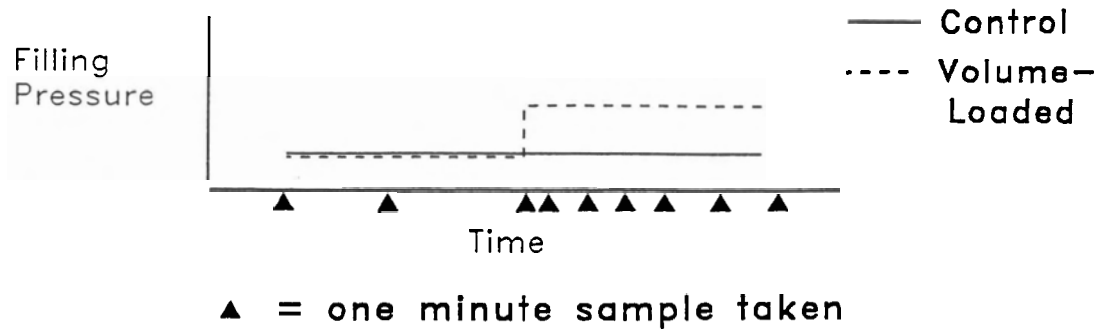
For both the VL and PL conditions, the heart perfusate was collected in one minute fractions for 30 minutes and quickly frozen. Unlike the VL group, ANF secretion in the PL group was expected to be similar to that in the CP group. In addition, at the completion of each experiment the heart was removed and assayed for ir-ANF.

### **(B) Is ANF Release Graded or "All-or-None" in a Perfused Trout Heart?**

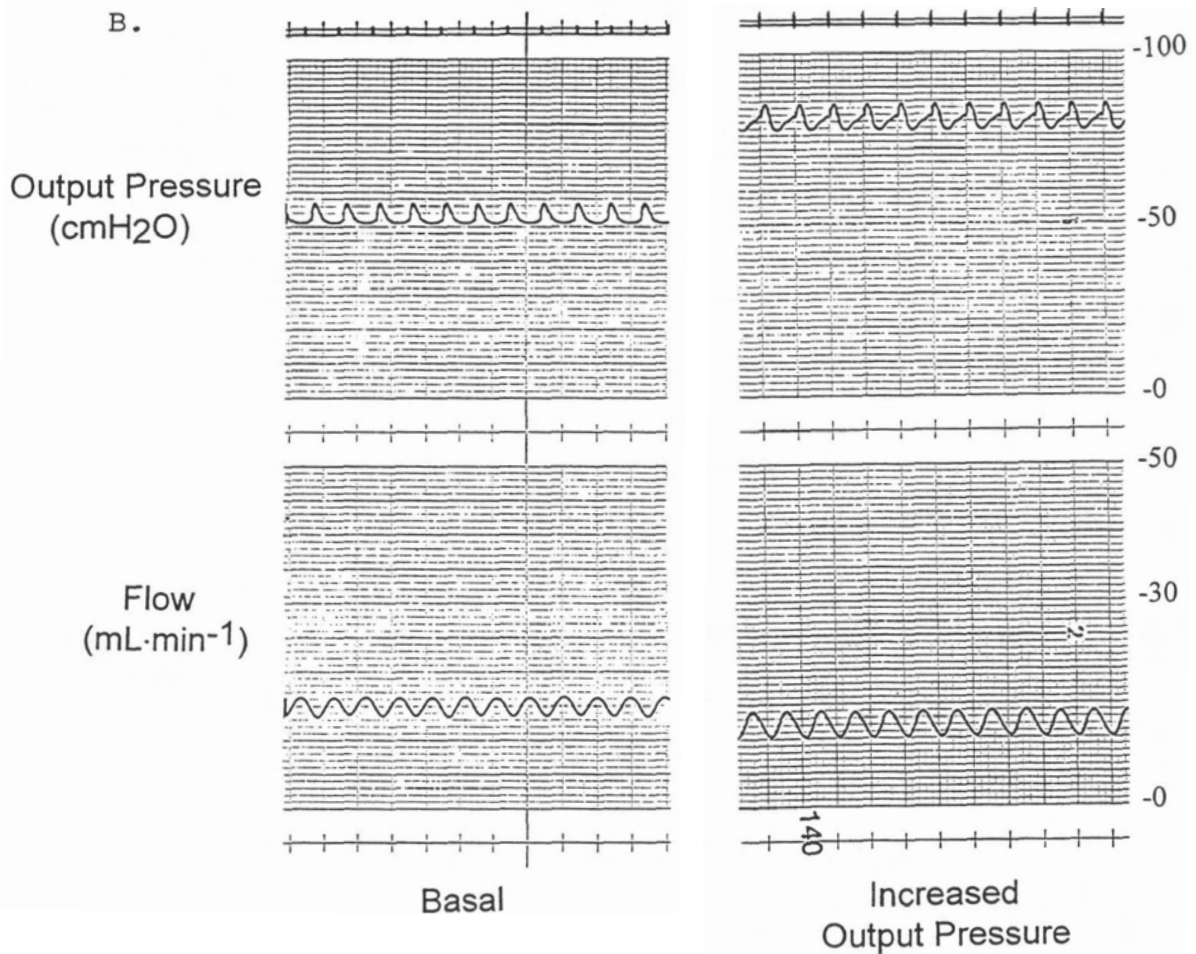
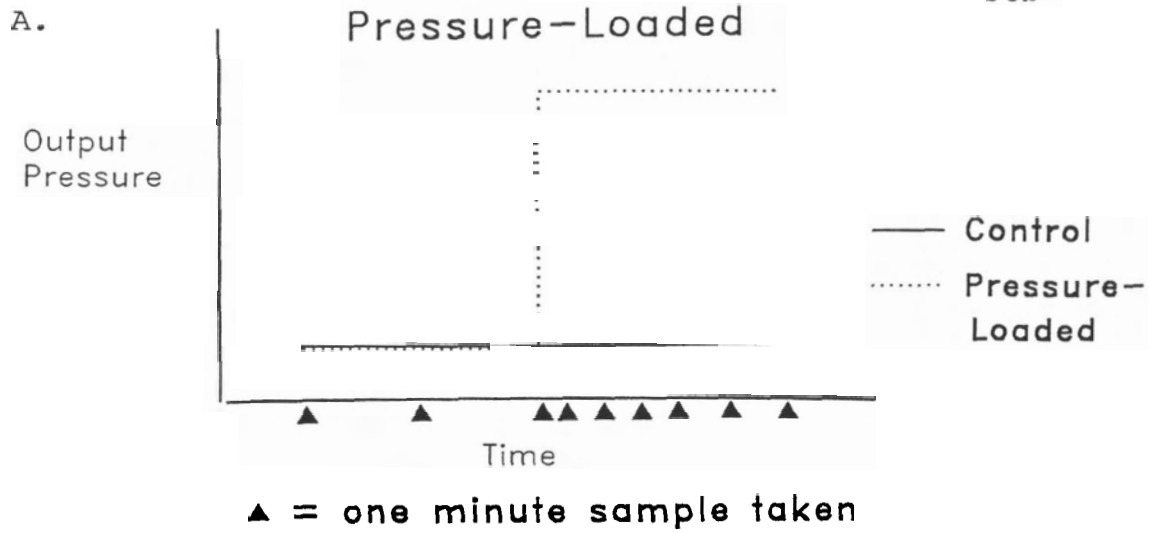
The second series of perfused heart experiments investigated if ANF release was a graded or an "all-or-none" response. Also, freshwater-acclimated rainbow trout hearts ( $N = 6$ ) were compared with seawater-acclimated rainbow trout hearts ( $N = 6$ ). As with the previous protocol (Section A), for each fish the cardiac output ( $\dot{Q}$ ) and  $P_o$  were set at a

**Figure 3.4.** A schematic diagram showing the experimental protocol for the volume-loaded perfused trout heart (A) and a corresponding original tracing (B). In the volume-loaded experiments, the filling pressure was set to the same level as in the control (approximately  $17 \text{ mL}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$  body mass). Then, at time zero, the filling pressure was increased to a stroke volume maximum. One minute samples were taken as indicated by the arrow heads. As shown in (B), both the flow (lower trace;  $\text{mL}\cdot\text{min}^{-1}$ ) and the output pressure (upper trace;  $\text{cmH}_2\text{O}$ ) increased with an increase in filling pressure. (Note that  $1 \text{ cmH}_2\text{O} = 0.0981 \text{ kPa}$ .)

## A. Volume-Loaded



**Figure 3.5.** A schematic diagram showing the experimental protocol for the pressure-loaded perfused trout heart (A) and a corresponding original tracing (B). In the pressure-loaded experiments, the output pressure was set to the same level as in the control (approximately 4.91 kPa). Then, at time zero, the output pressure was increased to a maximum value between 7.16 and 7.85 kPa. One minute samples were taken as indicated by the arrow heads. As shown in (B), the flow (lower trace; mL·min<sup>-1</sup>) slightly decreased while the output pressure (upper trace; cmH<sub>2</sub>O) increased. (Note that 1 cmH<sub>2</sub>O = 0.0981 kPa.)



control level for 30 minutes and the preparation was allowed to recover from the effects of the surgery. The experimental protocol for the freshwater and seawater groups was identical.

The  $P_i$  that would generate a maximum stroke volume and cardiac output was chosen based upon data from the previous VL experiments (section A). This maximum  $P_i$  value represented the upper stroke volume and cardiac output limit ( $\dot{Q}_{\max}$ ). In the range between the control level and  $\dot{Q}_{\max}$ , two intermediate filling pressures (i.e. 30%  $\dot{Q}_{\max}$  and 60%  $\dot{Q}_{\max}$ ) were also examined. One elevated input pressure was randomly selected and maintained for 10 minutes while one minute fractions of heart perfusate were collected and then individually and immediately frozen. The two other elevated  $P_i$  conditions were subsequently tested and perfusate samples similarly collected. It was predicted that step increases in filling pressure would result in similar increases in ANF release. In addition, statistical comparisons were made between ANF release from the freshwater and seawater perfused hearts, as it was predicted that there would be differences in ANF release between the freshwater and seawater perfused hearts.

### **Myocardial and Plasma Extracts**

The hearts from the VL ( $N = 7$ ) and PL ( $N = 5$ ) groups were removed and assayed for ir-ANF after the *in situ* experiments to determine the ANF content in the trout myocardium at the conclusion of the various experimental protocols. Comparisons were made between the VL and PL groups and three other heart samples. The first comparative samples were the extract control hearts (CH;  $N = 4$ ). The CH group was used to determine the ANF content in trout myocardial tissues before the *in situ* surgery. In these samples, trout were first anesthetized, weighed and then their hearts removed and placed into a saline-filled petri dish. The atrial and ventricular chambers were quickly dissected apart and blotted dry before being weighed. Cardiac tissues in a fourth group, SH ( $N = 7$ ),



were removed during the completion of the surgery. The SH group was used to determine if there was a change in myocardial ANF content after the surgery. A fifth group, CP ( $N = 6$ ), was used to determine the ANF content in the myocardial tissues after one hour at a control cardiac output in the *in situ* heart preparation. It was predicted that there would be differences in myocardial ir-ANF content between the VL and PL experimental groups. In addition, it was also predicted that there would be differences in myocardial ir-ANF content between the experimental groups and the CH, SH and CP groups.

The trout heart extracts were prepared in a similar manner as mammalian cardiac tissues (Gutkowska *et al.*, 1984; Wilson *et al.*, 1986). First, the cardiac tissues were homogenized with 1 mL cold 0.1 M acetic acid. The homogenized tissues were centrifuged (4 °C) at 17,000 g for 20 minutes. The resulting supernatants were drawn off and allowed to freeze for at least one hour. After thawing the samples, they were recentrifuged (4 °C) at 17,000 g for 20 minutes and then assayed for ir-ANF.

Plasma samples were obtained from the freshwater-acclimated and seawater-acclimated trout (Section B) immediately before the *in situ* surgery ( $N = 4$ ). These samples were taken from the caudal vein of each animal while under anesthetic and were individually treated to determine if there were any differences in ir-ANF plasma content between the freshwater-acclimated and seawater-acclimated trout. It was predicted that there would be differences in ir-ANF plasma content between the two groups.

To prepare the samples, a 1 mL sample of blood was first collected in a chilled heparinized syringe and then transferred into a centrifuge vial containing 1 mg of ethylene-diaminetetraacetic acid (EDTA). These samples were then centrifuged (1,700 g for 3 minutes at 4 °C) and the plasma supernatant aspirated off. The plasma samples were stored at -80 °C until they were assayed for ir-ANF. Blood samples were also taken from the dorsal aorta of the freshwater trout used in Section (A). Each blood sample was

centrifuged and the plasma supernatant aspirated off and pooled. This plasma pool was then used as the between-assay standard for the RIA.

### Calculations

Cardiac output ( $\text{mL}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$  body mass) was calculated as the product of heart rate ( $\text{beats}\cdot\text{min}^{-1}$ ) and stroke volume ( $\text{mL}\cdot\text{kg}^{-1}$  body mass). The maximum cardiac output ( $\dot{Q}_{\text{max}}$ ) was determined by increasing the filling pressure until no further increases in flow were noted. At this point, the heart was at the peak of the Frank-Starling curve and maximally volume-loaded. The cardiac power output ( $\text{mW}\cdot\text{g}^{-1}$  ventricular mass) was the amount of work the heart needed to do to pump blood against the arterial resistance.

Cardiac power output was calculated as follows:

$$\text{Cardiac Power Output} = [\text{Flow} \times (\text{Po}_{\text{true}} - \text{Pi}_{\text{true}}) \times k] / \text{ventricular mass}$$

where flow was in  $\text{mL}\cdot\text{min}^{-1}$ .  $\text{Po}_{\text{true}}$  was the output pressure, measured in kPa, adjusted for output cannula resistance ( $0.0116 \text{ kPa}\cdot\text{min}\cdot\text{mL}^{-1}$ ). Similarly,  $\text{Pi}_{\text{true}}$  was the input or the filling pressure, also measured in kPa and adjusted for the input cannula resistance ( $0.0059 \text{ kPa}\cdot\text{min}\cdot\text{mL}^{-1}$ ). The constant,  $k$ , equaled  $0.0162 \text{ mW}\cdot\text{min}\cdot\text{mL}^{-1}\cdot\text{kPa}^{-1}$  and the ventricular mass was measured in grams.

As previously described in the methods, perfusate samples were collected in one minute fractions, freeze dried and then reconstituted in 1 mL of the RIA buffer. This was done to concentrate ANF in each sample so that it could be detected in the RIA.

However, the perfusate collection volume varied between samples because the absolute  $\dot{Q}$  varied between protocols and fish of different size. For example, perfusate volumes collected at  $\dot{Q}_{\text{max}}$  were often three times greater than those collected at the control  $\dot{Q}$ .

Thus, ANF content in a one minute perfusate sample was corrected for the total volume collected using the following formula:

$$\text{ANF Release} = \text{ir-ANF (pg}\cdot\text{mL}^{-1} \text{ reconstituted perfusate)} \times \dot{Q} \text{ (mL}\cdot\text{min}^{-1}\cdot\text{kg}^{-1} \text{ body mass)}$$

where the ir-ANF concentration was obtained from the RIA. The calculated ANF release ( $\text{pg min}^{-1}\cdot\text{kg}^{-1}$  body mass) was corrected for differences in heart mass (in grams of wet weight) between the experimental animals to give an ANF release rate in  $\text{pg}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  wet heart weight.

### **Statistical Procedures**

A repeated-measures three-way analysis of variance (ANOVA) was used to compare the mean values for ANF release (Winer *et al.*, 1991). The significant differences were at the 95% confidence level. The test trials were run from a double Latin square design such that each treatment was preceded by every other treatment. This design controlled for the probability of a cross-over effect (Jones and Kenward, 1989). Thereafter, the mean values were subjected to an ANOVA. All cardiovascular mean values were subjected to multiple comparisons using a single factor ANOVA with the Student-Newman-Keuls method. In addition, statistical comparisons of morphometric mean values between the freshwater and seawater groups were carried out using a Student's *t*-test. Significant differences between the mean values were tested at the 95% confidence level.

## RESULTS

### Morphometrics

The morphometric comparisons between the control and treatment groups for sections (A) and (B) are presented in Table 3.3. For the rainbow trout sampled in Section (A), there were significant differences in the mean values between the control and treatment groups for fork length ( $p = 0.001$ ), body mass ( $p = 0.001$ ) and ventricular mass ( $p = 0.008$ ), but no significant differences were noted when either the atrial ( $p = 0.417$ ) or ventricular ( $p = 0.331$ ) mass was expressed as a percentage of body mass (RAM% or RVM%). In addition, no significant differences ( $p = 0.985$ ) were found in the condition factor mean values nor were there significant differences ( $p = 0.202$ ) found in the atrial mass values between the control and treatment groups. Therefore, the differences noted between the control and treatment groups for body and ventricular mass were not enough to affect the RVM ratio.

The morphometric comparisons between the freshwater-acclimated (FW) and seawater-acclimated (SW) rainbow trout are also presented in Table 3.3 (Section B). There were significant differences in the mean values between the groups for body mass ( $p = 0.032$ ). However, there were no significant differences found between the group means for fork length ( $p = 0.350$ ), atrial mass ( $p = 0.294$ ) or ventricular mass ( $p = 0.930$ ). In addition, there were no significant differences ( $p = 0.066$ ) noted between the treatment groups for the condition factor, RVM% or RAM% ( $p = 0.795$ ).

### Western Blot

The total volume of sample loaded into each well was 20  $\mu\text{L}$ . The human  $\alpha$ -ANF-99-126 standards and trout perfusate samples contained  $1.3 \pm 0.1$   $\mu\text{g}$  protein and  $1.1 \pm 0.9$   $\mu\text{g}$  protein, respectively (mean  $\pm$  SEM;  $N = 4$ ). The atrial extracts contained  $70.0 \pm 0.6$   $\mu\text{g}$

**Table 3.3** Morphometric data of rainbow trout used in the sections (A) and (B). The condition factor was calculated as:  $[(\text{Weight})/(\text{Length})^3] \times 100$  (Carlander, 1950). Both ventricular and atrial mass compared to the animal's body mass (RVM% and RAM%) were calculated as:  $[\text{heart mass}/\text{body mass}] \times 100$ . Significant differences ( $p < 0.05$ ) in mean values between the control and treatment groups for the rainbow trout used in section (A) are indicated superscripted. All values in section (A) are mean  $\pm$  S.E.M. ( $N = 7$ ). All values in section (B) are mean  $\pm$  S.E.M. ( $N = 6$ ). Significant differences between the freshwater-acclimated (FW) and seawater-acclimated (SW) trout mean values are indicated by an asterisk (\*).

Treatment Group	Atrial Mass (mg)	Ventricular Mass (g)	Body Mass (g)	Length (cm)	Condition Factor	RAM%	RVM%
<b>Section A</b>							
Atrial Control Hearts (CH)	44.75 <sup>a</sup> $\pm 11.66$	0.328 <sup>b</sup> $\pm 0.033$	372.08 <sup>b</sup> $\pm 23.07$	32.88 <sup>b</sup> $\pm 0.56$	1.01 <sup>a</sup> $\pm 0.03$	0.011 <sup>a</sup> $\pm 0.002$	0.096 <sup>a</sup> $\pm 0.006$
Surgery Control Hearts (SH)	50.78 <sup>a</sup> $\pm 4.82$	0.345 <sup>b</sup> $\pm 0.036$	376.71 <sup>b</sup> $\pm 32.86$	33.00 <sup>b</sup> $\pm 0.65$	1.03 <sup>a</sup> $\pm 0.05$	0.014 <sup>a</sup> $\pm 0.001$	0.092 <sup>a</sup> $\pm 0.004$
Control (CP)	79.85 <sup>a</sup> $\pm 15.01$	0.593 <sup>a</sup> $\pm 0.096$	509.57 <sup>a</sup> $\pm 37.26$	36.21 <sup>a</sup> $\pm 0.95$	1.06 <sup>a</sup> $\pm 0.01$	0.017 <sup>a</sup> $\pm 0.002$	0.113 <sup>a</sup> $\pm 0.011$
Volume-Loaded (VL)	58.23 <sup>a</sup> $\pm 5.34$	0.372 <sup>b</sup> $\pm 0.028$	385.43 <sup>b</sup> $\pm 12.96$	33.29 <sup>b</sup> $\pm 0.17$	1.04 <sup>a</sup> $\pm 0.03$	0.015 <sup>a</sup> $\pm 0.001$	0.093 <sup>a</sup> $\pm 0.005$
Pressure-Loaded (PL)	68.14 <sup>a</sup> $\pm 14.29$	0.484 <sup>a</sup> $\pm 0.051$	525.86 <sup>a</sup> $\pm 19.13$	36.93 <sup>a</sup> $\pm 0.33$	1.04 <sup>a</sup> $\pm 0.02$	0.014 <sup>a</sup> $\pm 0.002$	0.092 <sup>a</sup> $\pm 0.005$
<b>Section B</b>							
FW	78.45 $\pm 9.30$	0.481 $\pm 0.024$	511.50* $\pm 10.29$	34.17 $\pm 0.15$	1.28 $\pm 0.02$	0.015 $\pm 0.002$	0.094 $\pm 0.003$
SW	67.78 $\pm 4.82$	0.485 $\pm 0.077$	449.50* $\pm 22.56$	34.00 $\pm 0.0$	1.14 $\pm 0.06$	0.015 $\pm 0.001$	0.108 $\pm 0.006$

protein while the ventricle extracts contained  $66.1 \pm 1.3 \mu\text{g}$  protein. The plasma samples contained  $1.7 \pm 0.3 \mu\text{g}$  protein.

As shown in Figure 3.6, the range of values for the low molecular weight standard was between 106 to 18.5 kDa. For the SDS-PAGE stained gel and the corresponding western blot, both the human ANF-(99-126) standard and the trout perfusate sample displayed faint banding at approximately 5 kDa (Figs. 3.3 and 3.4). Although the plasma and tissue extract samples displayed several distinct protein bands at the higher molecular weights, no bands were evident at the 5 kDa range. The Western blot revealed that the trout perfusate sample cross-reacted with the human ANF antibody, however, no similar binding was noted for either the plasma or the tissue extracts (Fig. 3.6).

#### **(A) Effect of Volume-Loading and Pressure-Loading on ANF Release**

A filling pressure of  $-0.09 \pm 0.4$  kPa resulted in a control  $\dot{Q}$  of  $15.24 \pm 0.73$   $\text{mL}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$  body mass and an ANF release of  $29.54 \pm 6.78$   $\text{pg}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  wet heart weight (Table 3.4). After 60 minutes at control  $\dot{Q}$ , ANF release was  $30.68 \pm 4.76$   $\text{pg}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  wet heart weight.

In VL hearts, an increase in filling pressure from  $-0.08 \pm 0.02$  to  $0.36 \pm 0.01$  kPa resulted in an immediate and a highly significant ( $p < 0.001$ ), three-fold increase in stroke volume from  $0.27 \pm 0.02$  to  $1.09 \pm 0.04$   $\text{mL}\cdot\text{kg}^{-1}$  body mass. The increase in stroke volume could be maintained as long as the increase in filling pressure was maintained (Fig. 3.7). Consequently, both  $\dot{Q}$  and cardiac power output were also maintained at significantly higher levels ( $p < 0.001$ ). Cardiac output increased from a basal level of  $17.13 \pm 0.45$  to  $69.75 \pm 5.59$   $\text{mL}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$  body mass while cardiac power output increased from  $1.60 \pm 0.12$  to  $6.05 \pm 0.49$   $\text{mW}\cdot\text{g}^{-1}$  wet ventricular weight (Fig. 3.7). The intrinsic heart rate (Table 3.4), however, was not significantly affected by an increase in either  $\dot{Q}$  or power output ( $p = 0.838$ ). When stroke volume increased, due to the increase

in filling pressure, there was an increase in systolic output pressure and a modest increase in mean output pressure (Fig. 3.4B).

When the filling pressure was increased to stimulate  $\dot{Q}_{\max}$ , ANF release immediately and significantly increased from a control level of  $29.50 \pm 1.85 \text{ pg}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  wet heart weight to  $158.87 \pm 13.71 \text{ pg}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  wet heart weight ( $p < 0.001$ ). This elevated level was still present after 30 minutes at an elevated filling pressure; ANF release was  $189.43 \pm 29.74 \text{ pg}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  wet heart weight (Fig. 3.8). For one preparation, subsequent samples were collected at 60, 90 and 150 minutes. These samples revealed that, for at least two and half hours, when an elevated filling pressure was maintained ANF secretion could be maintained at an elevated rate (Fig. 3.9).

In the PL group, an increase in output pressure from  $5.23 \pm 0.05$  to  $7.84 \pm 0.04$  kPa resulted in a slight reduction in the basal stroke volume from  $0.30 \pm 0.01$  to  $0.24 \pm 0.02 \text{ mL}\cdot\text{kg}^{-1}$  body mass (Figs. 3.5B and 3.7). The intrinsic heart rate was not affected by the increase in output pressure (Table 3.4), but  $\dot{Q}$  significantly decreased from  $17.09 \pm 0.39$  to  $13.24 \pm 0.53 \text{ mL}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$  body mass ( $p = 0.01$ ). Although  $\dot{Q}$  decreased with the increase in output pressure, cardiac power increased slightly from  $1.68 \pm 0.11$  to  $1.92 \pm 0.15 \text{ mW}\cdot\text{g}^{-1}$  wet ventricular weight ( $p = 0.013$ ; Fig. 3.7c).

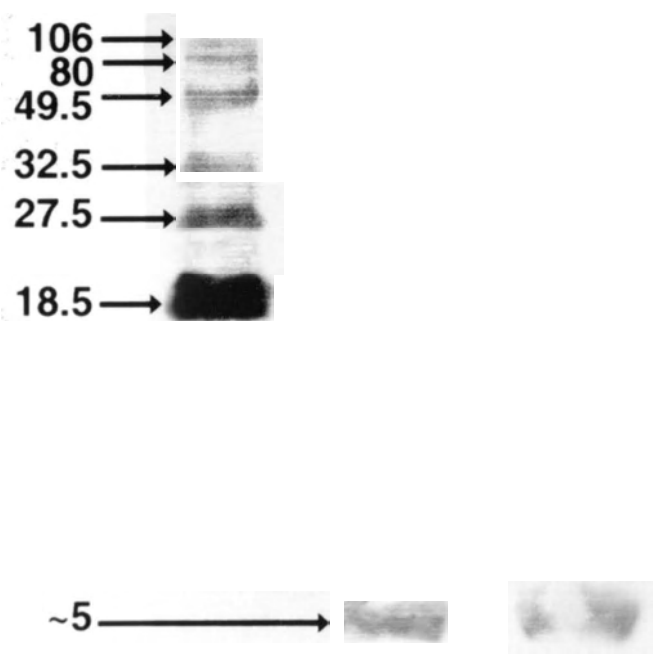
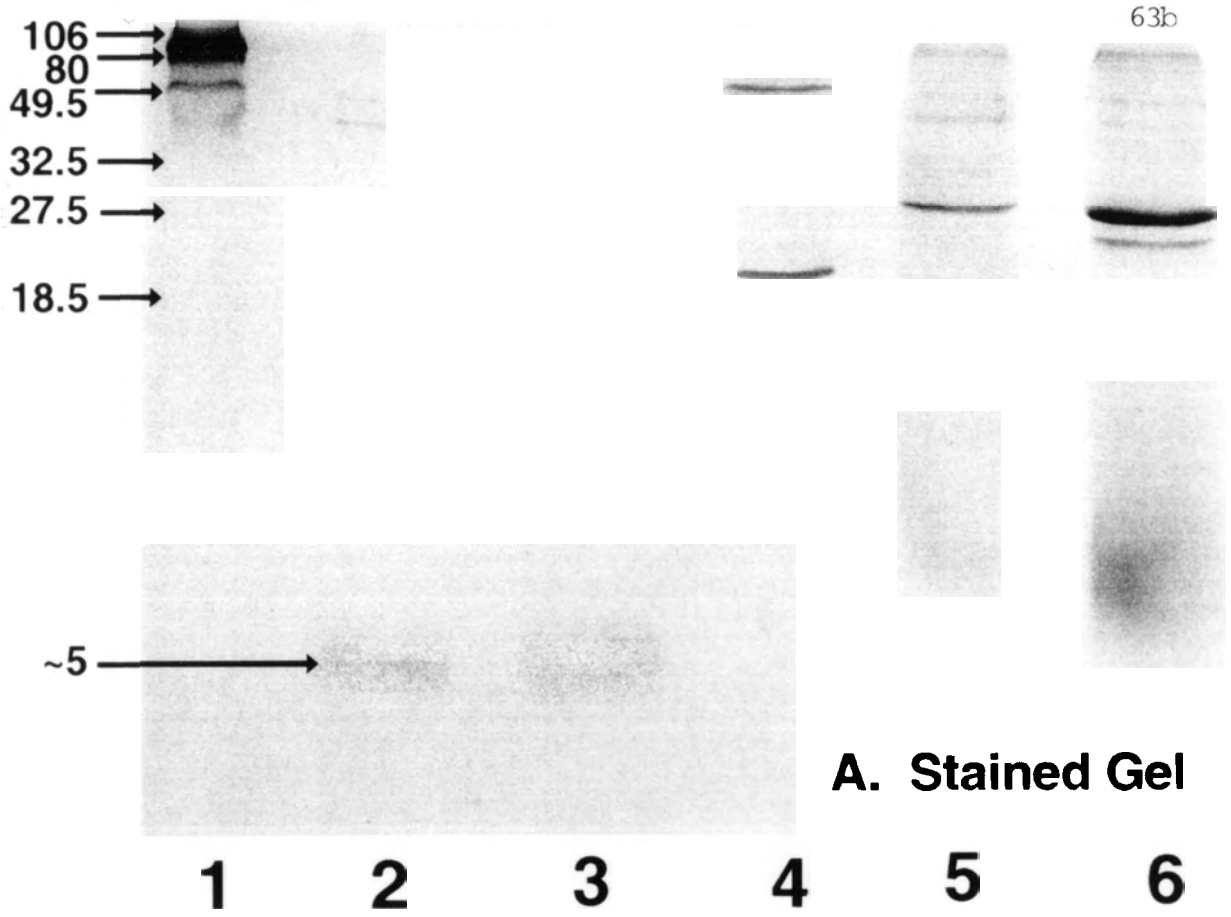
ANF release in the PL group was not significantly different ( $p = 0.669$ ) from the control despite the slightly lower  $\dot{Q}$  (Table 3.4). In the pressure-loaded experiments at the basal level, ANF release was  $34.98 \pm 5.56 \text{ pg}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  wet heart weight compared to a control level of  $29.54 \pm 6.78 \text{ pg}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  wet heart weight (Fig. 3.8). After 30 minutes at an elevated output pressure, ANF release was  $22.63 \pm 2.48 \text{ pg}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  wet heart weight (Table 3.4). This level was slightly lower than the control level of  $30.68 \pm 4.76 \text{ pg}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  wet heart weight. These results suggest that increased systolic pressure associated with volume-loading probably had very little effect on ANF release *in situ*.

**Table 3.4.** Perfusate ANF release ( $\text{pg}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  wet heart weight.) and cardiovascular variables for rainbow trout hearts after one hour of perfusion as an *in situ* preparation. Control values are mean  $\pm$  S.E.M. ( $N = 4$ ) while treatment values are mean  $\pm$  S.E.M. ( $N = 7$ ). Statistically significant differences ( $p < 0.05$ ) between control and treatment means are indicated by an asterisk (\*). The experimentally altered variables, filling pressure in the VL group and output pressure in the PL group, are indicated by the boxed values. All significant changes occurred as a result of the experimental changes.

	CONTROL	VOLUME- LOADED	PRESSURE- LOADED
<b>Perfusate ANF Concentration</b> ( $\text{pg}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ heart)			
<b>Time = Zero</b>	29.54 $\pm$ 6.78	29.50 $\pm$ 1.85	34.98 $\pm$ 5.56
<b>Time = +30 minutes</b>	30.68 $\pm$ 4.76	189.43 $\pm$ 29.74*	22.63 $\pm$ 2.48
<b>Filling Pressure</b> (kPa)	-0.09 $\pm$ 0.04	0.36 $\pm$ 0.01*	-0.17 $\pm$ 0.04
<b>Output Pressure</b> (kPa)	5.17 $\pm$ 0.03	5.29 $\pm$ 0.09	7.84 $\pm$ 0.04*
<b>Heart Rate</b> (beats $\cdot\text{min}^{-1}$ )	61.3 $\pm$ 4.0	61.7 $\pm$ 3.6	57.6 $\pm$ 3.4
<b>Stroke Volume</b> ( $\text{mL}\cdot\text{kg}^{-1}$ )	0.24 $\pm$ 0.01	1.09 $\pm$ 0.04*	0.24 $\pm$ 0.02
<b>Cardiac Output</b> ( $\text{mL}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$ )	15.24 $\pm$ 0.73	62.90 $\pm$ 5.25*	13.24 $\pm$ 0.53
<b>Power</b> ( $\text{mW}\cdot\text{g Vt}\cdot^{-1}$ )	1.19 $\pm$ 0.21	5.76 $\pm$ 0.37*	1.92 $\pm$ 0.15*

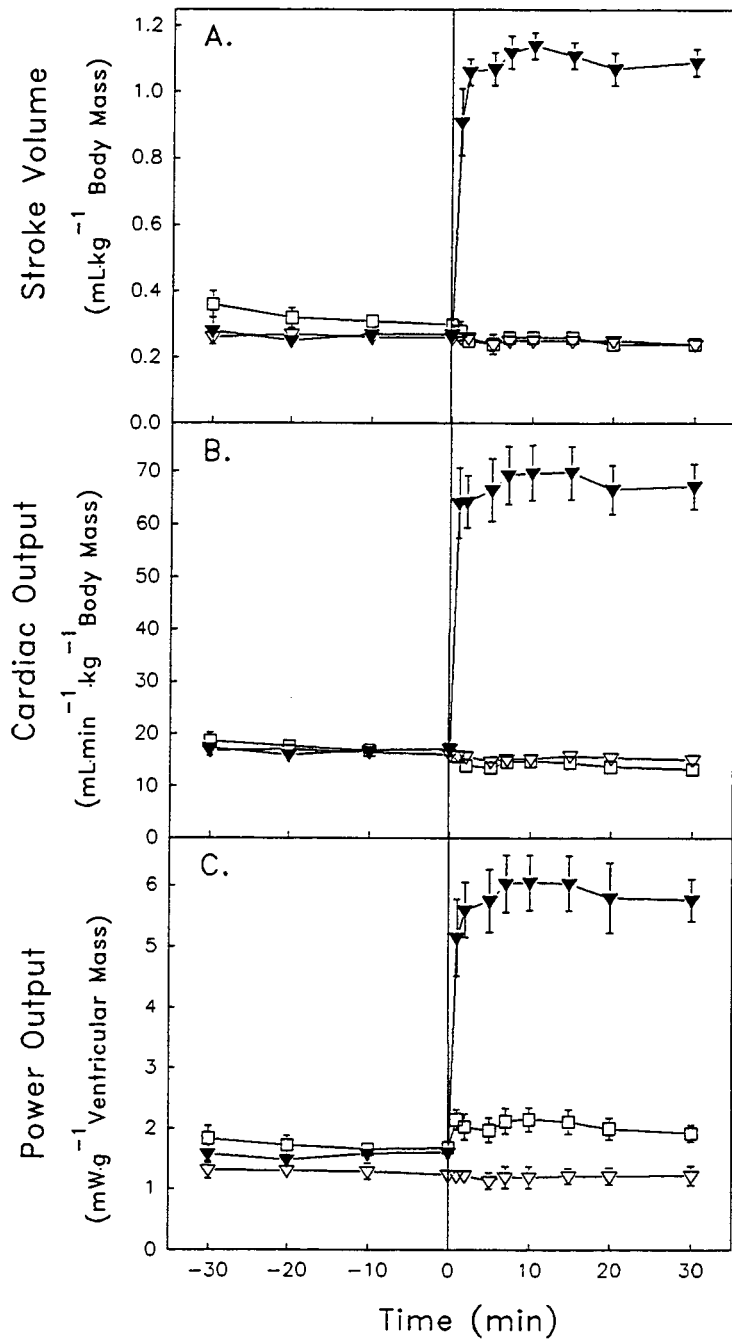


**Figure 3.6.** SDS-PAGE stained gel (A) and corresponding western blot (B). The samples in each loading well are as follows: Lane (1) low weight molecular standard in kDa; Lane (2) human  $\alpha$ -ANF-(99-126) standard; Lane (3) rainbow trout perfusate sample; Lane (4) rainbow trout plasma extract; Lane (5) rainbow trout atrial extract; and, Lane (6) rainbow trout ventricular extract. The low weight molecular standard ranged between 106 to 18.5 kDa. On the stained gel, the  $\alpha$ -ANF-(99-126) standard appeared at approximately 5 kDa and was detected on Lane (2) of the western blot. Note a similar band for the trout perfusate sample (Lane 3) on both the gel and western blot.

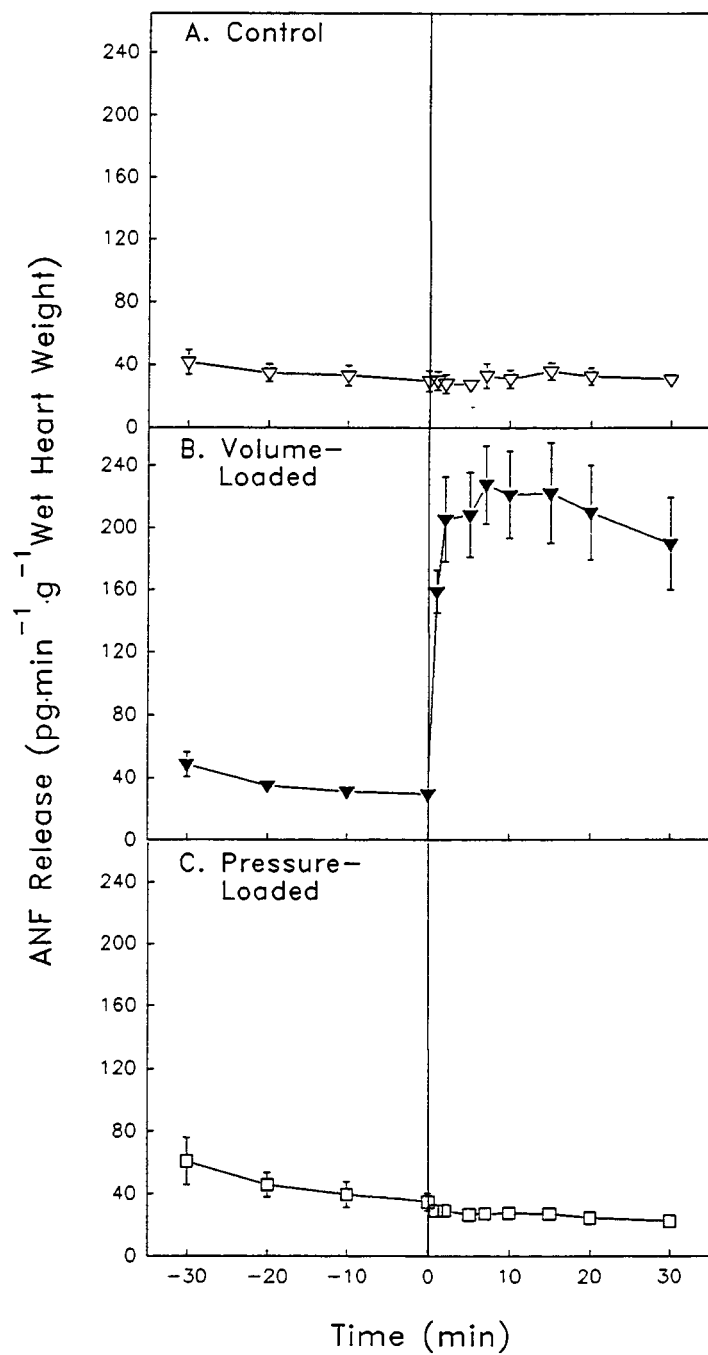


**B. Western Blot**

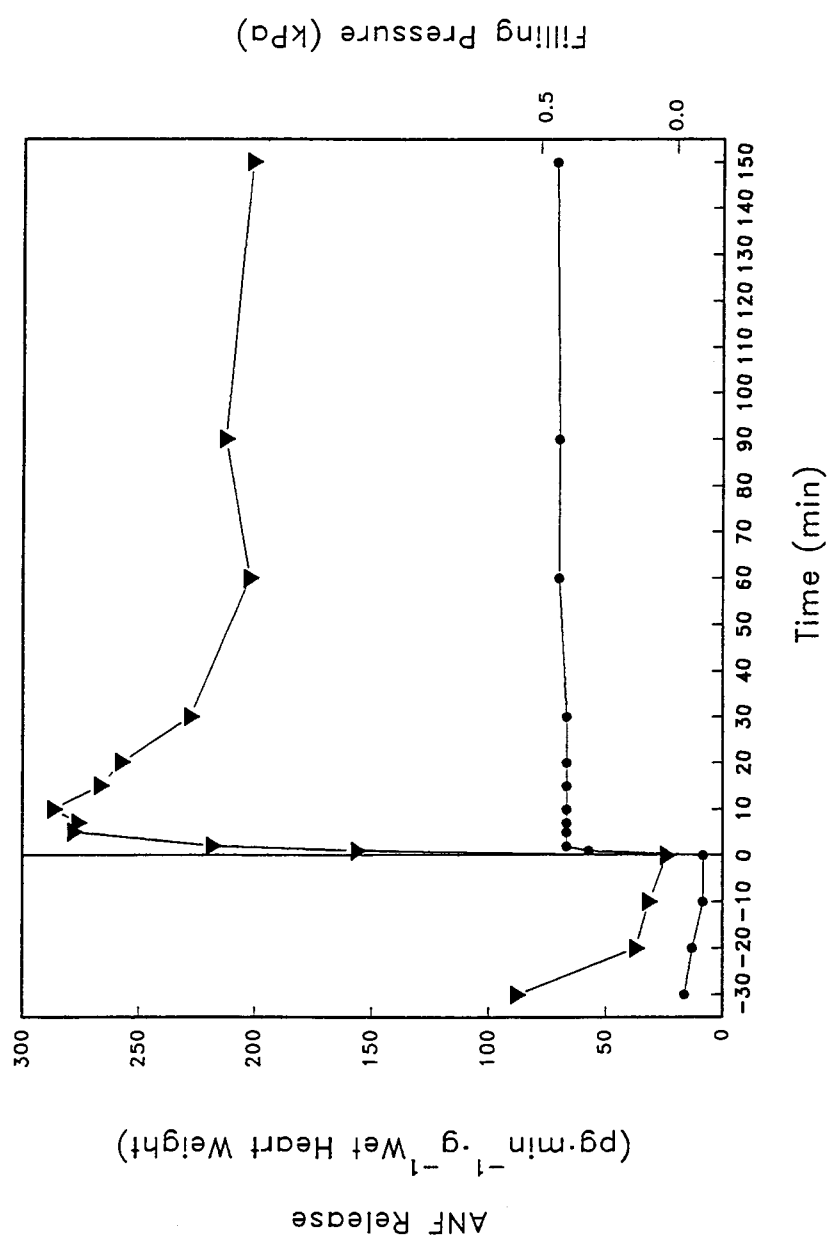
**Figure 3.7.** Three graphs illustrating the stability of the cardiovascular variables, such as stroke volume (A), cardiac output (B) and power output (C) under control ( $\nabla$ ), volume-loaded ( $\blacktriangledown$ ) and the pressure-loaded ( $\square$ ) experimental conditions. Time -30 minutes to time 0 is the recovery time for the surgery. At time 0 a stimulus was applied and maintained for 30 minutes. With the volume-loaded group, a step increase in filling pressure gave an immediate and stable increase in stroke volume ( $\text{mL}\cdot\text{kg}^{-1}$  body mass), cardiac output ( $\text{mL}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$  body mass) and cardiac power ( $\text{mW}\cdot\text{g}^{-1}$  wet ventricular weight). This change in the experiment condition could be maintained for 30 minutes. With the pressure-loaded group, a step increase in output pressure did not significantly effect stroke volume ( $p = 0.273$ ) or cardiac output ( $p = 0.437$ ), but did increase the power output. The control group was left unaltered for the entire 60 minute duration of the experiment. All values are mean  $\pm$  S.E.M. (control  $N = 4$  and treatments  $N = 7$ ).



**Figure 3.8.** Three graphs showing ANF release ( $\text{pg}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  wet heart weight) in the perfusate leaving the heart plotted against time (min) for the control (A), volume-loaded (B) and pressure-loaded (C) groups. Even though the state of the cardiovascular variables were stable during the initial 30 minutes of the experiments, the figures clearly show that this period of time was required for ANF release to reach a steady level, such that perfusate ANF release rates were approximately  $30 \text{ pg}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  wet heart weight in all groups. In the control and pressure-loaded groups, no subsequent change in ANF release was noted; however, the volume-loaded group gave an immediate and sustained increase in ANF release. All values are mean  $\pm$  S.E.M. (control  $N = 4$  and treatments  $N = 7$ ).



**Figure 3.9.** A graph showing ANF release ( $\blacktriangledown$ ;  $\text{pg}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  wet heart weight) and filling pressure ( $\bullet$ ; kPa) plotted against time (min) for one volume-loaded perfused trout heart. Time -30 minutes to time 0 was the recovery time for the surgery. An increase in filling pressure was applied at time 0 and maintained for 150 minutes. With the increase in filling pressure, ANF release increased 10-fold in the first five minutes. At the seventh minute, ANF release began to decline to a steady level of approximately  $220 \text{ pg}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  wet heart weight which was maintained for the duration of the stimulus.





### **(B) Is ANF Release Graded or "All-or-None" in a Perfused Trout Heart?**

At the control filling pressure, ANF release in both the FW and SW groups ( $33.86 \pm 2.09$  and  $29.99 \pm 1.89$   $\text{pg}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  wet heart weight, respectively) were similar to the control value reported in the previous section (A). In both the FW and SW perfused trout hearts, randomized, step increases in filling pressure, lasting 10 minutes each, resulted in proportional increases in both the stroke volume and ANF release (Figs. 3.10 and 3.11).

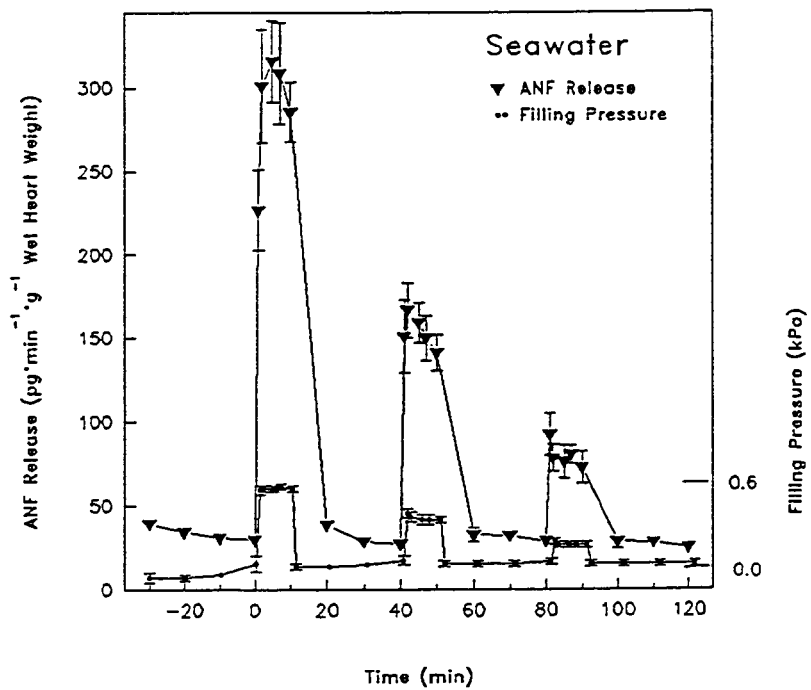
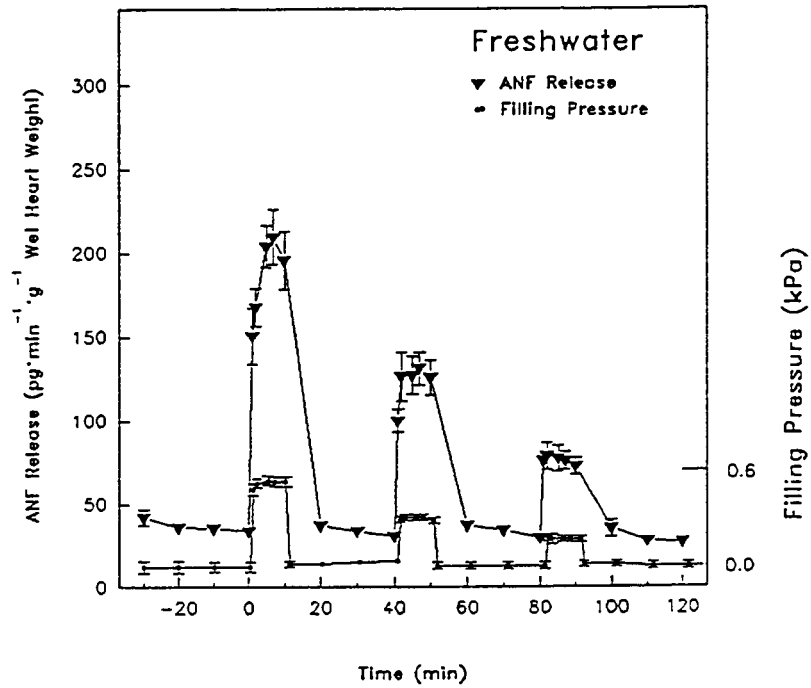
At the highest filling pressure, the SW perfused hearts had significantly higher ( $p = 0.016$ ) cardiac outputs and than those found for the FW perfused hearts. In the FW group, an increase in the filling pressure from the control level ( $0.03 \pm 0.03$  kPa) to the highest value ( $0.58 \pm 0.03$  kPa) resulted in significant increases ( $p = 0.022$ ) in stroke volume,  $\dot{Q}$  and power output (Table 3.5). In the SW group, an increase in the filling pressure from  $0.02 \pm 0.02$  to  $0.58 \pm 0.03$  kPa also resulted in significant increases in stroke volume,  $\dot{Q}$  and power output ( $p < 0.05$ ; Table 3.5). However, in the SW perfused hearts, this increase in stroke volume was significantly higher ( $p = 0.022$ ) than that found for the FW hearts, even though the filling pressures between the two groups were not significantly different ( $p = 0.384$ ; Table 3.5).

Accompanying the step increases in filling pressure were proportional increases in ANF release (Fig. 3.11). In both the FW and SW perfused hearts, for each step increase in filling pressure ANF release immediately increased to a new and higher level of release and, with a constant stimulus, this increase in ANF secretion did not change appreciably over time (Fig. 3.13). Therefore, both FW and SW perfused hearts had a similar sensitivity to increases in filling pressure, in terms of the increases in stroke volume, and in ANF release (Figs. 3.11 and 3.12). At the highest filling pressure the SW hearts released ANF at significantly higher rates ( $p = 0.006$ ) than those of the FW hearts (Table 3.5; Fig. 3.13). Also, for any given cardiac parameter, the ANF release in the seawater hearts was more variable than that for the freshwater hearts (Figs. 3.11 and 3.12).

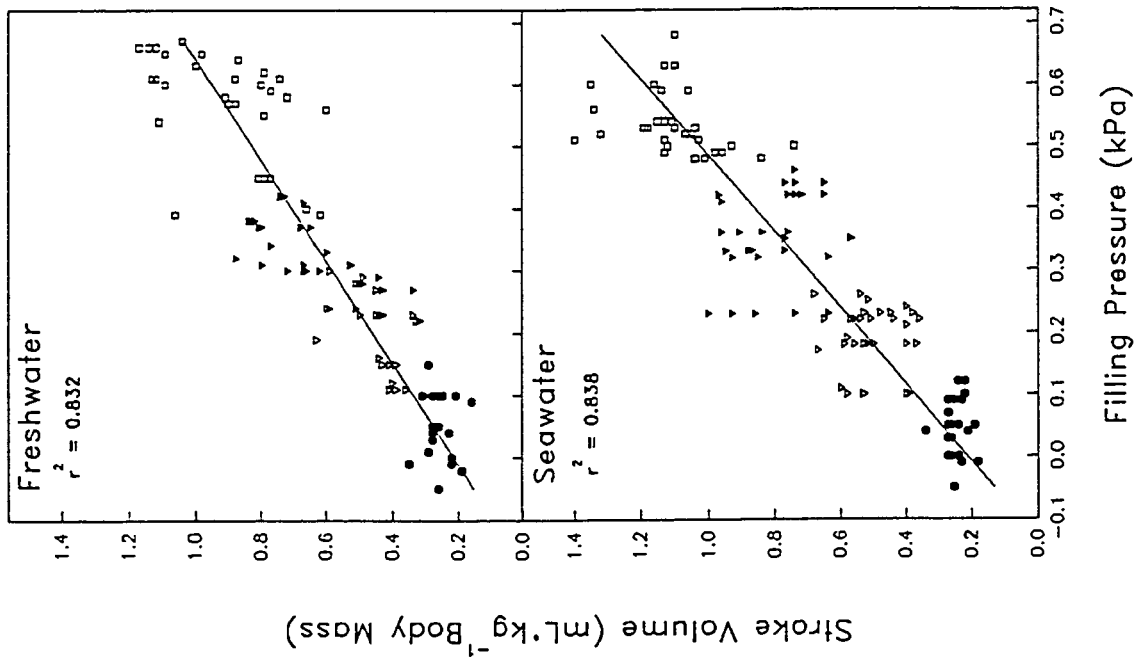
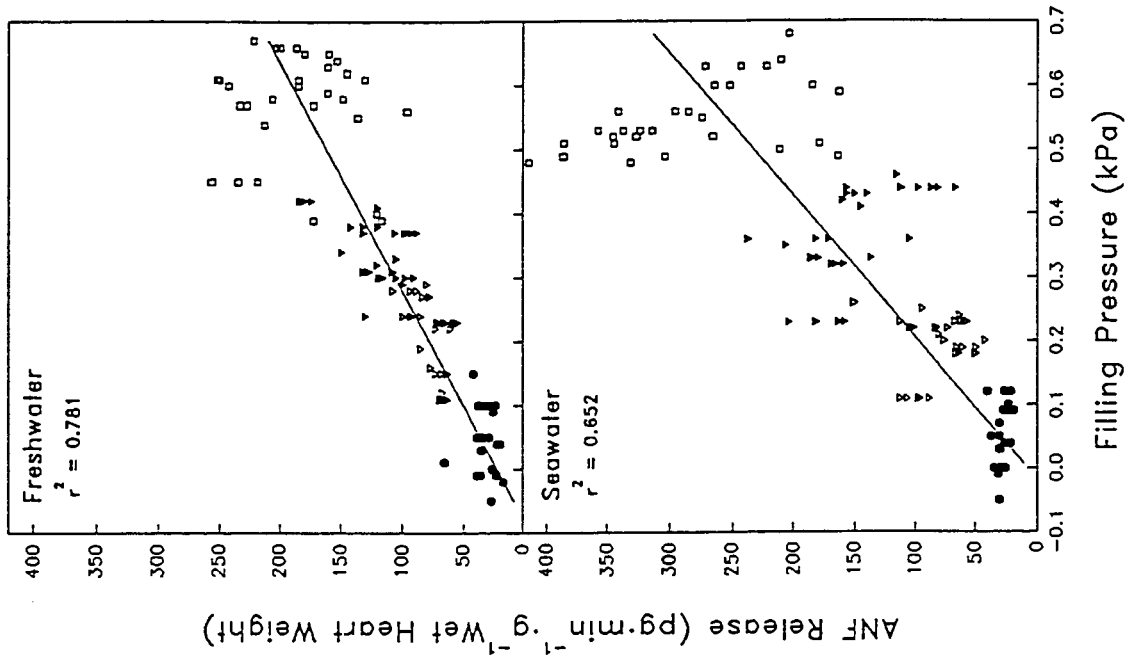
**Table 3.5.** Perfusate ANF release ( $\text{pg}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  wet heart weight) and cardiovascular variables for freshwater and seawater rainbow trout hearts at four cardiac outputs. The basal cardiac output ( $\dot{Q}$ ) represents the control filling pressure while the maximum cardiac output ( $\dot{Q}_{\text{max}}$ ) represents the filling pressure at the peak of the Starling curve. All values are mean  $\pm$  S.E.M. ( $N = 6$ ). Statistically significant differences ( $p < 0.05$ ) between the freshwater and seawater mean values at the  $\dot{Q}_{\text{max}}$  state are indicated by an asterisk (\*). Dissimilar superscript letters indicate significant differences ( $p < 0.05$ ) in values within each group.

	FRESHWATER TROUT				SEAWATER TROUT			
	Basal	30% $\dot{Q}_{\text{max}}$	60% $\dot{Q}_{\text{max}}$	$\dot{Q}_{\text{max}}$	Basal	30% $\dot{Q}_{\text{max}}$	60% $\dot{Q}_{\text{max}}$	$\dot{Q}_{\text{max}}$
ANF Release ( $\text{pg}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ )	33.86 <sup>a</sup> $\pm 2.09$	72.61 <sup>b</sup> $\pm 5.46$	125.74 <sup>c</sup> $\pm 11.51$	195.82 <sup>d*</sup> $\pm 19.10$	29.99 <sup>a</sup> $\pm 1.89$	80.44 <sup>b</sup> $\pm 5.74$	141.29 <sup>c</sup> $\pm 11.90$	285.38 <sup>d*</sup> $\pm 19.63$
Filling Pressure (kPa)	0.03 <sup>a</sup> $\pm 0.04$	0.21 <sup>b</sup> $\pm 0.02$	0.33 <sup>c</sup> $\pm 0.03$	0.58 <sup>d</sup> $\pm 0.03$	0.02 <sup>a</sup> $\pm 0.02$	0.19 <sup>b</sup> $\pm 0.02$	0.35 <sup>c</sup> $\pm 0.03$	0.55 <sup>d</sup> $\pm 0.02$
Output Pressure (kPa)	5.23 <sup>a</sup> $\pm 0.06$	5.30 <sup>a</sup> $\pm 0.08$	5.38 <sup>b</sup> $\pm 0.06$	5.62 <sup>c</sup> $\pm 0.11$	5.18 <sup>a</sup> $\pm 0.04$	5.29 <sup>a</sup> $\pm 0.04$	5.56 <sup>b</sup> $\pm 0.12$	5.67 <sup>b</sup> $\pm 0.09$
Heart Rate (beats $\cdot\text{min}^{-1}$ )	58.2 <sup>a</sup> $\pm 2.4$	62.0 <sup>a</sup> $\pm 3.6$	59.8 <sup>a</sup> $\pm 2.7$	59.8 <sup>a</sup> $\pm 2.8$	64.7 <sup>a</sup> $\pm 2.5$	65.2 <sup>a</sup> $\pm 1.1$	62.2 <sup>a</sup> $\pm 0.8$	59.7 <sup>a</sup> $\pm 1.8$
Stroke Volume ( $\text{mL}\cdot\text{kg}^{-1}\cdot\text{bw}$ )	0.30 <sup>a</sup> $\pm 0.01$	0.45 <sup>b</sup> $\pm 0.04$	0.68 <sup>c</sup> $\pm 0.04$	0.90 <sup>d*</sup> $\pm 0.08$	0.27 <sup>a</sup> $\pm 0.02$	0.49 <sup>b</sup> $\pm 0.03$	0.80 <sup>c</sup> $\pm 0.04$	1.15 <sup>d*</sup> $\pm 0.04$
Cardiac Output ( $\text{mL}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}\cdot\text{bw}$ )	17.03 <sup>a</sup> $\pm 0.14$	31.93 <sup>b</sup> $\pm 4.98$	38.51 <sup>c*</sup> $\pm 1.57$	53.40 <sup>d*</sup> $\pm 4.11$	17.07 <sup>a</sup> $\pm 0.29$	32.00 <sup>b</sup> $\pm 2.15$	49.95 <sup>c*</sup> $\pm 2.81$	68.11 <sup>d*</sup> $\pm 3.01$
Power ( $\text{mW}\cdot\text{g}\cdot\text{Vt}^{-1}$ )	1.57 <sup>a</sup> $\pm 0.08$	2.44 <sup>b</sup> $\pm 0.08$	3.50 <sup>c</sup> $\pm 0.22$	5.02 <sup>d</sup> $\pm 0.56$	1.35 <sup>a</sup> $\pm 0.09$	2.53 <sup>b</sup> $\pm 0.14$	4.12 <sup>c</sup> $\pm 0.29$	5.64 <sup>d</sup> $\pm 0.35$

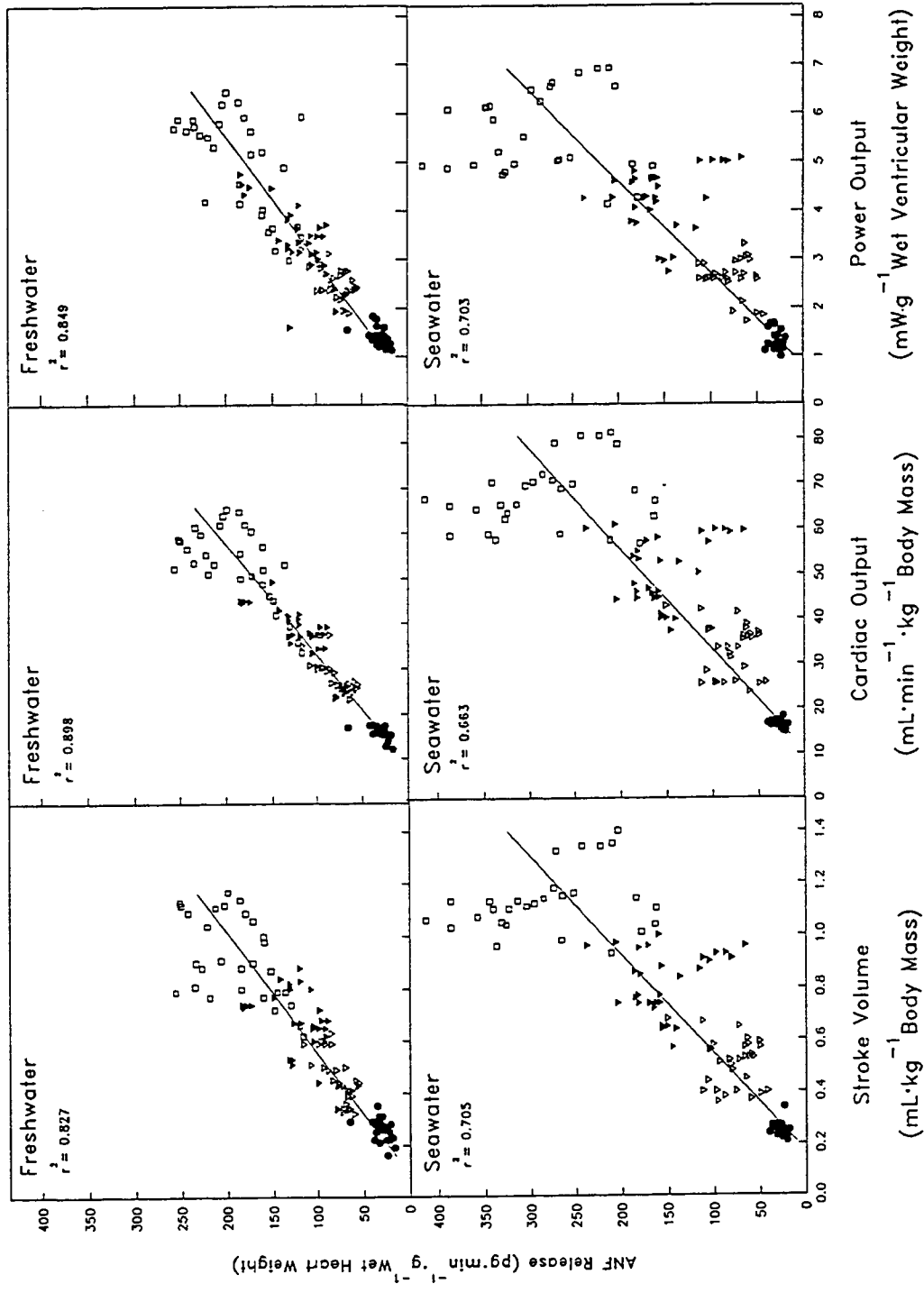
**Figure 3.10.** Two graphs showing the graded response of ANF release ( $\text{pg}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  wet heart weight) with step increases in filling pressure (kPa) for both freshwater-acclimated and seawater-acclimated perfused trout hearts. Time -30 minutes to time 0 was the recovery time for the surgery. A step increase in filling pressure was applied at times 0, +40, and +80 minutes and maintained for 10 minutes each. The filled circles ( $\bullet$ ) represent the filling pressure and the filled triangles ( $\blacktriangledown$ ) represent ANF release. All values are mean  $\pm$  S.E.M. ( $N = 6$ ).



**Figure 3.11.** Four graphs showing the relationship between filling pressure (kPa) and stroke volume ( $\text{mL}\cdot\text{kg}^{-1}$  body mass) and ANF release ( $\text{pg}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  wet heart weight) in perfusate for both the freshwater-acclimated and the seawater-acclimated trout hearts. For both groups, the experimental changes were step increases in filling pressure. As expected, an increase in filling pressure resulted in a linear increase in stroke volume (FW  $r^2 = 0.832$ ; SW  $r^2 = 0.838$ ). In addition, the 3-fold increase in stroke volume was associated with a 10-fold increase in ANF release in the perfusate that was also described by a linear regression (FW  $r^2 = 0.781$ ; SW  $r^2 = 0.652$ ). The symbols are represented as follows: filled circles ( $\bullet$ ) = resting  $\dot{Q}$ ; hollow triangles ( $\nabla$ ) = 30%  $\dot{Q}_{\text{max}}$ ; filled triangles ( $\blacktriangledown$ ) = 60%  $\dot{Q}_{\text{max}}$ ; and the hollow squares ( $\square$ ) =  $\dot{Q}_{\text{max}}$ .

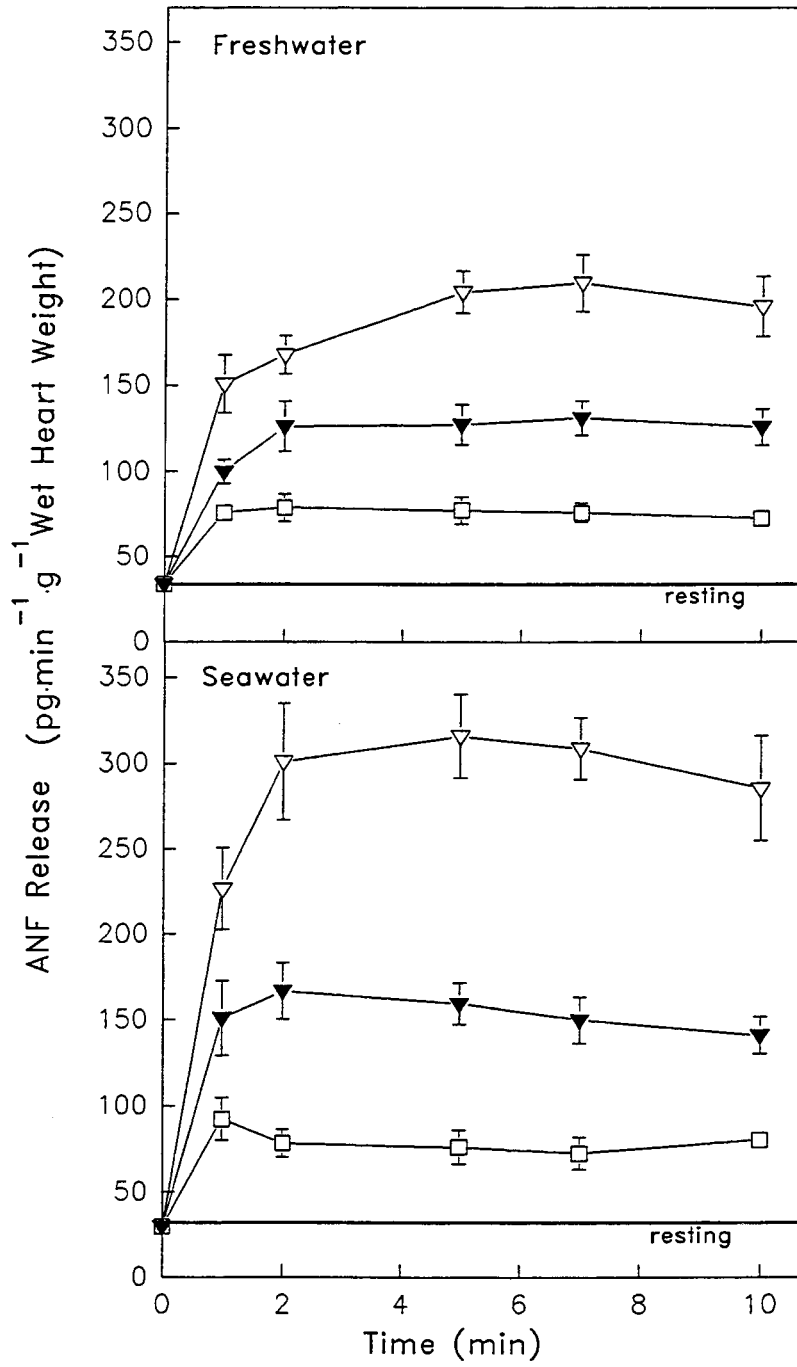


**Figure 3.12.** Six plots showing the relationship between stroke volume ( $\text{mL}\cdot\text{kg}^{-1}$  body mass), cardiac output ( $\text{mL}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$  body mass) and power output ( $\text{mW}\cdot\text{g}^{-1}$  wet ventricular weight) and ANF release ( $\text{pg}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  wet heart weight) in perfusate for both the freshwater-acclimated and the seawater-acclimated trout hearts. For both groups, the experimental changes were step increases in filling pressure. Both stroke volume and ANF release increase linearly with increases in filling pressure (Figure 3.11). Consequently, ANF release also increases linearly with increases in stroke volume (FW  $r^2 = 0.827$ ; SW  $r^2 = 0.705$ ), cardiac output (FW  $r^2 = 0.898$ ; SW  $r^2 = 0.663$ ) and power output (FW  $r^2 = 0.849$ ; SW  $r^2 = 0.703$ ). The symbols are represented as follows: filled circles ( $\bullet$ ) = resting  $\dot{Q}$ ; hollow triangles ( $\nabla$ ) = 30%  $\dot{Q}_{\text{max}}$ ; filled triangles ( $\blacktriangledown$ ) = 60%  $\dot{Q}_{\text{max}}$ ; and the hollow squares ( $\square$ ) =  $\dot{Q}_{\text{max}}$ .





**Figure 3.13.** Two graphs showing ANF release ( $\text{pg}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  wet heart weight) plotted against time (min) for both the freshwater-acclimated and seawater-acclimated trout perfused hearts. An increase in filling pressure was applied at time 0 and maintained for 10 minutes. The bold solid line represents the rate of ANF release at control  $\dot{Q}$ . The hollow triangles ( $\nabla$ ) represent the mean rate of ANF release at an increase in filling pressure to  $\dot{Q}_{\text{max}}$ . The filled triangles ( $\blacktriangledown$ ) represent the mean rate of ANF release at 60%  $\dot{Q}_{\text{max}}$  and the squares ( $\square$ ) represent the mean rate of ANF release at 30%  $\dot{Q}_{\text{max}}$ . For both groups, the rate of ANF release increases to a proportionally higher level for each step increase in cardiac output. These rates of ANF release are above that for the resting rate of ANF release. At  $\dot{Q}_{\text{max}}$ , the rate of ANF release in the perfusate for the seawater group was significantly higher than that for the freshwater group ( $p < 0.001$ ). All values are mean  $\pm$  S.E.M. ( $N = 6$ ).



### **Immunoreactive-ANF in the Trout Plasma and Myocardium**

The measurement of ir-ANF content in the trout plasma and tissue extracts, plus ir-ANF in the *in situ* perfusate samples, were obtained using a direct radioimmunoassay specific for human ANF-(99-126) (see Fig. 3.1). The plasma ir-ANF content in the freshwater-acclimated trout was  $17.25 \pm 1.45$  pg·mL<sup>-1</sup>. This value was not significantly different from the seawater-acclimated trout ( $p = 0.473$ ;  $N = 4$ ), such that the ir-ANF plasma content in the seawater trout was  $15.75 \pm 1.32$  pg·mL<sup>-1</sup>.

As established in Chapter 2, the trout myocardial tissues were found to cross-react with the human ANF-(99-126). Using the relative weights of the atrium and ventricle, it was possible to calculate that the atrium contained 92% of the total ir-ANF stored in the trout heart. The ventricle contained  $0.04 \pm 0.01$  pg·mg<sup>-1</sup> wet weight. The measurements of ir-ANF in the trout heart extracts are presented in Table 3.6. Total ir-ANF content in the atrial tissues ranged between  $2.13 \pm 0.28$  to  $4.38 \pm 1.71$  pg·mg<sup>-1</sup> wet weight, and although the controls contained a higher concentration of ANF, no significant differences were noted between the controls and treatment groups ( $p = 0.248$ ).

**Table 3.6.** Ir-ANF content ( $\text{pg}\cdot\text{mg}^{-1}$ ) in rainbow trout atrial extracts. Control hearts (CH) were removed from the animals prior to surgery while surgery control hearts (SH) were sampled immediately during or immediately after the cannulation. Control, volume- and pressure-loaded hearts were subjected to one hour in the *in situ* preparation. Values are mean  $\pm$  S.E.M. Numbers in brackets indicate sample size.

		<b>Atrial ANF Content (<math>\text{pg}\cdot\text{mg}^{-1}</math>)</b>
<b>Controls</b>	CH (4)	$4.38 \pm 1.71$
	SH (7)	$3.10 \pm 0.24$
<b><i>In situ</i> Preparation</b>	Control (6)	$2.13 \pm 0.28$
	Volume-loaded (7)	$2.57 \pm 0.23$
	Pressure-loaded (5)	$2.34 \pm 0.35$

## DISCUSSION

Although there were significant differences in morphometric values between the trout used in this study, the relative health of the fish in each treatment group was not significantly different (Table 3.3). The health of each fish in this study was estimated through a condition factor that varied between  $1.01 \pm 0.03$  and  $1.28 \pm 0.02$  (Tables 3.3 and 3.5). These condition factor values were well over 0.7, which represents the threshold value for healthy rainbow trout (Reimers, 1963). Also, the fish were found to be brightly coloured and rigorously feeding at the time of sampling. Consequently, the performance of the fish in the *in situ* preparation was not compromised by poor health.

In the trout perfusate samples, the Western blotting technique showed that human ANF-(99-126) antibodies labeled the trout ANF antigens (Fig. 3.6). A band in the trout perfusate lane was identified on the SDS-PAGE gel and labeled on the Western blot at approximately the 5 kDa range. This molecular weight agrees with the molecular weight found for human ANF-(99-126) (Napier *et al.*, 1984). However, no cross-reactivity was noted between the human ANF antibodies and the trout ANF plasma and myocardial antigens. In addition, on the SDS-PAGE gel, no bands appeared in the 5 kDa range for either the plasma or myocardial samples. There are two possible explanations for these findings.

First, and most importantly, plasma and myocardial ANF-(99-126) are most likely bound to other proteins and this could have interfered with the Western blotting. For instance, the extraction of ANF from plasma by phase column separation has been reported to eliminate the binding interference by unknown plasma components (Richards *et al.*, 1897; Genest and Cantin, 1988). As described in the methods, phase columns were not used to extract ANF from the trout plasma samples before they were assayed. The trout perfusate samples, on the other hand, were free of the normal plasma protein

constitutes. I conclude that ANF-(99-126) in the perfusate samples was detected by the immunoblotting, whereas the ANF-(99-126) in the plasma samples was not detected due to possible binding interference by plasma proteins.

Chapter 2 of this study demonstrated that trout atrial and ventricular tissues contained ir-ANF in secretory vesicles in the form of a prohormone. In mammalian and non-mammalian species, ir-ANF has also been found in secretory vesicles (Chapeau *et al.*, 1985; Kawata *et al.*, 1985; Reinecke *et al.*, 1985). According to mammalian literature, these myocardial vesicles contain proANF and upon release ANF-99-126 is cleaved from the prohormone (Martin *et al.*, 1990). Thus, in the trout myocardial samples, ANF-(99-126) was most likely bound to the amino-terminus of the prohormone and this could have interfered with the immunoblotting.

In addition, the immunoblotting technique may not be sensitive enough to detect ir-ANF in trout plasma and myocardial tissues. Both the plasma and myocardial tissues contained proteins other than ANF and some of these proteins were exceptionally large (Fig. 3.6a). As the heavier proteins were not extracted from the samples, this could have interfered with the migration and separation of smaller proteins, such as ANF-(99-126), through the acrylamide gel. Also, ir-ANF was not detected in the perfusate samples until they were concentrated at least four times with cold acetic acid. As the heavier proteins in the plasma and myocardial samples were not extracted prior to the immunoblotting, these samples could not be concentrated without equally concentrating the heavier proteins. As a result, when the plasma and myocardial samples were loaded on the SDS-PAGE gel, the ANF-(99-126) concentration in these samples may have been too low to be detected.

Unlike the Western blot, the RIA, which was also specific for human ANF-(99-126), detected ir-ANF in the trout myocardial, plasma and perfusate samples. The RIA between-assay coefficient of variation was 12.33% which was within the acceptable parameters of 6 and 15% (Chard, 1990). The standard curves were reproducible (Fig. 3.1)

and the  $ED_{50}$  equaled  $10.7 \pm 0.7 \text{ pg}\cdot\text{mL}^{-1}$ . This  $ED_{50}$  value was also similar to the values reported from other non-mammalian studies using the same commercially available RIA specific for human ANF (Baeyens *et al.*, 1989; Ryu *et al.*, 1992; Palmer *et al.*, 1994). In addition, both the plasma and atrial extract dilution curves were relatively parallel to the human ANF standard curve. These findings suggest that the human ANF antibodies bound specifically with trout ANF antigens (Fig. 3.1).

This was the first study to quantitatively describe the ir-ANF content in the trout heart using a RIA. The ir-ANF content obtained from the RIA in rainbow trout atrial and ventricular tissue was  $3.10 \pm 0.24 \text{ pg}\cdot\text{mg}^{-1}$  and  $0.04 \pm 0.01 \text{ pg}\cdot\text{mg}^{-1}$ , respectively. While values are considerably lower than those reported for mammalian myocardial tissues, they are in the same range as those found for other fish species (Table 1.1). Using the relative weights of the atrium and ventricle, it was possible to calculate that the atrium contained 92% of the total ir-ANF stored in the heart. This finding, that the trout atrium contains the majority of the total ANF store, complements the findings found in Chapter 2 of this study and the findings from both mammalian and non-mammalian studies (Chapeau *et al.*, 1985; Reinecke *et al.*, 1985; Larsen *et al.*, 1994). The present study, however, is not the first to conclude that the rainbow trout atrium contained the majority of the total ANF store. A microscopic study by Reinecke *et al.* (1985) was the first to suggest that the atrium was the primary source of ANF in the trout heart; however, they did not quantitatively determine the atrial contribution to the total ANF store in the trout heart.

As well as being the site for ANF production and storage, the heart also functions to pump blood with the rhythmic contractions of the myofibrils. In mammals, an increase in end-diastolic volume, or cardiac filling pressure, results in a greater force of contraction and a greater stroke volume. This intrinsic response of cardiac muscle is called the Frank-Starling mechanism. In mammalian hearts, the ventricle does not completely empty at end-systole. Also, mammals tend to vary cardiac output by varying heart rate rather than

stroke volume. Consequently, in mammalian hearts, the stroke volume remains relatively constant even when the filling pressure varies.

In contrast to the mammalian heart, a fish heart completely empties at end-systole and is much more sensitive to increases in filling pressure (Farrell, 1991). Therefore, in a fish heart, relatively small changes in filling pressure can result in rather large changes in stroke volume. This was demonstrated in the trout *in situ* heart preparation. The rainbow trout heart was very sensitive to changes in filling pressure, such that an increase of only 0.4 kPa tripled resting stroke volume (Fig. 3.7). This was true for both the freshwater and seawater perfused hearts (Table 3.5; Fig. 3.11). This effect of filling pressure on seawater-acclimated rainbow trout hearts has not been studied previously.

While very little is known with regards to ANF release from non-mammalian hearts, the possible mediators for ANF release from mammalian hearts have been studied extensively. For instance, many mammalian studies have shown that an increase in atrial stretch results in an increase in ANF release (Dietz, 1984; Lang *et al.*, 1985; Ledsome *et al.*, 1985; Anderson *et al.*, 1986; Agnoletti *et al.*, 1989). Increases in atrial pressure were also thought to result in the release of ANF, until this was investigated using a clinical condition called cardiac tamponade. Cardiac tamponade is a condition in which a hemorrhage into the pericardial space increases atrial pressure while restricting atrial expansion. Clinical studies using anesthetized dogs demonstrated that during cardiac tamponade ANF was not released into the circulation (Edwards *et al.*, 1988). Interestingly, ANF release from a mammalian atria is very different than that from the ventricle, such that a stretch-induced atrial release can be sustained whereas the ventricular release lasts for only 10 minutes (Kinnunen *et al.*, 1992). In addition, increased ventricular pacing of a mammalian heart can also result in increased ANF release (Ngo *et al.*, 1989). In rats, endothelin, a vasoactive peptide, enhances the stretch-induced release of ANF while other vasoactive factors, such as arginine vasopressin and



angiotensin II, do not affect ANF release (Dietz, 1988; Schiebinger and Greening 1992). In mammals, both  $\text{Na}^+$  and  $\text{Ca}^{2+}$  can either increase (Weidmann *et al.*, 1986; Yamamoto *et al.*, 1988b), have no effect (Takayanagi *et al.*, 1985; Dietz, 1987), or hamper ANF release (Jin *et al.*, 1988; DeBold and DeBold, 1989). Thus, in mammals, it appears that factors, such as  $\text{Na}^+$ ,  $\text{Ca}^{2+}$  and endothelin, somehow modulate ANF release even though the mechanical stretch of the myocardium is the primary stimulus for ANF release.

The addition of adrenaline also modulates the release of ANF in both in mammals and fish. For example, 1 nM of isoproterenol (i.e., a  $\beta$ -receptor agonist) was reported to enhance the stretch-induced release of ANF from isolated rat hearts (Agnoletti *et al.*, 1992). In mammals, adrenaline increases both cardiac contractility (i.e., a positive inotropic effect) and heart rate (i.e., a positive chronotropic effect), thereby increasing  $\dot{Q}$ . Thus, in mammals, adrenaline may indirectly affect ANF release through its effects on cardiac contractility. This could also occur in the fish heart, as adrenaline can also cause both positive inotropic and chronotropic effects (Farrell, 1984). Throughout the *in situ* heart preparation, 5 nM of adrenaline was added into the perfusate at *in vivo* resting concentrations to provide a tonic stimulation of the heart and to maintain preparation viability (Farrell and Milligan, 1986). Thus, adrenaline was infused into the perfusate at a constant rate ( $50 \mu\text{L}\cdot\text{min}^{-1}$ ) to control for its possible effects on ANF release.

Each *in situ* trout heart was also allowed a 30 minute recovery time before a stimulus was applied. This recovery time was essential as the flow and filling pressure were not strictly controlled during the surgery or during the transfer of the preparation to the *in situ* bath. The fact that the ANF release at the start of each preparation was usually elevated suggests atrial distension occurred during the surgery and transfer to the *in situ* bath (Figs. 3.8, 3.9 and 3.10). Nevertheless, by the end of the 30 minute recovery period ANF release had decreased to a stable resting level of  $29.50 \pm 1.85 \text{ pg}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  wet heart

weight (Fig. 3.8). This stable resting level was unchanged in the control group after a further 30 minutes.

This was the first study to show that a volume-induced stretch resulted in the release of ANF from a perfused teleost heart and that this release occurred at a steady rate with a constant stimulus. The increase in filling pressure to an *in situ* trout heart resulted in an increase in atrial stretch with increases in stroke volume,  $\dot{Q}$ , power output. (Fig. 3.7). When the filling pressure to an *in situ* trout heart was increased to a maximum stroke volume and maintained over a 30 minute period, the ir-ANF release immediately increased 5-fold from the resting level, peaked after 7 minutes of stimulus, and then slowly declined to new steady release of  $189.43 \pm 29.74 \text{ pg}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  wet heart weight (Fig. 3.8). This rate of release was sustained for as long as the filling pressure was maintained (Fig.3.9). This suggests that there was an initial surge of ANF release in response to the increased filling pressure and then over a period of five to ten minutes this release fell back toward an elevated steady state. Thus, it appears that other circulating hormones or central neuronal pathways were not required for ANF release in a perfused teleost heart. However, the *in situ* preparation does not exclude the possibility that other local factors, such as neuroactive substances, within the myocardium could influence the stretch-induced release of ANF.

Although ANF was released with increases in filling pressure, it was not known whether this increased ANF release was due to changes in atrial or ventricular detension. According to Franklin and Davie (1992), when the afterload on an *in situ* trout heart is increased above 6.0 kPa without changing the filling pressure, the end-diastolic intraventricular volume/pressure increases without increasing atrial volume/pressure. In the present study, the maximum afterload on the pressure-loaded perfused trout heart was  $7.84 \pm 0.04 \text{ kPa}$  (Fig. 3.5B). In these pressure-loaded hearts, ANF release was not significantly different from the control group ( $p > 0.669$ ; Table 3.4). This suggests that

increases in afterload have no effect on ANF release and that the atrium, rather than the ventricle, was the primary ANF release source from the *in situ* trout heart. This was not surprising since the atrium contained 92% of the total ANF content in the rainbow heart. These results also suggest that ANF release from a trout heart is not dependent upon a negative feedback mechanism from the arterial side of the heart.

This study is also the first to demonstrate that ANF release in both freshwater and seawater perfused hearts is proportional to the magnitude of the atrial stretch. In both the freshwater and seawater perfused trout hearts, randomized step increases in filling pressure resulted in sustained and proportional increases in stroke volume,  $\dot{Q}$ , power output and ANF release (Fig. 3.11, 3.12 and 3.13). Cardiac performance and ANF release was also compared between the two freshwater groups; namely, the VL group in Section (A) and the freshwater graded response group in Section (B). At  $\dot{Q}_{\max}$ , the filling pressures, stroke volumes and  $\dot{Q}$  for the VL Section (A) perfused hearts were higher (Table 3.4) than those in the Section (B) freshwater hearts (Table 3.5). After 10 minutes of volume-loading the Section (A) hearts, ANF release was also higher ( $221.03 \pm 27.87$  pg·min<sup>-1</sup>·g<sup>-1</sup> wet heart weight) than the Section (B) freshwater hearts ( $195.82 \pm 17.43$  pg·min<sup>-1</sup>·g<sup>-1</sup> wet heart weight). This provided additional evidence that changes in filling pressure resulted in proportional changes in ANF release.

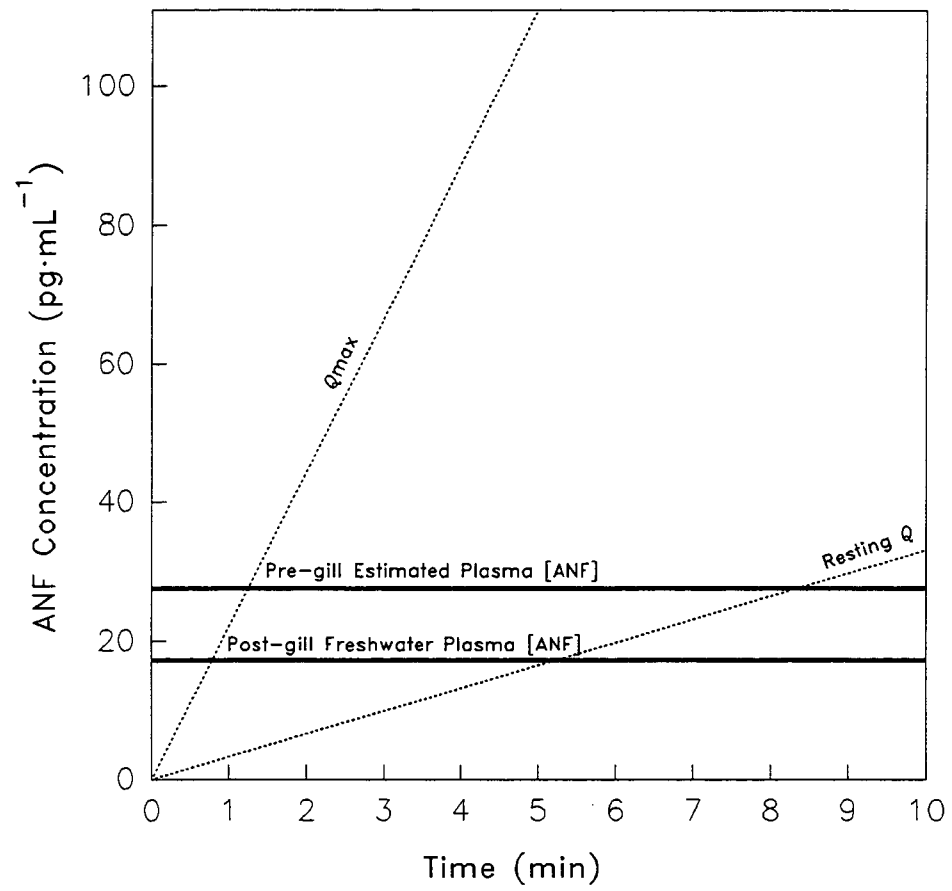
Interestingly, the seawater perfused hearts tended to perform better than the freshwater hearts at the higher filling pressures (Table 3.5). In addition, the maximum rate of ANF release from the seawater perfused hearts was also greater than that from the freshwater perfused hearts (Figs. 3.10 and 3.13). The ventricular mass and filling pressures were not significantly different between the freshwater and seawater groups ( $p = 0.930$  and  $p = 0.384$ , respectively), yet the seawater perfused hearts had greater stroke volumes. This suggests that the seawater hearts were more sensitive to the higher filling pressures. However, the mechanisms mediating this increased sensitivity are unknown.

This finding also suggests that ANF release in the perfused trout heart was driven more by volume changes rather than pressure changes.

Furthermore, for all cardiovascular parameters, ANF release was more variable in the seawater group than in the freshwater group (Figs 3.11 and 3.12). The trout used in this study were acclimated in seawater for 6 weeks before they were subjected to the *in situ* preparation. It also took an additional two weeks to complete the *in situ* experiments. Consequently, the first and last seawater trout tested had different acclimation times. Therefore, if seawater transfer resulted in temporary increases in the maximum rate of ANF release in response to increases in filling pressure, then ANF release from trout acclimated in seawater for a longer period may be different from trout acclimated for a shorter period and this could account for the high variability seen in ANF release between the seawater perfused hearts.

As this study provides the rates of ANF release, an assessment can now be made between the secretion rates and the ANF plasma levels measured *in vivo*. In this study, at resting  $\dot{Q}$ , ANF release was approximately  $30 \text{ pg}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$  body weight and at  $\dot{Q}_{\text{max}}$  this release could increase to approximately  $200 \text{ pg}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$  body weight. Also, the *in vivo* ANF plasma concentration in a freshwater trout was  $17.25 \pm 1.45 \text{ pg}\cdot\text{mL}^{-1}$  and the plasma volume in their primary circulation was estimated at approximately  $9 \text{ mL}\cdot\text{kg}^{-1}$  (Steffensen and Lomholt, 1992). Thus, given the *in situ* rates of ANF release, it would take 5.18 minutes at a basal  $\dot{Q}$  and 0.78 minutes at  $\dot{Q}_{\text{max}}$  to obtain the *in vivo* plasma ANF levels (Fig. 3.14). As *in vivo* plasma levels are normally relatively stable, this suggests that either plasma ANF is cleared as fast as it is released from the heart under basal conditions or a negative feedback system somehow reduces the basal rate of release from that observed *in situ*. The *in situ* heart by being a single pass perfusion system was in effect an open circuit without any negative feedback. At present, nothing is known about possible negative feedback in fish or mammals. However, there is an effective clearance

**Figure 3.14.** A model showing ANF secretion ( $\cdots$ ) and plasma levels ( $\text{---}$ ) plotted against time. This model was based on a 1 kg rainbow trout with a 1 g heart weight. This model assumes that the rate of secretion equals the rate of clearance without a feedback mechanism (i.e., there is no change in the ANF plasma levels). The ANF post-gill plasma concentration found in freshwater trout was  $17.25 \pm 1.45 \text{ pg}\cdot\text{mL}^{-1}$  and the total plasma concentration in the primary circulation was estimated at  $9 \text{ mL}\cdot\text{kg}^{-1}$  (Steffensen and Lomholt, 1992). The rates of ANF release from a freshwater perfused trout heart at resting  $\dot{Q}$  and at  $\dot{Q}_{\text{max}}$  were  $30 \text{ pg}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$  body weight and  $200 \text{ pg}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$  body weight, respectively. ANF secretion was calculated as follows:  $[17.25 \pm 1.45 \text{ pg}\cdot\text{mL}^{-1} \times 9 \text{ mL}\cdot\text{kg}^{-1}] / \text{pg}$  of ANF  $\cdot \text{min}^{-1}\cdot\text{kg}^{-1}$  body weight. At resting  $\dot{Q}$ , ANF release attains the known ANF plasma level in 5.18 minutes. When a gill clearance factor of approximately 60% (Olson and Duff, 1993) was incorporated into the model, then the resting  $\dot{Q}$  ANF release attains meets the estimated plasma level in 8.28 minutes. When the heart is maximally stretched (i.e.  $\dot{Q}_{\text{max}}$ ), then both the known and estimated ANF plasma levels are met within 2 minutes.



of ANF at the trout gill and at other sites in fish, such as the brain, gallbladder and gut (Olson and Duff, 1993).

Therefore, at this time, it seems reasonable to assume that the basal rate of ANF release equals the basal rate of ANF clearance, such that the plasma ANF concentration of  $17.25 \pm 1.45 \text{ pg}\cdot\text{mL}^{-1}$  was maintained with a release of  $30 \text{ pg}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$  body weight. In freshwater parr, plasma ANF *in vivo* values can still remain elevated at approximately  $123 \text{ pg}\cdot\text{mL}^{-1}$  ten hours after they are transferred to seawater (Smith *et al.*, 1991). This change in ANF concentration in the plasma was most likely achieved through an increased rate of release from the atrium rather than a change in clearance rate. If atrial stretch was the only stimulus, then at  $\dot{Q}_{\text{max}}$  a new plasma level would occur within six minutes given no other change. Also, as the transfer from freshwater to seawater appears to cause a temporary increase in ANF release at  $\dot{Q}_{\text{max}}$  (approximately  $340 \text{ pg}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$  body weight) from a perfused trout heart, then an elevated plasma level would be attained within three minutes. As shown in Figure 3.14, when the gill clearance factor was incorporated into the model, this resulted in slightly longer release times to meet the estimated pre-gill plasma level. The point of this crude modeling was that ANF secretion appears to be a rapid response system and one that could be sustained for long periods.

Several studies have demonstrated the effects of circulating ANF in fish by injecting a bolus of either rat or human ANF into trout. For instance, Olson and Duff (1986) found that  $10 \text{ }\mu\text{g}\cdot\text{kg}^{-1}$  body weight of synthetic rat ANF injected into a rainbow trout produced diuretic and natriuretic effects. Later, these same authors reported that  $300 \text{ ng}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$  body weight of synthetic rat ANF infused into trout decreased the mean arterial pressure and pulse pressure while increasing heart rate (Olson and Duff, 1992). They also reported that  $10 \text{ }\mu\text{g}\cdot\text{kg}^{-1}$  body weight of eel ANF injected into trout was half as potent as the rat ANF (Olson and Duff, 1992). Eddy *et al.* (1990) reported that  $10 \text{ }\mu\text{g}\cdot\text{kg}^{-1}$  body weight of human ANF injected into trout *in vivo* reduced pulse pressure by 60%, but

did not affect either blood pressure or heart rate. Interestingly, eel ANF injected into freshwater eels was 100-fold more potent than either rat or human ANF (Takei and Balment, 1993). This suggests that doses of rat and human ANF injected into trout to elicit responses are unusually high compared with trout plasma ANF levels because of possible non-specific binding between rat and human ANF to trout ANF receptors.

The trend for seawater trout to have higher ANF plasma concentrations than freshwater trout was not found in this study. In this study, the plasma ir-ANF content for the freshwater-acclimated trout was not significantly different ( $p = 0.473$ ) from the value for the seawater-acclimated trout ( $17.25 \pm 1.45 \text{ pg}\cdot\text{mL}^{-1}$  and  $15.75 \pm 1.32 \text{ pg}\cdot\text{mL}^{-1}$ , respectively). A study by Smith *et al.* (1991) found similar ir-ANF plasma concentrations for freshwater trout ( $21.87 \pm 2.0 \text{ pg}\cdot\text{mL}^{-1}$ ), but they reported a much higher ir-ANF plasma value for their seawater-acclimated trout ( $75.16 \pm 6.8 \text{ pg}\cdot\text{mL}^{-1}$ ). Their blood samples were taken from the dorsal aorta of trout that had acclimated to seawater for three weeks. In contrast, blood samples in this study were taken from the caudal veins of trout that had acclimated for 6 to 8 weeks. Thus, the plasma ir-ANF values in this study may be lower because they were obtained from the caudal vein, rather than the dorsal aorta, and ANF may have been taken up or cleared by specific receptors in the systemic system.

Furthermore, upon transfer to seawater from freshwater, trout plasma ANF levels may not stabilize in the same manner as other plasma components. For instance, when rainbow trout are transferred from freshwater to seawater their plasma  $[\text{Na}^+]$  and  $[\text{Cl}^-]$  stabilize to new levels after only 7 days (Johnston and Cheverie, 1985), but plasma ANF and renin concentrations are still at elevated levels three weeks later (Smith *et al.*, 1991). Also, ten hours after freshwater parr are abruptly transferred to seawater, their ir-ANF plasma levels dramatically increase 4-fold and then, over a period of three days, this elevated ir-ANF plasma level decreases back toward the basal level (Smith *et al.*, 1991). Another study has shown that the number of  $\text{Na}^+\text{-K}^+\text{-Cl}^-$  cotransporter proteins in



freshwater trout also increase after they have been acclimated to seawater for a prolonged time (Flik *et al.*, 1993). As ANF is known to bind and activate Na<sup>+</sup>-K<sup>+</sup>-Cl<sup>-</sup> cotransporter proteins (O'Grady *et al.*, 1985), perhaps if the trout in the Smith *et al.*, (1991) study had been given a longer period for seawater-acclimation, such as six weeks, ANF plasma levels in their seawater-acclimated trout would not have been different from the levels found in freshwater trout.

Although trends in ANF plasma concentrations can be found using a direct RIA, ir-ANF plasma values are higher and less variable when ANF is extracted from plasma using either reverse phase columns (Richards *et al.*, 1987) or ethanol (Winters *et al.*, 1989). Ethanol extraction of ANF results in even higher recoveries than that of the column extraction (Winters *et al.*, 1989). In the Smith *et al.* (1991) study, ANF was extracted from the plasma using phase column separation before being assayed while in this study the ir-ANF plasma concentrations were obtained by direct RIA.. Thus, the ANF plasma concentrations found in this study may be lower than those reported by Smith *et al.* (1991) because ANF was not extracted from the plasma before being assayed. This was supported by a freshwater rainbow trout ANF plasma value of  $30.63 \pm 3.19$  pg·mL<sup>-1</sup> ( $N = 4$ ) which was obtained by ethanol extraction (Cousins and Vesely, unpublished observation). This value was almost double to the value determined on the same plasma samples by the direct RIA.

## Summary

This study answered four primary questions. It is the first study to describe and quantify the secretion of ir-ANF-(99-126) from a perfused teleost heart using a human antibody. Thus, I confirmed that the human ANF antibody binds specifically with the trout ANF antigen. This suggests a high sequence homology for this peptide may exist between the two species. The second question I answered was that the primary stimulus for ANF release in the trout heart is atrial detension. As this is also true for mammalian hearts, the mechanisms controlling ANF release may be highly conserved among vertebrates. What does differ between fish and mammalian hearts is the sensitivity of the fish heart to atrial detension in response to changes in filling pressure. As ANF is primarily located in the atrium and as the trout heart is sensitive to changes in venous return, subtle changes in venous pressure may have profound effects on ANF release *in vivo*. The third question I answered was that step increases in atrial filling pressure result in proportional increases in  $\dot{Q}$  and ANF release. Thus, ANF release from a perfused trout heart is a graded response. In addition, this release of ANF in response to volume-loading was rapid and could be sustained with a constant atrial detension. Even though it is now known that atrial stretch is the primary stimulus for ANF release in both mammals and fish, it is still unknown how the stretching of a cardiac myocyte stimulates the release. The final question I answered was that both freshwater and seawater perfused trout hearts have similar basal release rates of ANF; however, the seawater perfused hearts had higher rates of ANF release at the higher filling pressures than those from the freshwater hearts. This suggests that the acclimation to seawater may have somehow caused an increase in ANF release when the myocytes were stretched to the peak of the Frank-Starling curve. Thus, in the context of humoral controls in fish, ANF-mediated responses should be viewed as potentially belonging to a rapid and prolonged response system.

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