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RELEASE OF ATRIAL NATRIURETIC FACTOR PROHORMONE PEPTIDES 1-30, 31-67 AND 99-126 FROM FRESHWATER- AND SEAWATER-ACCLIMATED PERFUSED TROUT (*ONCORHYNCHUS MYKISS*) HEARTS

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Summary

Atrial natriuretic factor (ANF), a 28-amino-acid peptide hormone produced in the heart, circulates in both freshwater and seawater rainbow trout. In mammals, two other peptide hormones, proANF 1-30 and proANF 31-67, derived from the same 126-amino-acid prohormone as ANF (amino acids 99–126), circulate and have natriuretic and diuretic properties. It has never been determined whether these peptides circulate in fish. The present investigation was designed to determine (1) whether proANF 1-30 and/or proANF 31-67 circulate in perfused hearts from freshwater- and seawater-acclimated rainbow trout (*Oncorhynchus mykiss*) *in situ*, and (2) if they do, to determine whether increasing the filling pressure of the heart causes their release in trout as it does in mammals. High-performance gel-permeation chromatography of fish plasma revealed that both proANF 1-30 and 31-67 circulate in freshwater- and seawater-acclimated trout plasma at threefold higher concentrations than does ANF. The basal rates of release of ANF and proANF 1-30 and 31-67 were similar in both freshwater and seawater trout, with the rate of release of proANF 1-30 being 10 times higher and that of proANF 31-67 20 times higher than that of ANF. When the filling pressure was increased to the peak of the Starling curve ($\dot{Q}_{max}$), the rate of release of ANF and proANFs 1-30 and 31-67 increased fivefold for each peptide in the freshwater trout, while in seawater trout the rates of release increased six- to ninefold. We conclude that proANF 1-30 and 31-67, as well as ANF, circulate in both freshwater-and seawater-acclimated trout and do so at concentrations higher than that of ANF. Increasing the filling pressure to the trout heart was found to cause a similar increase in the release rates for each of these peptides, but the maximal increase was higher in the seawater-acclimated trout, apparently because they showed a larger increase in cardiac output.

Key words: cardiac hormones, atrial natriuretic factor, prohormone, proANF and ANF release, rainbow trout, *Oncorhynchus mykiss*.

Introduction

Atrial natriuretic factor (ANF) is a hormone produced and secreted from the heart (DeBold *et al.* 1981; Dietz, 1984). In mammals, ANF and three other peptide hormones are derived from a common prohormone (Flynn *et al.* 1985; Seidman *et al.* 1985; Vesely *et al.* 1989). In humans, ANF is the carboxy-terminal peptide resulting from protease cleavage of the 126-amino-acid prohormone at position arginine–serine (98–99) (Imada *et al.* 1988). Three other peptides are derived from the amino-terminal portion of the prohormone and are biologically active (Vesely *et al.* 1987). Furthermore, their effects are both more potent and longer-lasting than that of ANF (Martin *et al.* 1990; Vesely *et al.* 1994). These three proANF peptide hormones, consisting of amino acids 1–30, 31–67 and 79–98, have been tentatively named long-acting natriuretic peptide, vessel dilator and kaliuretic peptide, respectively, based upon their most potent biological properties (Vesely *et al.* 1994). Like ANF, these hormones circulate in the blood until they act at various receptor sites to cause natriuretic (increased Na^+ excretion), diuretic (increased urine production) and hypotensive (low blood pressure) effects (Vesely *et al.* 1992, 1994; Martin *et al.* 1990). Thus, natriuretic peptides are intimately involved in blood volume regulation.

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An extensive literature exists regarding natriuretic peptides and their release from the heart (see Vesely, 1992, for a review), but the majority of this is based on mammalian systems. ANF appears to be a highly conserved peptide for blood volume regulation, being present in several taxa, invertebrate and vertebrate alike. For instance, both ANF and proANFs have been found in the oyster (*Crassostrea virginica*; Vesely *et al.* 1991; Palmer *et al.* 1994) and the red-ear turtle (*Pseudemys scripta*; Baeyens *et al.* 1989), while Baranowski and Westenfelder (1989) demonstrated that ANF was released from isolated ventricular myocytes obtained from a fish heart. In mammals, 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), and the same appears to be true for a non-mammalian system since ANF release increases during atrial distension of a perfused rainbow trout heart (Cousins and Farrell, 1996). ANF has been identified in the plasma of many fish species (Evans *et al.* 1989; Uemura *et al.* 1990; Takei *et al.* 1990; Westenfelder *et al.* 1988; Uva *et al.* 1993), but the release of proANF peptides has not been previously documented.

Although it appears that the mechanism for ANF release may be highly conserved among the vertebrates, such that atrial distension results in ANF release in both mammals (Dietz, 1984) and fish (Cousins and Farrell, 1996), it is not clear whether an ionic mediator, such as Na^+, can influence ANF production and release. A decrease in dietary Na^+ in rats results in a decrease both in atrial ANF content (DeBold, 1979) and in levels of ANF and proANF peptides in the circulation (Dietz *et al.* 1992). Increases in dietary salt levels, however, do not result in an increase in atrial ANF content (Takayanagi *et al.* 1985; Dietz, 1987) even though the level of plasma ANF rises (Tanaka *et al.* 1984; Sagnella *et al.* 1985; Weidmann *et al.* 1986). This suggests that higher salt levels increase ANF release without simultaneously stimulating ANF synthesis. When rainbow trout are fed high-salt diets, their blood and pulse pressures increase (Eddy *et al.* 1990), but it is not known whether the levels of plasma ANF also change. Although several studies have shown that seawater fish tend to have higher ANF plasma concentrations than freshwater fish (Westenfelder *et al.* 1988; Evans *et al.* 1989; Smith *et al.* 1991), it is not known whether ANF production and release of ANF differ between animals in these two environments. Interestingly, when a euryhaline species is transferred to sea water, as shown by Eddy and Chang (1993) for Atlantic salmon parr (*Salmo gairdineri*), plasma ANF levels increase.

While atrial stretch, which is associated with hypervolemia, is the main stimulus for ANF release from the perfused heart of freshwater trout (Cousins and Farrell, 1996), it is not known how an osmotic challenge would affect this secretion. We have, therefore, investigated the differences in the regulation of ANF secretion in the perfused hearts of freshwater- and seawater-acclimated rainbow trout. In addition, we also investigated the possibility that fish hearts release other ANF-related cardiac peptides and whether the acclimation of freshwater trout to sea water affected the release of these ANF-related peptides.

Materials and methods

All rainbow trout [*Oncorhynchus mykiss* (Walbaum)] in this study were obtained from a local supplier (West Creek Trout Farms, Aldergrove, British Columbia) and weighed 300–600 g. The experimental animals were fed a maintenance diet (1 % body mass) of commercial trout chow (Silver Cup Fish Food, Murray, UT) on a daily basis. The freshwater trout were held indoors in 2000 l fiberglass tanks supplied with flowing, dechlorinated fresh water (13 °C; 12 h:12 h L:D photoperiod). A group of 12 fish was transferred to a tank supplied with recirculating sea water (salinity 28 ‰). These trout were allowed to acclimate to the sea water for at least 6 weeks and showed no signs of distress during this time. There were no mortalities during acclimation.

Perfused hearts from rainbow trout were used *in situ* to investigate how changes in filling pressure (P_I) affected ANF release. By subjecting both seawater-acclimated trout (SW; N=6) and freshwater trout (FW; N=6) to an identical protocol, the effects of salinity on ANF release could be examined. The perfused trout heart preparation utilized in this study was similar to that described by Farrell *et al.* (1986, 1988) and Cousins and Farrell (1996). In brief, an animal was randomly selected and anesthetized using a buffered MS222 solution (1:5000 w/v). The animal was then measured, weighed and transferred to an operating sling. During surgery, the gills were irrigated with chilled and aerated buffered MS222 solution (1:10 000 w/v). At this time, plasma samples were obtained from the blood drawn from the caudal vein. An input cannula was inserted into the sinus venosus through a hepatic vein, and the pericardium was secured around the cannula. An output cannula was inserted into the bulbus arteriosus through the ventral aorta and, finally, the ducti Cuvier were ligated to prevent backflow and to ensure that the heart received input only from the cannula. The total time for the surgery was approximately 20 min.

During the experiment, the circulated perfusate was collected from the output tubing at 1 min intervals and the fractions were then frozen by immersion in an ethanol and dry ice mixture. The perfusion saline for the FW trout was identical to the saline used by Cousins and Farrell (1996) and contained (in mmol l^{-1}): NaCl, 124.1; KCl, 3.1; $CaCl_2$, 2.5; $MgSO_4$, 0.9: dextrose, 0.5. The perfusion saline for the SW trout consisted of (in mmol l^{-1}): NaCl, 150; KCl, 5; $CaCl_2$, 2.3; $MgSO_4$, 2.0; dextrose, 0.5 (modified from Farrell *et al.* 1985). Both perfusate recipes were buffered with 10 mmol l^{-1} Tes with a pH of 7.85 at 13 °C. The osmolality of the freshwater perfusion solution was 281 mosmol kg^{-1} and that of the seawater solution was 328 mosmol kg^{-1}, which simulated their ambient osmolalities. For all preparations, adrenaline (5 nmol l^{-1}; bitartrate salt, Sigma Chemical, St Louis, MO) was added at a constant rate (50 µl min^{-1}) to the perfusate solution to provide

a resting tonus to heart rate and to reduce deterioration of the preparations (Farrell and Milligan, 1986).

Once the surgery was completed, the heart was left to pump under control conditions for 30 min to ameliorate any effects associated with surgical trauma. We have previously demonstrated that ANF release, which is initially high after surgery, falls rapidly to a stable, basal rate that can be maintained for several hours if perfusion conditions are constant (Cousins and Farrell, 1996). Control conditions consisted of (1) a filling pressure (P_I) set to maintain a cardiac output ($\dot{Q}$) of 17 ml min^{-1} kg^{-1} body mass and (2) an afterload or output pressure (P_O) set at 4.91 kPa, which resemble conditions established for resting trout *in vivo* (Graham and Farrell, 1989; Kiceniuk and Jones, 1977). Since changes in heart rate are typically small, $\dot{Q}$ and stroke volume were adjusted by changes in P_I. We measured ANF release under four conditions, namely, $\dot{Q}$ (the value for $\dot{Q}$ measured under resting conditions), 30 % $\dot{Q}_{max}$, 60 % $\dot{Q}_{max}$ and $\dot{Q}_{max}$ (maximum cardiac output), where $\dot{Q}_{max}$ was estimated from previous studies (e.g. Cousins and Farrell, 1996) and from the body mass of the animal. The order of testing the elevated $\dot{Q}$ conditions was randomized to control for possible cross-over effects. Each level of $\dot{Q}$ was maintained for 10 min with intervening periods when $\dot{Q}$ was maintained at control levels for 30 min. At the end of these test periods, fractions of heart perfusate were collected for a period of 1 min and immediately frozen, with the exception of the initial equilibration period when 1 min fractions were collected every 10 min. Once completely frozen, the samples were freeze-dried for approximately 24 h and then stored at room temperature (25 °C) in a powdered form until reconstituted with 1 ml of the radioimmunoassay buffer and assayed for immunoreactive ANF (ir-ANF).

Natriuretic peptides utilized for the radioimmunoassays described below were obtained from Peninsula Laboratories, Inc. (Belmont, CA, USA). The measurement of ir-ANF in the FW- and SW-acclimated trout plasma and reconstituted perfusate samples was accomplished using a radioimmunoassay (RIA) specific for human α-ANF-(99-126). Samples of FW and SW-acclimated trout plasma were obtained from blood drawn from the caudal vein and were collected into chilled tubes containing EDTA (1 mg ml^{-1} plasma) to prevent proteolytic breakdown of any peptides that might be present. These samples were transported on ice and immediately centrifuged at 3000 ***g*** at 4 °C for 15 min. After centrifugation, each sample was extracted with 100 % ethanol (1:2 dilution), vortexed, and allowed to stand at 4 °C for 30 min (Cousins and Farrell, 1996). Radioimmunoassays to measure peptides from the N terminus of the ANF 126-amino-acid prohormone were developed to amino acids 1–30 (proANF 1-30) and 31–67 (proANF 31-67) of this prohormone as previously described by Winters *et al.* (1989) and Vesely *et al.* (1989, 1994). For each RIA, the extracted plasma and/or perfusate sample was first reconstituted in 100 µl of 0.1 mol l^{-1} phosphate buffer (pH 7.4) containing 0.05 mol l^{-1} NaCl, 0.1 % bovine serum albumin, 0.1 % Triton X-100 and 0.01 % NaN_3. To the redissolved sample, 100 µl (0.03 mg) of rabbit IgG plus 100 µl of the respective antisera were added and the sample was incubated for 24 h. Then, 100 µl of ^{125}I-labeled peptide (10 000 cts min^{-1}) was added and the mixture was vortexed and incubated for 18 h at 4 °C. The antibody-bound tracer was then precipitated by adding 100 µl of goat anti-rabbit globulin and incubating this mixture for 2 h at room temperature (25 °C). Each tube was then centrifuged at 3000 ***g*** for 20 min. The supernatant was carefully aspirated off the remaining pellet. Each of these assays was performed in triplicate. The inter-assay coefficient of variation for the RIAs was 4.8 % for proANF 1-30, 5.3 % for proANF 31-67 and 5.7 % for ANF. The intra-assay coefficient of variation was 8.0 % for proANFs 1-30 and 31-67; ANF intra-assay variation was 6.9 %. Recovery was examined by adding synthetic unlabeled proANFs 1-30 and 31-67 and ANF at 100, 200 and 400 pg ml^{-1} to pooled plasma. Recovery for proANF 1-30 was 83.5±13.2 %, for proANF 31-67 100.9±8.9 % and for ANF 92.0±11.0 % (means ± S.E.M., N=10).

The molecular forms of the immunoreactive proANF peptides and ANF in trout plasma were determined using high-performance gel-permeation chromatography (HP-GPC). After centrifugation (3000 ***g***) for 30 min, 10 ml of trout plasma was extracted with 100 % ethanol (1:2 dilution) and then dried in a Speed-Vac A160 concentrator (Savant Instruments, Inc., Farmingdale, NY). The plasma extracts were then resuspended in 100 µl of HP-GPC column mobile phase (10 mmol l^{-1} trifluoroacetic acid containing 0.3 mol l^{-1} sodium chloride and 30 % acetonitrile) for HP-GPC assay (as described previously in Vesely *et al.* 1994). These 100 µl samples were applied to a TSK-Gel G2000SW column [7.5 mm×600 mm equipped with a 7.5 mm×75 mm guard column (Toyo Soda, Tokyo, Japan)] and eluted isocratically with HP-GPC column mobile phase at a flow rate of 0.3 ml min^{-1}. Fractions (0.3 ml) were collected, dried and then assayed for proANFs 1-30 and 31-67 and ANF by their respective RIAs described above. Blue Dextran (2×10^6 Da) was used to determine the void volume and *p*-aminohippuric acid (216 Da) the total volume of the column. To calibrate the column, the following molecular markers were used: carbonic anhydrase (29 000 Da), myoglobin (16 900 Da), cytochrome *c* (12 384 Da), vasoactive intestinal peptide (3300 Da) and (try^1)-somatostatin (1700 Da). The elution positions of proANF 1-30, proANF 31-67 and ANF were determined using ^{125}I-labeled synthetic peptides. Recoveries of these labeled peptides were as follows: 77 % for ANF, 74 % for proANF 1-30 and 82 % for proANF 31-67. High-performance gel-permeation chromatography was repeated three times for each of the RIAs.

If there were no changes in ANF release between the FW- and SW-acclimated groups, then step increases in filling pressure would result in similar increases in ANF release. Statistical comparisons were, therefore, made between the rates of ANF release from FW- and SW-acclimated groups. A repeated-measures three-way analysis of variance (ANOVA) was used to compare the mean values for the rate of ANF release at each P_I level (Winer *et al.* 1991) with significant

differences set at the 95 % confidence level (P<0.05). 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 possibility of a cross-over effect (Jones and Kenward, 1989). All cardiovascular and ANF release mean values were subjected to multiple comparisons using a single-factor ANOVA with the Student–Newman–Keuls method (SigmaStat Statistical Analysis System V.1, Jandel Corporation). 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

The morphometric comparisons between the FW- and SW-acclimated rainbow trout are presented in Table 1. There was a significant difference 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 noted between the treatment groups for the percentage of relative ventricular (P=0.066) or relative atrial (P=0.795) mass. In each treatment group, the sex was predominantly female. The freshwater group contained five females and one male, while the seawater group contained four females and two males.

ProANF 1-30 and proANF 31-67 were present in plasma (Fig. 1) and in the perfusate ejected from the perfused trout heart (Table 2). The ANF plasma content in the SW-acclimated group was slightly higher (37.20±2.80 pg ml^{-1}), but not significantly different (P=0.164) from that of the FW group (30.63±3.19 pg ml^{-1}). Both proANF 1-30 and 31-67 were found at plasma concentrations that were significantly higher than that for ANF (P=0.005; Fig. 1). In the FW group, the plasma contents of proANFs 1-30 and 31-67 were not significantly different (P=0.563); however, in the SW-acclimated group, the plasma content of proANF 1-30 was significantly higher than that of proANF 31-67 (P=0.001).

Table 1. *Morphometric data for the rainbow trout used in this study*

	Freshwater trout	Seawater trout
Atrial mass (mg)	78.45±9.30	67.78±4.82
Ventricular mass (g)	0.481±0.024	0.485±0.077
Body mass (g)	511.50±10.29*	449.50±22.56*
Fork length (cm)	34.17±0.15	34.00±0.0
RVM (%)	0.094±0.003	0.108±0.006
RAM (%)	0.015±0.002	0.015±0.001

All values are mean ± S.E.M. (N=6).

Relative ventricular mass (RVM) and relative atrial mass (RAM) were calculated as a percentage of the total body mass of the animal; (heart mass/body mass)×100.

Significant differences between the mean values for freshwater- and seawater-acclimated trout are indicated by an asterisk (P<0.05).

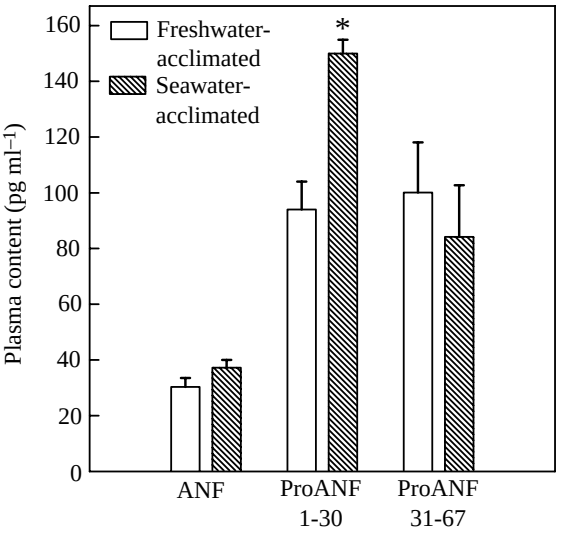

Fig. 1. The plasma content (in pg ml^{-1}) of ANF and proANFs 1-30 and 31-67 for freshwater (N=8) and seawater-acclimated rainbow trout (N=5). An asterisk denotes a significant difference from the freshwater group (P=0.001). Values are given as means + S.E.M.

At the control filling pressure, the rates of ANF release in the FW- and SW-acclimated groups were similar (Table 2). These values are similar to the control value for freshwater trout reported by Cousins and Farrell (1996) at 30.68±4.76 pg min^{-1} g^{-1} wet heart mass. ProANFs 1-30 and 31-67 were released at similar basal rates in both the FW- and SW-acclimated groups (Table 2). However, the basal release rate of proANF 1-30 was approximately 10 times higher (P<0.05) than that of ANF, and the basal release rate of proANF 31-67 was approximately 20 times higher (P<0.001) than the basal release rate of ANF (Table 2).

In the FW group, when the filling pressure to the perfused hearts was increased to elicit $\dot{Q}_{max}$, the release rates of proANFs 1-30 and 31-67 and ANF all increased by approximately fivefold compared with their basal rates of release (Table 2). Furthermore, randomized step increases in filling pressure, lasting 10 min each, resulted in proportional increases in both the stroke volume and the rate of ANF release (Table 3; Fig. 2). For each step increase in filling pressure, the rate of ANF release immediately increased to a new and higher level. At the highest filling pressure, the perfused hearts from the SW-acclimated group released proANF 1-30, proANF 31-67 and ANF at significantly higher rates (P=0.006) than those of the FW group (Table 2). Thus, the release of cardiac hormones occurred over the full range of filling pressures and was proportional to $\dot{Q}$.

Similar findings were made for the SW-acclimated group. However, increasing the filling pressure to elicit $\dot{Q}_{max}$ caused an even larger release (6- to 9.5 times their basal release rate) of proANFs 1-30 and 31-67 and ANF (Table 2) than in the FW group. For instance, at the highest filling pressure, the amount

Table 2. *Rate of release of proANFs and ANF into the perfusate for hearts of freshwater- and seawater-acclimated rainbow trout at basal and maximum cardiac outputs*

	Freshwater trout		Seawater trout	
	At $\dot{Q}$	At $\dot{Q}_{max}$	At $\dot{Q}$	At $\dot{Q}_{max}$
ANF (N=6)	33.86±2.09	195.82±19.10	30.00±1.89	285.3±19.63
ProANF 1-30 (N=3)	294.5±8.2	1426.5±74.9	296.9±20.0	1799.0±110.7
ProANF 31-67 (N=3)	570.1±69.8	2984.3±92.6	597.9±51.2	3851.6±332.5

The basal cardiac output ($\dot{Q}$) represents the control filling pressure, while the maximum cardiac output ($\dot{Q}_{max}$) represents the filling pressure at the peak of the Starling curve.

ProANFs 1-30, 31-67 and ANF were measured in the same trout with three extra trout being examined for ANF to confirm that the values were much lower at $\dot{Q}$ than for proANFs 1-30 and 31-67.

Values are given as (pg min^{-1} g^{-1} wet heart mass). All values are mean ± S.E.M.

of ANF secreted from a SW-acclimated trout heart in 1 min was 4.20 pg ml^{-1} perfusate, whereas the amount of ANF secreted from a FW-acclimated trout heart was 3.67 pg ml^{-1} perfusate. In addition to a higher maximum ANF release rate, the perfused hearts from the SW-acclimated group had significantly higher cardiac outputs (P=0.016) and stroke volumes (P=0.022) than those found for the FW group (Table 3). In the FW group, an increase in the filling pressure from the control level (0.03±0.04 kPa) to the highest value (0.58±0.03 kPa) significantly increased stroke volume, $\dot{Q}$ and power output by approximately threefold (P<0.05; Table 3). In the SW-acclimated group, a similar increase in filling pressure (from 0.02±0.02 to 0.55±0.02 kPa) also significantly increased stroke volume, $\dot{Q}$ and power output (P<0.05; Table 3), but to levels that were 10–15 % higher than those found for the FW group.

A typical standard curve for the proANF 1-30 RIA and the good parallelism of the circulating immunoreactive N-terminal ANF prohormone-like peptide(s) recognized by this assay in trout plasma, as well as in human plasma, is shown in Fig. 3.

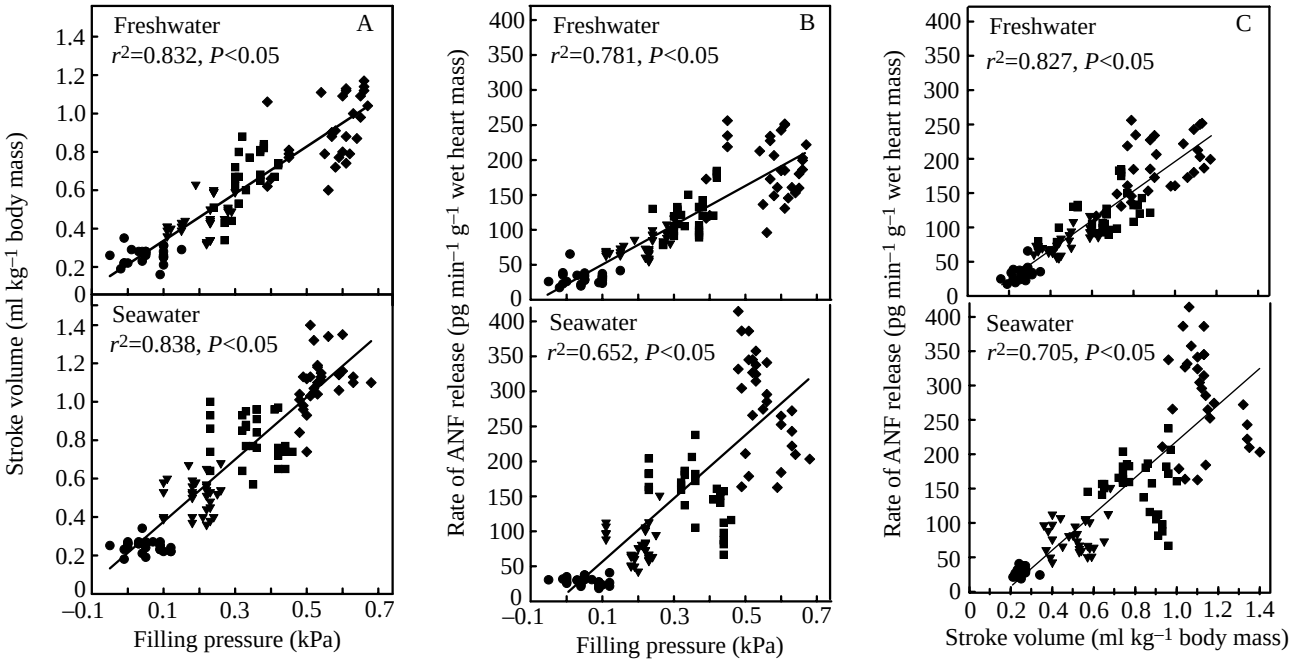

Fig. 2. Step increases in filling pressure to the perfused trout heart *in situ* result in step increases in stroke volume and the rate of ANF release. Stroke volume (A) is plotted as a function of filling pressure and illustrates the stability of the *in situ* preparation between the freshwater- and seawater-acclimated groups. The stroke volume (ml kg^{-1} body mass) increases in response to step increases in filling pressure (kPa) for the following cardiac outputs ($\dot{Q}$): ●=$\dot{Q}_{basal}$, ▼=30 % $\dot{Q}_{max}$, ■=60 % $\dot{Q}_{max}$ and ◆=$\dot{Q}_{max}$. The rate of ANF release (B) (pg min^{-1} g^{-1} wet heart mass) also increases with the same step increases in filling pressure (kPa). (C) For both the freshwater- and seawater-acclimated groups, the rate of ANF release (pg min^{-1} g^{-1} wet heart mass) is strongly correlated with increases in stroke volume (ml kg^{-1} body mass).

Table 3. *Rate of ANF release into the perfusate and cardiovascular variables for freshwater- and seawater-acclimated rainbow trout hearts at four levels of cardiac output*

	Freshwater trout				Seawater trout			
	Basal $\dot{Q}$	30 % $\dot{Q}_{max}$	60 % $\dot{Q}_{max}$	$\dot{Q}_{max}$	Basal $\dot{Q}$	30 % $\dot{Q}_{max}$	60 % $\dot{Q}_{max}$	$\dot{Q}_{max}$
Rate of ANF release (pg min^{-1} g^{-1} heart mass)	33.86±2.09[a]	72.61±5.46[b]	125.74±11.51[c]	195.82±19.10[d,*]	30.00±1.89[a]	80.44±5.74[b]	141.29±11.90[c]	285.38±19.63[d,*]
Filling pressure (kPa)	0.03±0.04[a]	0.21±0.02[b]	0.33±0.03[c]	0.58±0.03[d]	0.02±0.02[a]	0.19±0.02[b]	0.35±0.03[c]	0.55±0.02[d]
Output pressure (kPa)	5.23±0.06[a]	5.30±0.08[a]	5.38±0.06[b]	5.62±0.11[c]	5.18±0.04[a]	5.29±0.04[a]	5.56±0.12[b]	5.67±0.09[b]
Heart rate (beats min^{-1})	58.2±2.4[a]	62.0±3.6[a]	59.8±2.7[a]	59.8±2.8[a]	64.7±2.5[a]	65.2±1.1[a]	62.2±0.8[a]	59.7±1.8[a]
Stroke volume (ml kg^{-1} body mass)	0.30±0.01[a]	0.45±0.04[b]	0.68±0.04[c]	0.90±0.08[d,*]	0.27±0.02[a]	0.49±0.03[b]	0.80±0.04[c]	1.15±0.04[d,*]
Cardiac output (ml min^{-1} kg^{-1} body mass)	17.03±0.14[a]	31.93±4.98[b]	38.51±1.57[c,*]	53.40±4.11[d,*]	17.07±0.29[a]	32.00±2.15[b]	49.95±2.81[c,*]	68.11±3.01[d,*]
Power output (mW g^{-1} ventricular mass)	1.57±0.08[a]	2.44±0.08[b]	3.50±0.22[c]	5.02±0.56[d]	1.35±0.09[a]	2.53±0.14[b]	4.12±0.29[c]	5.64±0.35[d]

The basal cardiac output ($\dot{Q}$) represents the control filling pressure, while the maximum cardiac output ($\dot{Q}_{max}$) represents the filling pressure at the peak of the Starling curve.

All values are mean ± S.E.M. (N=6).

Statistically significant differences (P<0.05) between the freshwater and seawater mean values are indicated by an asterisk. Dissimilar superscript letters indicate significant differences (P<0.05) between values within each group of trout.

Evaluation of this assay revealed that it is sensitive, with an EC_{50} of 180 fmol per tube. The proANF 1-30 RIA is also specific, showing less than 0.5 % cross-reactivity with either proANF 31-67 or ANF (Fig. 3). The antiserum to proANF 1-30 had no detectable cross-reactivity with oxytocin, vasopressin, somatostatin, insulin or adrenocorticotrophic hormone.

The excellent parallelism of circulatory fluid concentrations of proANF 31-67 in trout and human plasma with a typical proANF 31-67 RIA standard curve is illustrated in Fig. 4. The proANF 31-67 RIA can detect as little as 35 fmol of synthetic proANF 31-67, and its EC_{50} of 120 fmol per tube shows that it is a sensitive assay. It is also specific, as shown by the dilution curves with proANF 1-30 and ANF (Fig. 4), showing that the cross-reactivity with these two peptides is less than 0.5 %. In addition, the proANF 31-67 antiserum, like the proANF 1-30 antiserum, had no detectable cross-reactivity with oxytocin, vasopressin, somatostatin, insulin or adrenocorticotropic hormone.

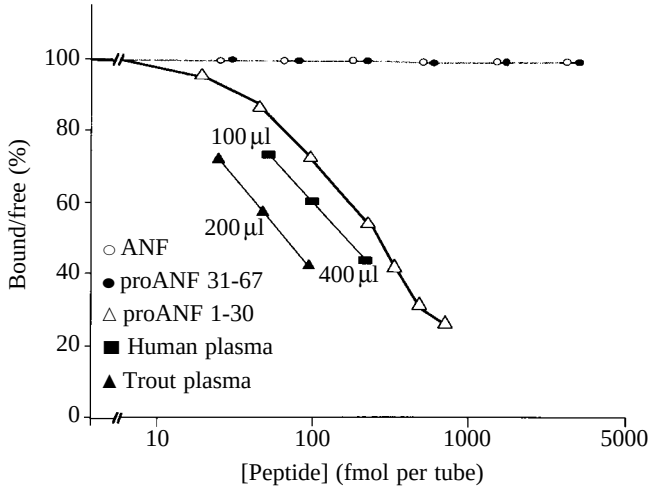

Fig. 3. Representative standard curve for the prohormone of atrial natriuretic factor (proANF) 1-30 radioimmunoassay with dilution curves of ANF, proANF 31-67, plus trout and human plasma. There was less than 0.5 % cross-reactivity with either ANF or proANF 31-67. Immunoreactivity paralleled this radioimmunoassay curve as demonstrated with 100, 200 and 400 µl of trout and human plasma. The EC_{50} of the assay was 180 fmol per tube.

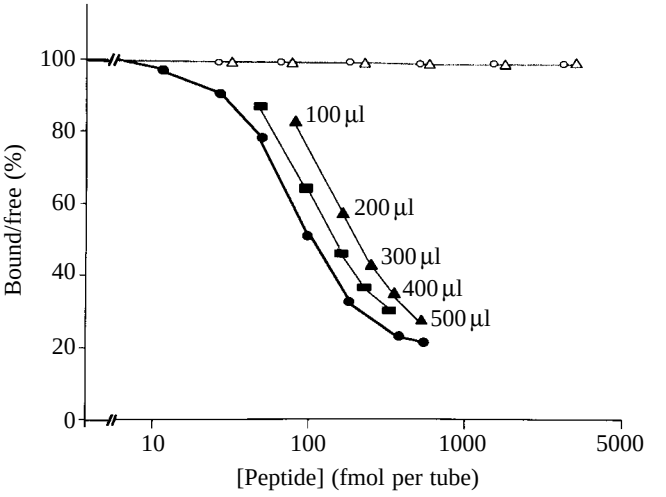

Fig. 4. Typical standard curve for the prohormone of atrial natriuretic factor (proANF) 31-67 radioimmunoassay (RIA) with dilution curves of ANF, proANF 1-30, plus trout and human plasma. Immunoreactivity of proANF 31-67 paralleled this RIA curve as observed with 100, 200, 300, 400 and 500 µl samples of trout and human plasma. There was less than 0.5 % cross-reactivity with either ANF or proANF 1-30. The EC_{50} of the assay was 120 fmol per tube. Symbols as in Fig. 3.

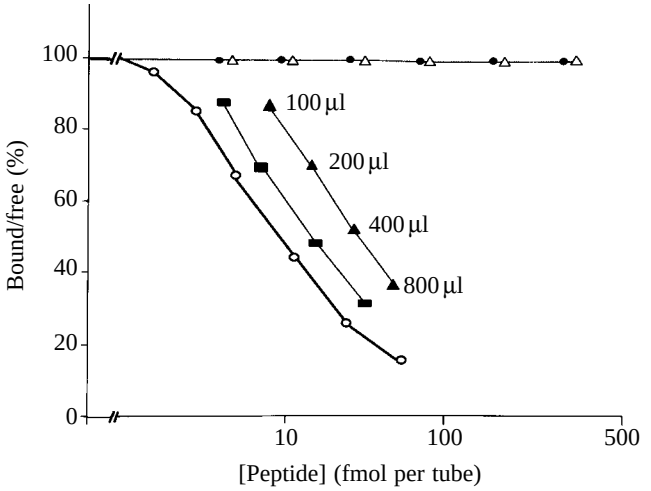

Fig. 5. A representative radioimmunoassay standard curve for atrial natriuretic factor. Immunoreactive ANF paralleled this curve as seen with 100, 200, 400 and 800 µl of trout and human plasma. There was less than 0.5 % cross-reactivity with either proANF 1-30 or proANF 31-67. The EC_{50} of the assay was 11 fmol per tube. Symbols as in Fig. 3.

The addition of 100, 200, 400 and 800 µl of trout and human plasma also revealed very good parallelism for circulating ANF-like peptides with the ANF RIA standard curve, as observed in Fig. 5. The ANF RIA had less than 0.5 % cross-reactivity with either proANF 1-30 or proANF 31-67 (Fig. 5) and is a sensitive assay able to detect as little as 1.4 fmol of ANF and with an EC_{50} of 11 fmol per tube.

Human synthetic proANF 1-30 was found in 0.3 ml fractions 70–74, with fraction 71 having the predominant peak when examined by HP-GPC (Fig. 6). The human synthetic form of proANF 31-67 was found in 0.3 ml fractions 67–73, with the major peak in fraction 69 (Fig. 6). The human synthetic form of ANF was in 0.3 ml fractions 74–79, with the peak in fraction 76 (Fig. 6). The elution profiles of [^{125}I]proANF 1-30 (peak of fraction 71), [^{125}I]proANF 31-67 (peak at fraction 70) and [^{125}I]ANF (peak of fraction 76) were nearly identical to those of the unlabeled peptides.

Analyses of trout plasma by HP-GPC followed by RIAs to amino acids 1–30, 31–67 and 99–126 (i.e. ANF) of the ANF prohormone indicated that proANFs 1-30, 31-67 and 1-98 each circulate in the blood (Fig. 7). The RIA for proANF 1-30 revealed that this assay recognized a peptide with a molecular mass of 9800 Da. This finding is consistent with the 98-amino-acid N terminus of the prohormone without an attached C terminus (i.e. ANF). This assay also had a peak where the human synthetic form of proANF 1-30 elutes (Fig. 7A), suggesting that proANF 1-30 exists in trout plasma as a separate entity from proANF 1-98. From the peaks shown in Fig. 7A, one can observe that approximately 50 % of the material recognized by the proANF 1-30 assay actually is proANF 1-30, while the other 50 % of the material recognized by the probe is circulating proANF 1-98. Similar HP-GPC evaluation of trout plasma followed by proANF 31-67 RIA of 0.3 ml fractions revealed only one peak where the human sequence of proANF 31-67 elutes (Fig. 7B). Neither proANF 1-98 nor the ANF prohormone (i.e. proANF 1-126) was

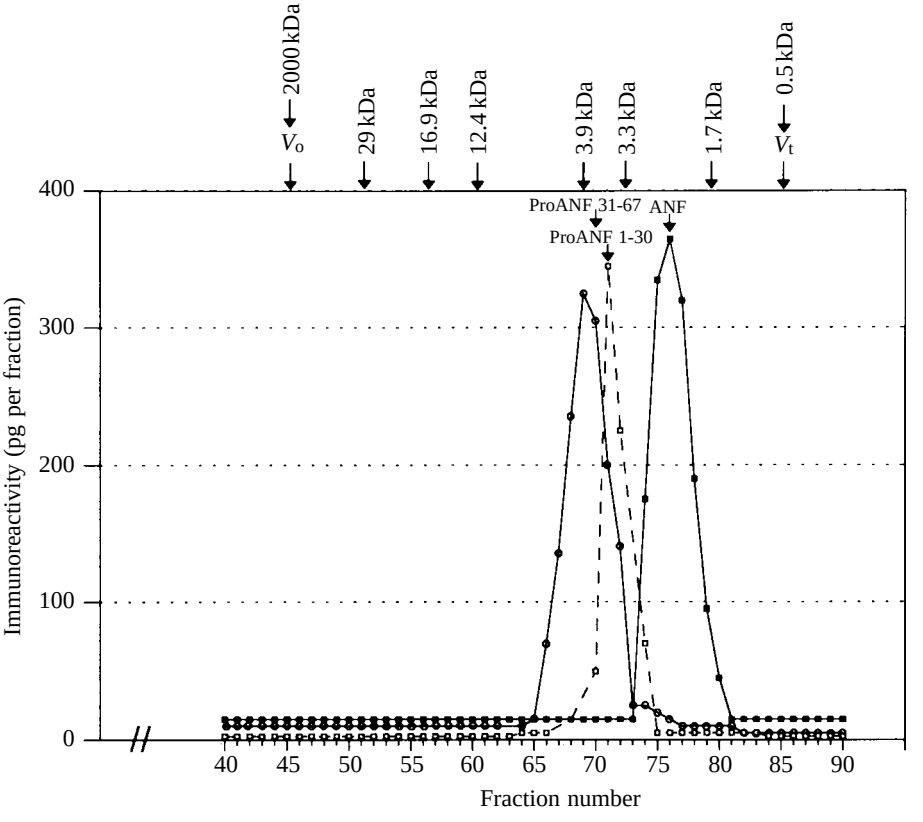

Fig. 6. High-performance gel-permeation chromatography of synthetic human sequence proANFs 1-30 and 31-67 and ANF. Also shown are the respective ^{125}I-labeled peptide elution profiles (arrows). V_o, void volume of column; V_t, total volume of column; fraction volume was 0.3 ml.

immunologically recognized in trout plasma by the proANF 31-67 assay. Evaluation of trout plasma by HP-GPC followed by ANF RIA also revealed only one peak, which eluted identically with the pure synthetic form of ANF (Fig. 7C).

Discussion

This is the first study to compare the effects of atrial stretch on ANF release rates in freshwater- and seawater-acclimated rainbow trout, and is only the second study to report ANF release rates in fish. The basal release rate for ANF from FW-acclimated perfused hearts is comparable to that reported earlier for the same type of preparation (Cousins and Farrell, 1996). We found a higher rate of release in the SW-acclimated group than in the FW group for the same level of cardiac filling pressure at the maximum cardiac output and, furthermore, the difference was significant relative to the overall changes that are possible in the ANF release rate. In the FW group, ANF release increased 5.8-fold (from 33.9 to 195.8 pg min^{-1} g^{-1} wet heart mass), whereas in the SW-acclimated group ANF release increased 9.5-fold (from 30.0 to 285.4 pg min^{-1} g^{-1} wet heart mass). Part of this difference can be attributed to the fact that the SW-acclimated group had a significantly greater $\dot{Q}_{max}$ (Table 3). Even though the ventricular mass (Table 1) and filling pressures were not significantly different between the FW- and SW-acclimated groups (P=0.930 and P=0.384, respectively), the SW-acclimated hearts still had greater stroke volumes (Table 3). Nevertheless, the SW-acclimated group released more ANF per milliliter of perfusate passing through the heart relative to the FW group (4.20 pg ml^{-1} *versus* 3.67 pg ml^{-1}). The consequence of this higher release rate is that the trout hearts in the SW-acclimated group were more sensitive to filling pressure in terms of both ANF release and the Starling response (Fig. 2). In fact, for a 0.1 kPa change in filling pressure, the change in ANF release for the SW-acclimated group (12.78 pg min^{-1} g^{-1} wet heart mass) is almost twice the value for the FW group (7.23 pg min^{-1} g^{-1} wet heart mass). We have no definitive explanation for these differences. We do know, however, that Thorarensen *et al.* (1996) reported higher *in vivo* maximum $\dot{Q}$ measured during swimming in SW than in FW trout. Our $\dot{Q}_{max}$ values for FW trout are virtually identical to the maximum $\dot{Q}$ reported by Kiceniuk and Jones (1977) (53.4 ml min^{-1} kg^{-1} body mass) during prolonged swim challenges. Thus, it appears that the acclimation to sea water somehow caused the hearts to have an increased sensitivity to the higher filling pressures, which in turn resulted in an increase in ANF release from the heart when the myocytes were stretched to the peak of the Frank–Starling curve. This finding also suggests that ANF release in the perfused trout

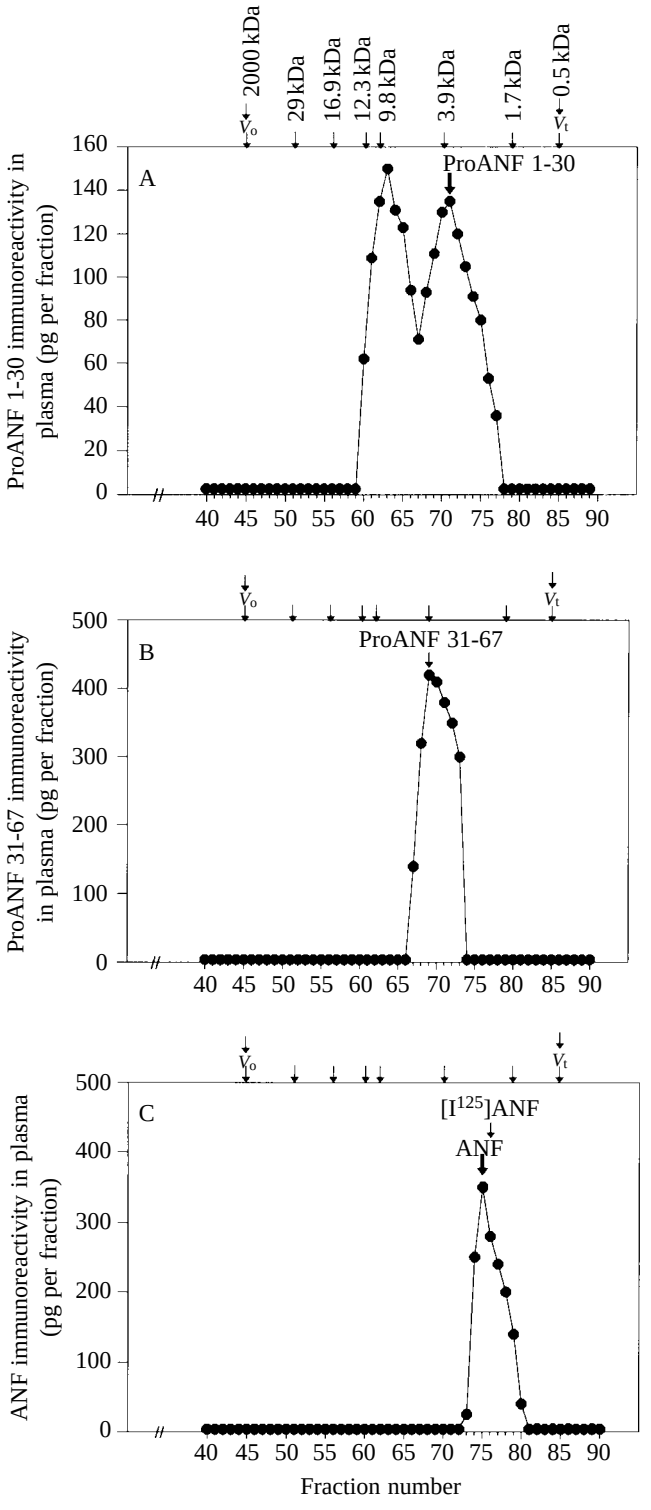

Fig. 7. (A) Evidence that proANF 1-30 exists as a separate entity in trout plasma when plasma is subjected to high-performance gel-permeation chromatography followed by proANF 1-30 radioimmunoassay. In the evaluation of trout plasma, two large sharp peaks were seen. One of these, at 9800 Da, was consistent with proANF 1-98, while a second peak at fractions 69–72 was seen in the region where the pure synthetic form of proANF 1-30 (arrow) elutes. (B) ProANF 31-67 exists in trout plasma as a distinct peptide. This elution of proANF 31-67 from plasma was identical with the elution of the pure human synthetic form of proANF 31-67. (C) Atrial natriuretic factor exists as a separate entity in trout plasma. This peak in trout plasma was identical with the elution of pure synthetic human sequence of ANF (arrow). V_o, void volume of column; V_t, total volume of column. Fraction size was 0.3 ml.

heart was driven more by volume changes than by pressure changes.

By comparing the osmotic and volume challenges faced by FW- and SW-acclimated trout, we can assess the significance of the greater release rate of ANF and the heightened sensitivity of release to the major release stimulus (atrial stretch). Marine fish are in hyperosmotic environments and are in danger of hypovolemia (i.e. low blood volume). Freshwater fish have serum Na^+ and Cl^- contents above that of their environment, subjecting them to the danger of hypervolemia (i.e. high blood volume). Euryhaline fish, such as the adult rainbow trout used in this study, can inhabit both freshwater and seawater environments while maintaining relatively constant blood volumes (Olson, 1992) and arterial blood pressures. The ventral aortic blood pressures of freshwater rainbow trout are reported to be only slightly higher (5.33 kPa, Stevens and Randall, 1967) than those of seawater-acclimated trout (4.55 kPa; Thorarensen, 1994). Since ANF is known to be released from the trout heart in response to elevated cardiac filling pressures (Cousins and Farrell, 1996) and given its known vasoactive, natriuretic and diuretic activities (Duff and Olson, 1986; Olson and Meisheri, 1989; Olson and Duff, 1992, 1993; Jensen and Olson, 1994), it seems reasonable to assume that ANF is intimately involved in the osmotic, ionic and blood volume regulation of the animal. As soon as ANF is released from the trout heart, it circulates through the primary osmotic and ionic regulating organ of the animal, namely, the gills. In freshwater trout, ANF-clearance gill receptors have been shown to remove at least 60 % of an arterial injection of synthetic ANF (Olson and Duff, 1992, 1993). Since SW-acclimated trout are continually faced with water loss and excess salts, their higher rates of ANF release may be related more to a natriuretic effect than to a diuretic effect. Whether the higher ANF release rate is also related to the slightly lower arterial blood pressures through vasoactive effects will require further studies to resolve.

The high-performance gel-permeation chromatography (HP-GPC) evaluation of fish plasma followed by ANF radioimmunoassay revealed that an ANF-like peptide in fish plasma had identical elution characteristics to those of the pure human sequence of ANF (Fig. 7A), indicating that ANF in trout is very similar to the human form of ANF. The amino acid sequence of ANF is known to be highly conserved, showing a 93 % homology when five species were compared (Vesely, 1992). This HP-GPC evaluation further revealed that the ANF assay recognized ANF in fish plasma and was not measuring ventricular natriuretic peptide (VNP). Since trout VNP is a 35-amino-acid peptide with a molecular mass of 3860 Da (Takei *et al.* 1994) and no peptide eluted at this molecular mass when the trout plasma was subjected to HP-GPC followed by the ANF radioimmunoassay, it appears that the human ANF assay does not immunologically recognize VNP in the trout plasma. It should also be noted that, using this evaluation, the ANF prohormone, which elutes at a molecular mass of 12 900 Da, does not circulate in the trout plasma (see Fig. 7). A similar finding has been reported in humans (Vesely *et al.* 1994) where ANF, proANF 1-30 and proANF 31-67 circulate, but the prohormone form from which they are derived does not.

In the present investigation, levels of circulating proANFs 1-30 and 31-67 in the plasma of a fish were measured for the first time. To assess whether the trout peptides identified by the proANF 1-30 and 31-67 RIAs were similar to the proANF 1-30 and 31-67 peptides in human plasma, the fish plasma was diluted and compared with human plasma using standard binding curves for these peptides. The excellent parallelism of trout plasma with the proANF 1-30 (Fig. 3) and proANF 31-67 (Fig. 4) binding curves suggests that the assays are specific for these peptides in trout plasma and that the amino acid sequences of the peptides in trout are similar to the sequences in humans. If the fish and human amino acid sequences were dissimilar, these peptides would not be recognized by antisera raised to the human sequences of proANF 1-30 and proANF 31-67. This is also true for ANF in trout plasma, which closely parallels the human ANF standard curve (Fig. 5). To characterize further proANF 1-30 and proANF 31-67 in fish plasma, HP-GPC of the plasma was followed by proANF 1-30 and proANF 31-67 RIAs. This technique revealed that trout plasma was contained a peptide that had identical elution characteristics to those of the pure human amino acid sequence of proANF 1-30. The HP-GPC evaluation of the trout plasma followed by a proANF 31-67 RIA also showed that only one peptide showed an immunological reaction and this co-eluted with the human form of proANF 31-67 (Fig. 7B). The proANF 31-67 assay does not immunologically recognize the N terminus of the ANF prohormone in trout plasma, and neither the proANF 1-30 nor the proANF 31-67 assay recognizes the ANF prohormone in the fish plasma, strongly suggesting that the ANF prohormone does not circulate in trout.

ProANFs 1-30 and 31-67 and ANF were found to be released proportionately with increasing filling pressure in perfused trout hearts from both the FW- and SW-acclimated groups, and the size of the increase at maximum filling pressure was similar for ANF, proANF 1-30 and proANF 31-67. Thus, it appears that an increase in circulating blood volume is important for the release of these cardiac peptide hormones in both freshwater and seawater fish. This parallels events in humans, where an increase in central blood volume simultaneously releases proANFs 1-30 and 31-67 and ANF (Vesely *et al.* 1989) and the relative increase in each is similar to that found in the FW trout. This also occurs in other mammals, where it has been found that an increase in atrial pressure of only 0.27–0.53 kPa in isolated rat hearts simultaneously releases proANFs 1-30 and 31-67 and ANF (Dietz *et al.* 1991). Thus, it appears that similar mechanisms for the cardiac release of proANFs and ANF may operate for trout and mammals.

The release of higher levels of ANF peptides in the SW-acclimated group could have been caused by an increase in plasma $[Na^+]$ and $[Cl^-]$ in those animals, which somehow improved their myocardial performance at the higher filling pressures. The enhanced release of these cardiac peptides in

the SW-acclimated trout exposed to high salt concentrations does have a theoretical advantage in that proANFs 1-30 and 31-67 and ANF are all potent stimulators of enhanced Na^+ excretion in mammals (Martin *et al.* 1990; Vesely *et al.* 1994) and may also have this role in fish. In this context, it has previously been shown in other euryhaline animals, such as the oyster (*Crassostrea virginica*), that the amount of salt in the external environment directly regulates the release of proANFs 1-30 and 31-67 and ANF, with increasing concentrations of peptides being released as Na^+ levels in their environment rise (Palmer *et al.* 1994).

Our findings for the effect of filling pressure on proANF release in trout are consistent with previous findings in mammals where the cardiac release of proANFs and ANF is enhanced by increasing central venous volume. Furthermore, the proportional increase in release of proANF and ANF is the same, even though basal release rates are quite different. These proANF peptide hormones, which cause prolonged natriuresis and diuresis compared with ANF in humans (Vesely *et al.* 1994), were found at threefold higher concentrations than ANF. A similar ratio of proANF to ANF concentration has been found in the hemolymph circulation of American oysters and blue crabs (*Callinectes sapidus*; Poulos *et al.* 1995), where proANF 31-67 levels are 2.5-fold (oyster) and fourfold (crab) higher than ANF levels. In mammals, the [proANF]:[ANF] ratio is even higher; the concentration of proANF 31-67 in the circulation is approximately seven times higher than the concentration of ANF in humans (Winters *et al.* 1989), dogs (Ngo *et al.* 1989) and rats (Martin *et al.* 1990). Whether variations in the [proANF]:[ANF] ratio are adaptations to living in aqueous as opposed to terrestrial habitats is not yet clear but, obviously, both terrestrial and seawater vertebrates face a similar danger of dehydration.

In summary, this study compares measurements of ANF release rates from the hearts of freshwater- and seawater-acclimated rainbow trout and demonstrates that ANF-related cardiac peptides (proANF 1-30 and proANF 31-67) are released from the fish heart and circulate in the blood at higher concentrations than ANF. The release of ANF from the perfused trout heart *in situ* was found to be a graded response to step increases in filling pressure. In both FW- and SW-acclimated trout, the ANF release rate changed by several orders of magnitude in direct proportion to increases in filling pressure, but this response was greatly enhanced in the SW-acclimated animals. The rate of release of both the proANFs 1-30 and 31-67 also increased when the filling pressure was increased to the maximum cardiac output. The sensitivity of ANF release in SW fish to filling pressure, however, was heightened compared with that of FW trout. It appears, therefore, that the secretion of proANFs and ANF in the trout heart may be regulated more by changes in volume than by changes in pressure.

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References

AGNOLETTI, G., FERRARI, R., SLADE, A. M., SEVERS, N. J. AND HARRIS, P. (1989). Stretch-induced centrifugal movement of atrial specific granules – a preparatory step in atrial natriuretic peptide secretion. *J. molec. cell. Cardiol.* **21**, 235–239.

ANDERSON, J. V., MILLAR, N. D., O'HARE, J. P., MACKENZIE, J. C., CORRALL, R. J. M. AND BLOOM, S. R. (1986). Atrial natriuretic peptide: Physiological release associated with natriuresis during water immersion in man. *Clin. Sci.* **71**, 319–322.

BAEYENS, D. A., PRICE, E., WINTERS, C. J. AND VESELY, D. L. (1989). Diving increases atrial natriuretic factor-like peptide in freshwater diving turtles. *Comp. Biochem. Physiol.* A **94**, 515–518.

BARANOWSKI, R. L. AND WESTENFELDER, C. (1989). Secretion of atrial natriuretic peptide (ANP) from fish atrial and ventricular myocytes in tissue culture. *Life Sci.* **44**, 187–191.

COUSINS, K. L. AND FARRELL, A. P. (1996). Stretch-induced release of atrial natriuretic factor (ANF) from the heart of rainbow trout (*Oncorhynchus mykiss*). *Can. J. Zool.* **74**, 380–387.

DEBOLD, A. J. (1979). Heart atrial granularity effects changes in water–electrolyte balance. *Proc. Soc. exp. Biol. Med.* **161**, 508–511.

DEBOLD, A. J., BORENSTEIN, H. B., VERESS, A. T. AND SONNENBERG, H. (1981). A rapid and potent natriuretic response to intravenous injections of atrial myocardial extracts in rats. *Life Sci.* **28**, 89–94.

DIETZ, J. R. (1984). Release of natriuretic factor from rat heart–lung preparation by atrial distension. *Am. J. Physiol.* **247**, R1093–R1096.

DIETZ, J. R. (1987). Control of atrial natriuretic factor release from a rat heart–lung preparation. *Am. J. Physiol.* **252**, R498–R502.

DIETZ, J. R., NAZIAN, S. J. AND VESELY, D. L. (1991). Release of ANF, pro-ANF (1-98) and pro-ANF (31-67) from isolated rat atria by atrial distension. *Am. J. Physiol.* **260**, H1774–H1778.

DIETZ, J. R., VESELY, D. L. AND NAZIAN, S. J. (1992). The effect of changes in sodium intake on atrial natriuretic factor (ANF) and peptides derived from the N-terminus of the ANF prohormone in the rat. *Proc. Soc. exp. Biol. Med.* **200**, 44–48.

DUFF, D. W. AND OLSON, K. R. (1986). Trout vascular and renal responses to atrial natriuretic factor and heart extracts. *Am. J. Physiol.* **215**, R639–R642.

EDDY, F. B. AND CHANG, Y. J. (1993). Effects of salinity in relation to migration and development in fish. In *The Vertebrate Gas Transport Cascade: Adaptations to Environment and Mode of Life* (ed. J. E. P. W. Bicudo), pp. 35–42. Boca Raton, FL: CRC Press.

EDDY, F. B., SMITH, N. F., HAZON, N. AND GRIERSON, C. (1990). Circulatory and ionoregulatory effects of atrial natriuretic peptide on rainbow trout (*Salmo gairdneri* Richardson) fed normal or high levels of dietary salt. *Fish Physiol. Biochem.* **8**, 321–327.

EDWARDS, B. S., ZIMMERMAN, R. S., SCHWAB, T. R., HEUBLEIN, D. M. AND BURNETT, J. C., JR (1988). Atrial stretch, not pressure, is the principal determinant controlling the acute release of atrial natriuretic factor. *Circulation Res.* **62**, 191–195.

EVANS, D. H., CHIPOURAS, E. AND PAYNE, J. A. (1989). Immunoreactive atriopeptin in plasma of fishes: its potential role in gill hemodynamics. *Am. J. Physiol.* **26**, R939–R945.

FARRELL, A. P., JOHANSEN, J. A. AND GRAHAM, M. S. (1988). The role of the pericardium in cardiac performance of the trout (*Salmo gairdneri*). *Physiol. Zool.* **61**, 213–221.

FARRELL, A. P., MACLEOD, K. R. AND CHANCEY, B. (1986). Intrinsic mechanical properties of the perfused rainbow trout heart and the effects of catecholamines and extracellular calcium under control and acidotic conditions. *J. exp. Biol*. **125**, 319–345.

FARRELL, A. P. AND MILLIGAN, C. L. (1986). Myocardial intracellular pH in a perfused rainbow trout heart during extracellular acidosis in the presence and absence of adrenaline. *J. exp. Biol*. **125**, 347–359.

FARRELL, A. P., WOOD, S., HART, T. AND DRIEDZIC, W. R. (1985). Myocardial oxygen consumption in the sea raven, *Hemitripterus americanus*: the effects of volume loading, pressure loading and progressive hypoxia. *J. exp. Biol.* **117**, 237–250.

FLYNN, T. G., DAVIES, P. L., KENNEDY, B. P., DEBOLD, M. L. AND DEBOLD, A. J. (1985). Alignment of rat cardionatrin sequences with the preprocardionatrin sequence from complementary DNA. *Science* **228**, 323–325.

GRAHAM, M. S. AND FARRELL, A. P. (1989). The effect of temperature acclimation and adrenaline on the performance of a perfused trout heart. *Physiol. Zool.* **62**, 38–61.

IMADA, T., TAKAYANAGI, R. AND INAGAMI, T. (1988). Atrioactivase, a specific peptidase in bovine atria for the processing of pro-atrial natriuretic factor. *J. biol. Chem.* **263**, 9515–9519.

JENSEN, J. AND OLSON, K. R. (1994). The effect of atrial natriuretic peptide on trout stomach motility. *Comp. Biochem. Physiol.* A **108**, 273–277.

JONES, B. AND KENWARD, M. G. (1989). *Design and Analysis of Cross-over Trials*. New York: Chapman & Hall.

KICENIUK, J. W. AND JONES, D. R. (1977). The oxygen transport system in trout (*Salmo gairdneri*) during sustained exercise. *J. exp. Biol*. **69**, 247–260.

LANG, R. E., THOELKEN, H., GANTEN, D., LUFT, F. C., RUSKOAHO, H. AND UNGER, T. (1985). Atrial natriuretic factor – a circulating hormone stimulated by volume loading. *Nature* **314**, 264–266.

LEDSOME, J. R., WILSON, N., COURNEYA, C. A. AND RANKIN, A. J. (1985). Release of atrial natriuretic peptide by atrial distension. *Can. J. Physiol. Pharmac*. **63**, 739–742.

MARTIN, D. R., PEVAHOUSE, J. B., TRIGG, D. J., VESELY, D. L. AND BUERKERT, J. E. (1990). Three peptides from the ANF prohormone NH_2-terminus are natriuretic and/or kaliuretic. *Am. J. Physiol*. **258**, F1401–F1408.

NGO, L., WYETH, R. P., BISSETT, J. K., HESTER, W. L., NEWTON, M. T., SALLMAN, A. L., WINTERS, C. J. AND VESELY, D. L. (1989). Prohormone atrial natriuretic peptides 1-30, 31-67 and 99-126 increase in proportion to right ventricular pacing rate. *Am. Heart J*. **117**, 385–390.

OLSON, K. R. (1992). Blood and extracellular fluid volume regulation: role of the renin–angiotensin system, kallikrein–kinin system and atrial natriuretic peptides. In *Fish Physiology*, vol. XIIB (ed. W. S. Hoar, D. J. Randall and A. P. Farrell), pp. 135–254. San Diego: Academic Press, Inc.

OLSON, K. R. AND DUFF, D. W. (1992). Atrial natriuretic peptide clearance receptors in trout: effects of receptor inhibition *in vivo*. *J. exp. Zool.* **262**, 343–346.

OLSON, K. R. AND DUFF, D. W. (1993). Single-pass gill extraction and tissue distribution of atrial natriuretic peptide in trout. *Am. J. Physiol.* **265**, R124–R131.

OLSON, K. R. AND MEISHERI, K. D. (1989). Effects of atrial natriuretic factor on isolated arteries and perfused organs of trout. *Am. J. Physiol*. **256**, R10–R18.

PALMER, P. A., FRIEDL, F. E., GIORDANO, A. T. AND VESELY, D. L. (1994). Alteration of environmental salinity modulates atrial natriuretic peptides concentrations in heart and hemolymph of the oyster, *Crassostrea virginica*. *Comp. Biochem. Physiol*. **108**A, 589–597.

POULOS, J. E., GOWER, W. R., JR, FRIEDL, F. E. AND VESELY, D. L. (1995). Atrial natriuretic peptide gene expression within invertebratic hearts. *Gen. comp. Endocr*. **100**, 61–68.

SAGNELLA, G. A., SHORE, A. C., MARKANDU, N. D. AND MACGREGOR, G. A. (1985). Effects of changes in dietary sodium intake and saline infusion on immunoreactive atrial natriuretic peptide in human plasma. *Lancet* **2**, 1208–1211.

SEIDMAN, C. E., BLOCH, K. D., ZISFEIN, J. B., SMITH, J. A., HABER, E., HOMCY, C. J., DUBY, A. D., CHOI, E., GRAHAM, R. M. AND SEIDMAN, J. G. (1985). Molecular studies of the atrial natriuretic factor gene. *Hypertension* **7**, 31–34.

SMITH, N. F., EDDY, F. B., STRUTHERS, A. D. AND TALBOT, C. (1991). Renin, atrial natriuretic peptide and blood plasma ions in parr and smolts of Atlantic salmon *Salmo salar* L. and rainbow trout *Oncorhynchus mykiss* (Walbaum) in fresh water and after short-term exposure to sea water. *J. exp. Biol.* **157**, 63–74.

STEVENS, E. D. AND RANDALL, D. J. (1967). Changes in blood pressure, heart rate and breathing rate during moderate swimming activity in rainbow trout. *J. exp. Biol.* **46**, 307–315.

TAKAYANAGI, R., TANEKA, I., MAKI, M. AND INAGAMI, T. (1985). Effects of changes in water–sodium balance on levels of atrial natriuretic factor messenger RNA and peptide in rats. *Life Sci.* **36**, 1843–1848.

TAKEI, Y., TAKANO, M., ITAHARA, Y., WATANABE, T. X., NAKAJIMA, K., CONKLIN, D. J., DUFF, D. W. AND OLSON, K. R. (1994). Rainbow trout ventricular natriuretic peptide: Isolation, sequencing and determination of biological activity. *Gen. comp. Endocr*. **96**, 420–426.

TAKEI, Y., TAMAKI, H. AND ANDO, K. (1990). Identification and partial characterization of immunoreactive and bioactive atrial natriuretic peptide from eel heart. *J. comp. Physiol.* B **160**, 119–126.

TANAKA, I., MISONO, K. S. AND INAGAMI, T. (1984). Atrial natriuretic factor in rat hypothalamus, atria and plasma: determination by specific radioimmunoassay. *Biochem. biophys. Res. Commun.* **124**, 663–668.

THORARENSEN, H. (1994). Gastrointestinal blood flow in chinook salmon (*Oncorhynchus tshawytscha*). PhD thesis, Simon Fraser University, Burnaby, B.C.

THORARENSEN, H., GALLAUGHER, P. AND FARRELL, A. P. (1996). Cardiac output in swimming rainbow trout, *Oncorhynchus mykiss*, acclimated to seawater. *Physiol. Zool.* **69**, 139–153.

UEMURA, H., NARUSE, M., HIROHAMA, T., NAKAMURA, S., KASUYA, Y. AND AOTO, T. (1990). Immunoreactive atrial natriuretic peptide in the fish heart and blood plasma examined by electron microscopy, immunohistochemistry and radioimmunoassay. *Cell Tissue Res.* **260**, 235–247.

UVA, B. M., MASINI, M. A., NAPOLI, L. AND DEVECCHI, M. (1993). Immunoreactive atrial natriuretic-like peptide in Antarctic teleosts. *Comp. Biochem. Physiol.* A **104**, 291–297.

VESELY, D. L. (1992). Atrial natriuretic hormones. In *Prentice Hall Endocrine Series* (ed. M. E. Hadley), p. 235. New Jersey: Prentice Hall, Inc.

VESELY, D. L., BAYLISS, J. M. AND SALLMAN, A. L. (1987). Human preproatrial natriuretic factors 26-55, 56-92, 104-123 increase renal guanylate cyclase activity. *Biochem. biophys. Res. Commun*. **143**, 186–193.

VESELY, D. L., DOUGLASS, M. A., DIETZ, J. R., GOWER, W. R., JR, MCCORMICK, M. T., RODRIGUEZ-PAZ, G. AND SCHOCKEN, D. D. (1994). Three peptides from the atrial natriuretic factor prohormone

amino terminus lower blood pressure and produce a diuresis, natriuresis and/or kaliuresis in humans. *Circulation* **90**, 1129–1140.

VESELY, D. L., GIORDANO, A. T. AND FRIEDL, F. E. (1991). Atrial natriuretic peptides in invertebrates: oyster ventricle, auricle and blood contain all 126 amino acids of the atrial natriuretic factor prohormone. *Clin. Res.* **39**, 365A.

VESELY, D. L., NORSK, P., WINTERS, C. J., RICO, D. M., SALLMAN, A. L. AND EPSTEIN, M. (1989). Increased release of the N-terminal and C-terminal portions of the prohormone of atrial natriuretic factor during immersion-induced central hypervolemia in normal humans. *Proc. Soc. exp. Biol. Med*. **192**, 230–235.

VESELY, D. L., SALLMAN, A. L. AND BAYLISS, J. M. (1992). Specific binding sites for pro atrial natriuretic factors 1-30, 31-67 and 99-126 on distal nephrons, proximal tubules, renal cortical and medullary membranes. *Renal Physiol. Biochem*. **15**, 23–32.

WEIDMANN, P., HELLMUELLER, B., UEHLINGER, D. E., LANG, R. E., GNADINGER, M. P., HASLER, L., SHAW, S. AND BACHMANN, C. (1986). Plasma levels and cardiovascular, endocrine and excretory effects of atrial natriuretic peptide during different sodium intakes in man. *J. clin. Endocr. Metab*. **62**, 1027–1036.

WESTENFELDER, C. F., BIRCH, M., BARANOWSKI, R. L., ROSENFELD, M. J., SHIOZAWA, D. K. AND KABLITZ, C. (1988). Atrial natriuretic factor and salt adaptation in the teleost fish *Gila atraria*. *Am. J. Physiol.* **255**, F1281–F1286.

WINER, B. J., BROWN, D. R. AND MICHELS, K. M. (1991). Three-factor experiment with repeated measures. In *Statistical Principles in Experimental Design*, pp. 547–550. New York: McGraw-Hill.

WINTERS, C. J., SALLMAN, A. L., BAKER, B. J., MEADOWS, J., RICO, D. M. AND VESELY, D. L. (1989). The N-terminus and a 4000 molecular weight peptide from the midportion of the N-terminus of the atrial natriuretic factor prohormone each circulate in man and increase in congestive heart failure. *Circulation* **80**, 438–449.