INFLUENCE OF ADRENERGIC STIMULATION AND CORONARY BLOOD FLOW ON IN VIVO CARDIAC PERFORMANCE IN RAINBOW TROUT (Oncorhynchus mykiss)

by

Anthony Kurt Gamperl

Submitted in partial fulfilment of the requirements

for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
February, 1994

National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

ISBN 0-315-93772-6

Canada

Name ANTHONY KURT GAMPERL

Dissertation Abstracts International is arranged by broad, general subject categories Please select the one subject which most nearly describes the content of your dissertation Enter the corresponding four-digit code in the spaces provided

BIOLOGY / PHYSIOLOGY
SUBJECT TERM

0433
SUBJECT CODE

U·M·I

Subject Categories

THE HUMANITIES AND SOCIAL SCIENCES

COMMUNICATIONS AND THE ARTS

Architecture	0729
Art History	0377
Cinema	0900
Dance	0378
Fine Arts	0357
Information Science	0723
Journalism	0391
Library Science	0399
Mass Communications	0708
Music	0413
Speech Communication	0459
Theater	0465

EDUCATION

General	0515
Administration	0514
Adult and Continuing	0516
Agricultural	0517
Art	0273
Bilingual and Multicultural	0282
Business	0688
Community College	0275
Curriculum and Instruction	0727
Early Childhood	0518
Elementary	0524
Finance	0277
Guidance and Counseling	0519
Health	0680
Higher	0745
History of	0520
Home Economics	0278
Industrial	0521
Language and Literature	0279
Mathematics	0280
Music	0522
Philosophy of	0998
Physical	0523
Psychology	0525
Reading	0535
Religious	0527
Sciences	0714
Secondary	0533
Social Sciences	0534
Sociology of	0340
Special	0529
Teacher Training	0530
Technology	0710
Tests and Measurements	0288
Vocational	0747

LANGUAGE, LITERATURE AND LINGUISTICS

Language	
General	0679
Ancient	0289
Linguistics	0290
Modern	0291
Literature	
General	0401
Classical	0294
Comparative	0295
Medieval	0297
Modern	0298
African	0316
American	0591
Asian	0305
Canadian (English)	0352
Canadian (French)	0355
English	0593
Germanic	0311
Latin American	0312
Middle Eastern	0315
Romance	0313
Slavic and East European	0314

PHILOSOPHY, RELIGION AND THEOLOGY

Philosophy	0422
Religion	
General	0318
Biblical Studies	0321
Clergy	0319
History of	0320
Philosophy of	0322
Theology	0469

SOCIAL SCIENCES

American Studies	0323
Anthropology	
Archaeology	0324
Cultural	0326
Physical	0327
Business Administration	
General	0310
Accounting	0272
Banking	0770
Management	0454
Marketing	0338
Canadian Studies	0385
Economics	
General	0501
Agricultural	0503
Commerce Business	0505
Finance	0508
History	0509
Labor	0510
Theory	0511
Folklore	0358
Geography	0366
Gerontology	0351
History	
General	0578
Ancient	0579
Medieval	0581
Modern	0582
Black	0328
African	0331
Asia, Australia and Oceania	0332
Canadian	0334
European	0335
Latin American	0336
Middle Eastern	0333
United States	0337
History of Science	0585
Law	0398
Political Science	
General	0615
International Law and Relations	0616
Public Administration	0617
Recreation	0814
Social Work	0452
Sociology	
General	0626
Criminology and Penology	0627
Demography	0938
Ethnic and Racial Studies	0631
Individual and Family Studies	0628
Industrial and Labor Relations	0629
Public and Social Welfare	0630
Social Structure and Development	0700
Theory and Methods	0344
Transportation	0709
Urban and Regional Planning	0999
Women s Studies	0453

THE SCIENCES AND ENGINEERING

BIOLOGICAL SCIENCES

Agriculture	
General	0473
Agronomy	0285
Animal Culture and Nutrition	0475
Animal Pathology	0476
Food Science and Technology	0359
Forestry and Wildlife	0478
Plant Culture	0479
Plant Pathology	0480
Plant Physiology	0817
Range Management	0777
Wood Technology	0746
Biology	
General	0306
Anatomy	0287
Biostatistics	0308
Botany	0309
Cell	0379
Ecology	0329
Entomology	0353
Genetics	0369
Limnology	0793
Microbiology	0410
Molecular	0307
Neuroscience	0317
Oceanography	0416
Physiology	0433
Radiation	0821
Veterinary Science	0778
Zoology	0472
Biophysics	
General	0786
Medical	0760

EARTH SCIENCES

Biogeochemistry	0425
Geochemistry	0996
Geodesy	0370
Geology	0372
Geophysics	0373
Hydrology	0388
Mineralogy	0411
Paleobotany	0345
Paleoecology	0426
Paleontology	0418
Paleozoology	0985
Palynology	0427
Physical Geography	0368
Physical Oceanography	0415

HEALTH AND ENVIRONMENTAL SCIENCES

Environmental Sciences	0768
Health Sciences	
General	0566
Audiology	0300
Chemotherapy	0992
Dentistry	0567
Education	0350
Hospital Management	0769
Human Development	0758
Immunology	0982
Medicine and Surgery	0564
Mental Health	0347
Nursing	0569
Nutrition	0570
Obstetrics and Gynecology	0380
Occupational Health and Therapy	0354
Ophthalmology	0381
Pathology	0571
Pharmacology	0419
Pharmacy	0572
Physical Therapy	0382
Public Health	0573
Radiology	0574
Recreation	0575
Speech Pathology	0460
Toxicology	0383
Home Economics	0386

PHYSICAL SCIENCES

Pure Sciences

Chemistry	
General	0485
Agricultural	0749
Analytical	0486
Biochemistry	0487
Inorganic	0488
Nuclear	0738
Organic	0490
Pharmaceutical	0491
Physical	0494
Polymer	0495
Radiation	0754
Mathematics	0405
Physics	
General	0605
Acoustics	0986
Astronomy and Astrophysics	0606
Atmospheric Science	0608
Atomic	0748
Electronics and Electricity	0607
Elementary Particles and High Energy	0798
Fluid and Plasma	0759
Molecular	0609
Nuclear	0610
Optics	0752
Radiation	0756
Solid State	0611
Statistics	0463

Applied Sciences

Applied Mechanics	0346
Computer Science	0984
Engineering	
General	0537
Aerospace	0538
Agricultural	0539
Automotive	0540
Biomedical	0541
Chemical	0542
Civil	0543
Electronics and Electrical	0544
Heat and Thermodynamics	0348
Hydraulic	0545
Industrial	0546
Marine	0547
Materials Science	0794
Mechanical	0548
Metallurgy	0743
Mining	0551
Nuclear	0552
Packaging	0549
Petroleum	0765
Sanitary and Municipal	0554
System Science	0790
Geotechnology	0428
Operations Research	0796
Plastics Technology	0795
Textile Technology	0994

PSYCHOLOGY

General	0621
Behavioral	0384
Clinical	0622
Developmental	0620
Experimental	0623
Industrial	0624
Personality	0625
Physiological	0989
Psychobiology	0349
Psychometrics	0632
Social	0451

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

Page

ABSTRACT

In fish, the adrenergically–mediated stimulation of cardiac performance is an important physiological response to stress. However, few studies have evaluated the *in vivo* dose–response relationship for cardiovascular variables at physiological catecholamine (CA) levels, and no studies have evaluated whether repeated CA exposure reduces cardiac adrenergic sensitivity. In addition, information is lacking on how coronary flow (CF) and adrenergic stimulation interact to determine *in vivo* cardiac performance.

The ED_{50} values for epinephrine–stimulated cardiac output (Q) and dorsal aortic pressure (P_{DA}) were 0.22 μg kg^{-1} and 0.48 μg kg^{-1}, respectively, with Q approaching a maximum at the highest dose (2.0 μg kg^{-1}). Removal of the coronary artery had no effect on cardiac performance at any epinephrine dose. These results suggest that small increases in plasma CA levels can cause large increases in Q, and that O_2 delivery from the luminal blood to the myocardium is not limited during normoxia. Measurements of Q, P_{DA}, and CF during hypoxia and following epinephrine injection revealed that increases in CF are important for determining Q during hypoxia, and that vasoconstriction of the coronary artery may limit cardiac performance during hypoxic conditions.

Using a new micropunch technique, it was found that cell–surface β–receptors in the trout ventricle represent a single population of β_2–type receptors with a K_d of 0.25 nM and a density of 12,000 sites per cell. The repeated injection of CAs did not alter ventricular β–receptor density or K_d. Neither acute nor chronic injection of epinephrine affected CA clearance and metabolism. The CA clearance/metabolism and β–receptor experiments indicate that the duration and/or the magnitude of CA–stimulated cardiovascular effects are unlikely to be altered by previous CA exposure.

LIST OF ABBREVIATIONS

ANOVA	analysis of variance
B_{max}	maximum binding
CA	catecholamine
CF	coronary flow
CGP	[^{3}H] CGP-12177
Ci	curies
COMT	catecholamine-O-methyl-transferase
DPM	disintegrations per minute
ED_{50}	dose at which 50% of maximal response is achieved
EDTA	ethylenediamine-tetraacetic acid
f_H	heart rate
FW	freshwater
GLM	general linear model
h	hours
Hb	haemoglobin
Hct	haematocrit
Hg	mercury
HPLC	high performance liquid chromatography
i.d.	inside diameter
IC_{50}	concentration of inhibitor at which ligand binding is depressed by 50%

K_d	concentration of ligand at which receptor binding is 50% of maximum
K_m	Michaelis–Menten constant
kPa	kilopascals
M	molar
MAO	monoamine oxidase
min	minutes
MOPEG	3–methoxy–4–hydroxy–phenol glycol
MS 222	tricaine methane sulphonate
NSB	non–specific binding
O_2	oxygen
P_{DA}	dorsal aortic pressure
P_{VA}	ventral aortic pressure
PO_2	partial pressure of oxygen
P_aO_2	partial pressure of oxygen in arterial blood
P_vO_2	partial pressure of oxygen in venous blood
P_wO_2	partial pressure of oxygen in water
P_{50}	partial pressure of oxygen at which haemoglobin is 50% saturated
PE	polyethylene
Q	cardiac output
Q_{max}	maximum cardiac output
Q_{10}	temperature quotient

R_{cor}	coronary resistance
sec	seconds
S.E.	standard error of the mean
SW	seawater
V_s	stroke volume
VMA	vanillyl-mandelic acid

ACKNOWLEDGEMENTS

Without the assistance, cooperation and technical expertise of numerous individuals, the completion of this project would have been impossible. I would like to thank, Roy Grant, Mary Primrose, Dawn Josey, Charlie Thompson, Jessica Meijer, Nancy Irwin, Wade Blanchard, Alice Giles, Grant Pogson and Brian Dixon for providing technical assistance. I am indebted to Anna Metaxas, Dr. Alan Pinder, Dr. Yong Tang, Dr. J.A. Nelson, Dr. Guy Claireaux, Dr. Tony Farrell, and Nancy Irwin for constructively editing various parts of this thesis. I thank the Canadian Red Cross for supplying human blood and Dr. Ron O'Dor for being on my supervisory committee.

Foremost, I would like to thank Drs. R.G. Boutilier, Alan Pinder and M. Wilkinson who have been a constant source of support and inspiration over the past four years. I thank Dr. R.G. Boutilier for allowing me to pursue my research interests independent of what my proposal stated, Dr. Alan Pinder for providing much needed encouragement, patience, and constructive criticism in the absence of my supervisor, and Dr. M. Wilkinson for showing tremendous enthusiasm for my project.

Finally, I would like to thank my wife Kathy, and friends Don Webb and Anna Metaxas, whose love and patience enabled me to overcome the many obstacles that I encountered during the course of this project.

This project was supported by an N.S.E.R.C. operating grant to Dr. R.G. Boutilier, a New Brunswick Heart and Stroke Foundation Grant to Dr. M. Wilkinson, and funds made available through the Patrick Lett Fund. AKG was supported by an N.S.E.R.C. PGS-4 Graduate Scholarship and a Dalhousie Graduate Fellowship.

GENERAL INTRODUCTION

Catecholamines (epinephrine and norepinephrine) are released from the chromaffin tissue of fish in response to a wide range of experimentally induced stressors (e.g. air exposure, hypoxia, exhaustive exercise, hypoxemia, handling, hypercapnic acidosis)(Mazeaud *et al.*, 1977; Ristori and Laurent, 1985; Tang and Boutilier, 1988; Vermette and Perry, 1989, Perry *et al.*, 1989; Fievet *et al.*, 1990). Once mobilised they promote respiratory, metabolic and circulatory adjustments that facilitate oxygen delivery to tissues with high oxygen demand, and the mobilization of energy stores. These adjustments include: the release of erythrocytes from the spleen (Nilsson and Grove, 1974; Kita and Itazawa, 1989; Perry and Kinkead, 1989); the regulation of erythrocyte pH thereby preserving oxygen carrying capacity (Nikinma *et al.*, 1984; Primmett *et al.*, 1986); an increase in ventilatory frequency (Vermette and Perry, 1988); an increase in gill oxygen diffusing capacity due to increased functional surface area and/or increased epithelial permeability to oxygen (Perry *et al.*, 1985); increased branchial net H^+ equivalent flux, facilitating restoration of post-exercise blood pH and thus blood carrying capacity (Tang *et al.*, 1988); positive chronotropic and inotropic effects on the heart (Holmgren, 1977; Cameron and Brown, 1981; Farrell, 1984; Hipkins *et al.*, 1985); and the maintenance or elevation of plasma glucose levels through increased hepatic glycogenolysis and/or gluconeogenesis (Ottolenghi *et al.*, 1984; Mommsen *et al.*, 1988; Perry *et al.*, 1988; Wright *et al.*, 1989).

Although several authors have reported the effects of acute *in vivo* catecholamine

administration on respiratory, circulatory or metabolic parameters (Randall and Stevens, 1967; Larsson, 1973, Booth, 1979; Pettersson and Nilsson, 1980; Peyraud-Waitzenegger *et al.*, 1980, Wright *et al.*, 1989 etc.), few have studied the effects of chronic catecholamine exposure on fish physiology. Recent studies have used chronically elevated levels of epinephrine (6×10^{-8} M; 12-24 h infusion) to elucidate the effect of prolonged catecholamine exposure on fish acid-base and blood respiratory status (Vermette and Perry, 1987; Perry and Vermette, 1988; Vermette and Perry, 1988). However, these results must be viewed with caution since: (1) the effects of catecholamines seem to depend on spurt-like increases rather than blood titre (Epple and Nibbio, 1985); (2) catecholamine specific desensitization can occur within minutes in some tissues that are continuously exposed to adrenergic agonists (Su *et al.*, 1980); and (3) exogenously administered catecholamines have *in vivo* clearance times from 3 - 10 minutes (Dashow and Epple, 1983; Nekvasil and Olson, 1986). Chronic stress would presumably involve the pulsatile release of catecholamines from the chromaffin tissue. Therefore, investigations on the effect of chronic catecholamine elevation should adopt a similar protocol.

The repeated elevation of catecholamines during chronic stress *in vivo* may reduce the effect of subsequent catecholamine surges on various physiological parameters as: (1) target tissues become desensitized through reductions in adrenoreceptor number or binding affinity (Su *et al.*, 1980; Vatner [illegible] 1989); (2) the amount of catecholamines released from the chromaffin tissue decre[illegible]nd/or (3) catecholamine clearance or metabolism is enhanced. In addition, these alterations may undermine the fish's ability to maximally compensate for further environmental perturbations and therefore decrease its chance of

survival.

The proposed research investigates whether the exposure of fish to repeated elevations in circulating catecholamine levels has the potential to decrease the magnitude or duration of a tissue's response to further catecholamine exposure by: (1) establishing whether the relationship between catecholamine dose and tissue response is strong enough to suggest that small changes in circulating catecholamine levels or adrenergic sensitivity could significantly alter adrenergically-mediated responses; (2) the determination of whether the "acute" and or "chronic" exposure to repeated catecholamine injections alters the clearance and/or metabolism of blood borne catecholamines; and (3) the examination of whether the repeated exposure to elevated catecholamines causes a decrease in β-adrenoreceptor number or binding affinity.

In this study, the heart was chosen as a model for investigating the effects of catecholamine exposure on tissue adrenergic sensitivity, β-adrenoreceptor density and binding affinity, for numerous reasons. First, catecholamine-mediated alterations in cardiovascular performance (Q, V_s, f_H, and P_{DA}) are easily measured *in vivo* (Wood and Shelton, 1980a). Second, evidence in the mammalian literature indicates that myocardial β-adrenoreceptors are sensitive to *in vivo* perturbations such as chronic catecholamine exposure (Vatner *et al.*, 1989), hypoxia (Voekel *et al.*, 1981; Mader *et al.*, 1991) and ischemia (Watson-Wright *et al.*, 1989). Third, the effects of catecholamines on fish cardiac performance have been extensively studied (Holmgren, 1977; Wood *et al.*, 1979; Wood and Shelton, 1980; Farrell, 1981; Farrell *et al.*, 1982; Farrell, 1984; Hipkins, 1985; Farrell *et al.*, 1986; Axelsson, 1988; Graham and Farrell, 1988; Franklin and Davie,

1992).

In addition to the above reasons, the heart was chosen as a model because studies on catecholamine-stimulated cardiac performance would allow for an *in vivo* evaluation of the importance of the coronary circulation in determining cardiovascular function in fish. Unlike the situation in mammals, there are few studies of the coronary physiology of fishes. Although recent measurements of *in vivo* coronary flow in the coho salmon (*Oncorhynchus kisutch*)(Axelsson and Farrell, 1993) have shown that coronary blood flow increases during exercise, following the injection of adrenergic drugs, and upon exposure to hypoxia, conflicting evidence exists as to whether these alterations in coronary blood flow are necessary for maintaining cardiac function under these conditions. Daxboeck (1982) showed that rainbow trout survive coronary ligation and that the critical swimming speed of trout was unaffected by coronary ablation. In contrast, Farrell and Steffensen (1987) found that acute coronary ligation reduced maximum prolonged swimming speed in chinook salmon (*Oncorhynchus tshawytscha*) by 32%, and Steffensen and Farrell (1993) found that coronary ablated rainbow trout could only maintain swimming performance if cardiac oxygen demand was reduced through the homeometric lowering of blood pressure. To further elucidate the role of the coronary circulation in determining cardiac function in fish, I examined: (1) whether coronary artery ablation reduced epinephrine-stimulated cardiac performance; and (2) how hypoxia, myocardial oxygen demand and adrenoreceptor-mediated vasoconstriction interact to control coronary blood flow.

In these experiments, intra-arterial injections of epinephrine and/or norepinephrine

were used to elevate circulating catecholamine levels because the levels of plasma catecholamines can be tightly controlled and because the temporal pattern of catecholamine elevation following bolus injections is similar to that seen in fish following acute stress (exhaustive exercise, handling, air exposure; see Gamperl *et al.*, in press c for a review).

Chapter 1

EFFECT OF CORONARY ABLATION AND ADRENERGIC STIMULATION ON *IN VIVO* CARDIAC PERFORMANCE IN TROUT

INTRODUCTION

The chronotropic and inotropic effects of epinephrine on fish cardiac performance have been studied *in vitro* (Holmgren, 1977; Granam and Farrell, 1989), *in situ* (Farrell *et al.*, 1982; Stuart *et al.*, 1983; Farrell *et al.*, 1986) and *in vivo* (Pettersson and Nilsson, 1980; Wood and Shelton, 1980a; Farrell, 1981; Hipkins, 1985). *In vivo* studies proved especially valuable in elucidating the complex interactions between direct adrenergic effects (observed *in vitro and in situ*), pressor-stimulated vagal cardio-inhibitory tone, and pressor-mediated decreases in stroke volume (systolic emptying). However, these *in vivo* studies have provided only limited information on dose-dependent cardiovascular changes. Investigators usually report dose-response relationships for dorsal aortic pressure (P_{DA})(Randall and Stevens, 1967; Wood and Shelton, 1980a) and when data are included for other variables (Q, V_s, f_H) only peak values are shown (e.g. Pettersson and Nilsson, 1980). In the present study, detailed information on the magnitude and time course for epinephrine stimulated, dose-dependent, changes in cardiac performance have been collected. This information will be essential in future studies for predicting the effect of elevated circulating catecholamine levels on *in vivo* cardiovascular performance and for selecting epinephrine doses to achieve a desired change in cardiovascular function.

The role of the coronary circulation in salmonid fishes is unclear. However, experimental evidence from *in vitro* heart perfusions (Davie and Farrell, 1991a; Davie *et al.*, 1992) and from measurements of swimming performance in trout with intact or ablated coronary arteries (Steffenson and Farrell, 1993) suggests that the coronary blood supply is essential for maintaining cardiac output only when the heart must work against elevated output pressures. To test this hypothesis further, *in vivo* measurements of dose-dependent changes in cardiovascular performance were made on rainbow trout (*Oncorhynchus mykiss*) with intact or ablated coronary arteries. Adrenergic stimulation is an appropriate model for studying the role of the coronary circulation in the maintenance of *in vivo* cardiac performance because epinephrine increases both cardiac output (through positive chronotropic and inotropic effects) and arterial blood pressure. The concomitant elevation in cardiac power output increases oxygen demand by the myocardium (Graham and Farrell, 1990), and may accentuate the reliance of the heart upon oxygen delivered by the coronary circulation.

MATERIALS AND METHODS

Rainbow trout (480 - 760g) were obtained from Merlin Fish Farms (Wentworth, Nova Scotia) and held in tanks (1 x 1 x 1.5 m) supplied with seawater at 4-6 °C. Fish were fed daily, to satiation, on a diet of commercially prepared feed pellets, but were fasted for 48 hours prior to surgery. Photoperiod was 12h light : 12h dark. All experiments were conducted between March 15 and June 15, 1991.

Surgical Procedures

Trout were anaesthetized (0.1 g l^{-1} tricaine methane sulphonate, MS 222), and placed supine in a wetted chamois leather sling. The fish were quickly fitted (~45 sec) with a dorsal aortic cannula (PE 50, Clay Adams)(Smith and Bell, 1964) after which retrograde irrigation with seawater containing anaesthetic (0.05 g l^{-1} MS 222) was begun. A 3–4 cm midventral incision was made through the skin and muscle at a position overlying the ventral aorta and anterior aspect of the pericardium. After cutting through the pectoral girdle and expanding the resultant cavity with tissue spreaders, the anterior portion of the pericardium was cut to expose the ventral aorta and coronary (hypobranchial) artery. The connective tissue from the anterior portion of the ventral aorta was subsequently removed to aid in placement of a Doppler flow probe (see later description). In fish where the coronary artery was ligated and electrocauterized, the Doppler flow probe was placed around the ventral aorta without further surgical procedures. However, when the coronary was left intact, placement of the Doppler flow probe necessitated that the connective tissue which adheres the coronary artery to the ventral aorta and the anterior bulbus arteriosus be removed. This allowed the flow probe to be placed underneath the freed coronary, thus maintaining normal coronary perfusion. Once the flow probe was in place, the musculature and skin were closed using continuous silk sutures. The integrity of the pericardium was not restored by suturing because of time constraints. The Doppler probe leads were secured to the body wall at the anterior apex of the incision, and at a position just posterior to the pectoral fins. The operation generally took between 45 and 60 minutes, and bleeding was usually minimal. Some fish

were fitted only with a dorsal aortic cannula (N = 9). Comparisons between this group, and those that underwent surgery ("operated" fish) allowed for the evaluation of flow probe placement on cardiovascular responses.

Once surgery was completed, fish were given an intraperitoneal injection of tetracycline (5 mg kg^{-1}) and placed into black perspex boxes (45 x 8 x 15 cm) to recover. Boxes were supplied with aerated seawater (4.5 - 6.2 °C) at a flow rate of 1 l min^{-1}.

Probe Design

Flow probes were constructed by implanting a piezoelectric crystal with 80 cm leads (Crystal Biotech, Hopkinton MA) into a 5 mm length of PE 200 (i.d. 1.4 mm) or PE 240 (i.d. 1.67 mm) Intramedic® tubing (Clay Adams). The tubing was split to facilitate placement on the ventral aorta and had a small (0.5 x 1.0 mm) notch to allow for crystal attachment. The piezoelectric crystal was secured in the notch with cyanoacrylate cement after thoroughly roughening the area with a scalpel blade. Probes were not fitted with tie-strings to prevent tube diameter from increasing at elevated ventral aortic pressures because PE tubing is extremely rigid at 5 °C.

Experimental Protocol

After 48 hrs of recovery, fish were sequentially injected with 0.2, 0.5, 1.0 and 2.0 μg kg^{-1} epinephrine at 1.5 hour intervals. The inter-injection period allowed cardiovascular variables to return to "resting" levels for approximately 1 hour (results from preliminary experiment). All doses of epinephrine (Sigma Chemical Co., St. Louis,

MO) were injected slowly (over approximately 15 sec) through the dorsal aortic cannula in a concentrated form using a 0.2 - 0.4 ml carrier volume of saline. Heart rate (f_H), dorsal aortic pressure (P_{DA}), and ventral aortic flow (cardiac output; Q) were continuously recorded from 5 minutes prior to epinephrine injection ("resting" levels) to 20 minutes post-injection. Trout with irregular heart rates, low haematocrits (< 10%), or which continued to struggle periodically within the black perspex boxes were not used for data collection.

Recording Systems

P_{DA} was measured by attaching the dorsal aortic cannula to a Gould Statham (Model P 23-10) pressure transducer, connected to a Molytec amplifier-recorder (Model 3501-MS). Mean P_{DA} was calculated as: (systolic pressure + 2 (diastolic pressure)) / 3 (Burton, 1972; Wood *et al.*, 1979). Pressure calibrations were performed daily against a static water column. f_H at rest, or at a particular time post-injection, was determined by measuring the number of systolic peaks during a 30 second interval; the interval being 15 seconds on either side of the desired time.

Mean ventral aortic flow (Q) was measured by connecting a pulsed Doppler flowmeter (Model 545c-4; Bioengineering, University of Iowa) to an RC-integrator, and an amplifier recorder (Asea Brown and Boveri; Model SE-120). In order to determine absolute flow rates (ml min^{-1}), an in *situ*, post-mortem, calibration of each flow probe was performed at physiologically relevant pressures using a peristaltic pump (Gilson, Minipuls® II) and human blood (approximate haematocrit 20%). To accomplish this, after

removal of the sinus venosus and atrium, the ventricle was bisected laterally and the peristaltic pump outflow tubing (PE 160) was tied into the ventricular lumen. Because of the high flow rates required (> 35 ml min^{-1}), 2 pieces of Viton® tubing (4 mm i.d.) were attached to the peristaltic pump; and PE 160 tubing connected the peristaltic pump to the ventricle. Doppler flow probes were calibrated over a range of flows from 5 to 38 ml min^{-1}, 5 ml min^{-1} being the minimal flow at which the ventral aorta adequately filled the probe lumen. Blood pressure was monitored through a side-arm in the peristaltic pump outflow tubing, and was found to increase linearly (approximate range 2-7 kPa) with elevations in blood flow [pressure = -0.44 + 0.54 (flow), $r^2 = 0.995$] .

Although coronary blood flow was not measured in animals with intact vessels, coronary patency was verified by visual inspection, and by following the progression of methylene blue up the coronary artery. Methylene blue was injected into the dorsal aorta of anaesthetized fish, just prior to sacrifice for flow probe calibration.

Analysis

Stroke volume (V_s) was calculated from Q/f_H. Barostatic gain (normalized gain, $\%\Delta\ f_H$ per unit change in mean arterial pressure, Smith *et al.*, 1981) was determined from pre-injection and 2 minute post-injection values of f_H and P_{DA}. ED_{50} (dose of epinephrine required to elicit 50% of maximal effect) values for maximum P_{DA} and Q_{max}, for each group (type 1, type 2, fish only with cannulae) were calculated from Eadie-Hofstee plots of mean values.

Statistical differences ($p < 0.05$) between means, for resting and post-injection

cardiovascular parameters were determined using a 2 ("type") x 2 (intact/ablated) factorial with repeated measures, followed by multiple contrasts (Proc GLM; SAS Institute Inc.). To facilitate the determination of whether surgical procedures (installation of flow probe) affected resting f_H and P_{DA}, means for fish with only cannulae were compared to those for "operated" fish using a one way analysis of variance (Proc GLM). Previous statistical analysis (2 x 2 factorial) had shown that the "type" of cardiovascular response pattern, and the presence/absence of a coronary artery had no effect on resting f_H and P_{DA}. Post-injection increases in P_{DA} were compared between fish with only cannulae and "operated" fish using a one way analysis of variance (Proc GLM) with repeated measures, followed by multiple contrasts. Again previous statistical analysis had shown that the presence/absence of the coronary artery and response type had no effect on the magnitude of post-injection P_{DA} increases.

RESULTS

Resting Cardiovascular Variables

Cardiac output, f_H, P_{DA} and V_s were the same in trout with intact or ablated coronary arteries at 48 hours post-surgery, averaging 18 ml min^{-1} kg^{-1}, 42 beats min^{-1}, 2.2 kPa, and 0.42 ml kg^{-1}, respectively (Table 1.1). In addition, no significant differences in resting cardiovascular parameters were observed between fish exhibiting "type 1" and "type 2" (see below) cardiac adrenergic responses. "Operated" trout had a significantly higher f_H, and a significantly lower P_{DA} when compared to individuals fitted only with dorsal aortic cannulae. Mean haematocrit levels were not significantly different between

any of the groups, and ranged from 16.8 to 21.3% (Table 1.1). Although it appears from Table 1.1 that differences in P_{DA} between groups may be correlated with haematocrit, analysis of the relationship between vascular resistance (P_{DA}/Q) and haematocrit, for "operated fish", failed to reveal a significant trend (VR = 0.102 + 0.001 Hct; $r^2 = 0.022$).

Table 1.1. Haematocrit and resting cardiovascular parameters in trout fitted with both a ventral aortic flow probe and a dorsal aortic cannulae ("operated" fish), and trout possessing only dorsal aortic cannulae. "Type" refers to the cardiovascular response to epinephrine injection (see text). (*) Indicates a significant difference ($p < 0.05$) between "operated" trout and those only fitted with dorsal aortic cannulae. Values are mean ± S.E. Numbers in brackets indicate sample size.

	Haematocrit	Q	f_H	V_s	P_{DA}
Type 1					
Coronaries					
Ablated (9)	19.8 ± 1.7	19.0 ± 2.2	42.9 ± 0.8	0.44 ± 0.05	2.21 ± 0.09
Intact (4)	16.8 ± 1.2	16.0 ± 1.5	40.3 ± 4.2	0.42 ± 0.07	1.97 ± 0.21
Type 2					
Groups (7) Combined	19.2 ± 0.8	18.4 ± 1.1	43.3 ± 1.1	0.41 ± 0.03	2.33 ± 0.20
Fish only with DA Cannulae (8)	21.3 ± 1.9	—	38.6 ± 2.6*	—	2.84 ± 0.17*

Variations in the Response to Epinephrine Injection

Injection of epinephrine into fish with intact or ablated coronary arteries resulted in two "types" of responses in cardiac output (Fig. 1.1). In the type 1 response, Q began to increase shortly (< 30 sec) after epinephrine injection and reached maximum levels at 4–10 minutes post–injection (time required to reach Q_{max} increasing with epinephrine dose). In the type 2 response, alterations in Q were biphasic. In the initial minutes post–injection, Q fell gradually and reached a minimum at 1 to 2 minutes. Thereafter Q gradually increased, reaching pre–injection levels at approximately 4 to 6 minutes and maximum levels at approximately 10 – 12 minutes. Both trout with ablated coronary arteries and those with intact coronary arteries showed the two response types. The ratio (Type 1:Type 2) was 9:4 in coronary ablated fish, and 4:3 in fish with intact coronaries. Response type, for individuals, was conserved across dose levels (Fig. 1.2); e.g. fish exhibiting type 1 alterations in Q at the lowest dose (0.2 μg kg^{-1}), also displayed type 1 responses at 0.5, 1.0 and 2.0 μg kg^{-1}. Although some trout with type 1 response patterns did show a transition to a type 2 response at high epinephrine doses (1.0 and 2.0 μg kg^{-1})(Fig. 1.2), the decrease in Q was small when compared to fish displaying the more typical type 2 responses.

The form of the pressor response varied slightly between fish, but remained consistent for individual fish. Wood and Shelton (1980a) separated P_{DA} responses into four basic configurations. However, I view these alterations to be part of a continuum, with variations being of minimal physiological importance. In both type 1 and type 2 cardiac responses, P_{DA} always rose quickly following injection and reached maximum

Figure 1.1. Continuous recordings of cardiac output (Q) in trout showing the two different responses, type 1 and type 2, to the injection of 0.5 μg kg^{-1} epinephrine. Arrowheads indicate the point of maximum cardiac output (Q_{max}). The point of injection is marked with an arrow.

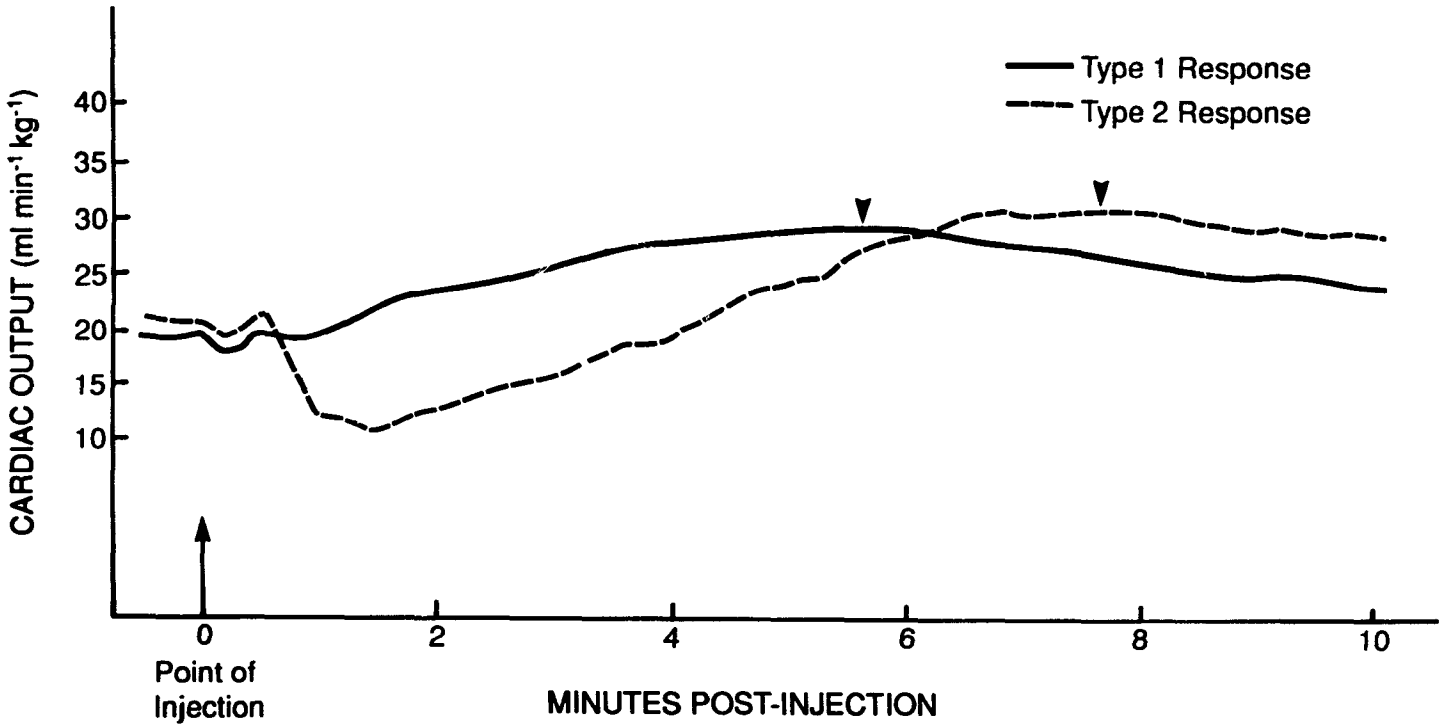
Type 1 Response
Type 2 Response
CARDIAC OUTPUT (ml min-1 kg-1)
40
35
30
25
20
15
10
0
Point of Injection
2
4
6
8
10
MINUTES POST-INJECTION

Figure 1.1

Figure 1.2. Recordings of cardiac output (Q) in a type 1 trout following the injection of various doses of epinephrine. Arrowheads indicate the point of maximum cardiac output (Q_{max}). The point of injection is marked with an arrow.

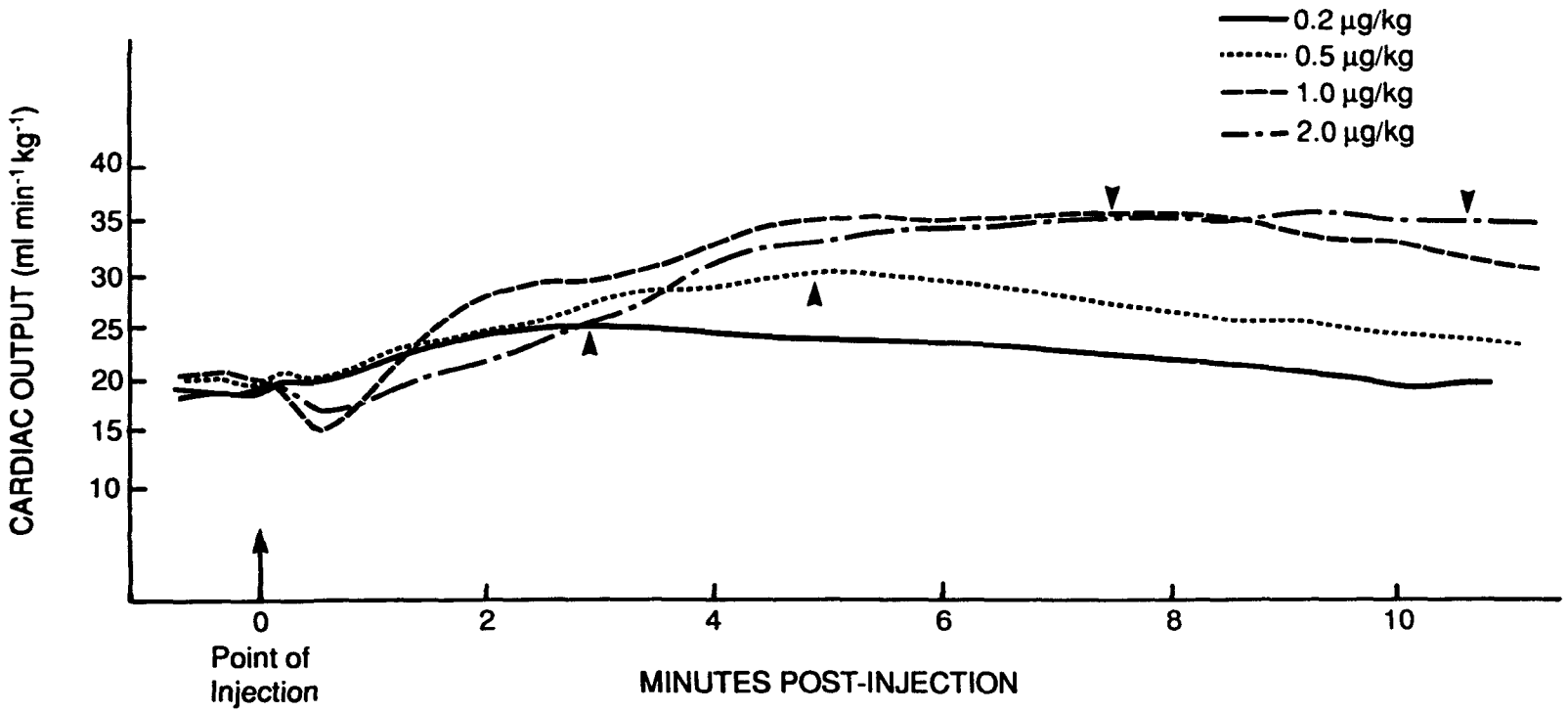
0.2 µg/kg
0.5 µg/kg
1.0 µg/kg
2.0 µg/kg
CARDIAC OUTPUT (ml min-1 kg-1)
40
35
30
25
20
15
10
0
2
4
6
8
10
Point of Injection
MINUTES POST-INJECTION

Figure 1.2

levels within 1–4 minutes (generally < 2 min.); for example, at 0.2 μg kg^{-1} the time required to reach peak P_{DA} was 123 ± 29 sec for type 2 fish and 99 ± 13 sec for type 1 fish. No obvious relationship between Q response pattern and P_{DA} response pattern was evident.

In both type 1 and type 2 fish f_H decreased immediately post injection (Figs. 1.3, 1.4, 1.5). However, statistical analysis (2 x 2 factorial, repeated measures) of the drop in f_H between pre-injection and 2 minutes post-injection revealed that the degree of bradycardia was significantly greater in trout displaying type 2 responses at 0.2 0.5 and 1.0 μg kg^{-1} ($p < 0.05$), but not at 2.0 ug kg^{-1} ($p = 0.07$). Because maximum P_{DA} was not significantly different between type 1 and type 2 fish at any dose (see next section), differences in barostatic gain (%Δ f_H per unit P_{DA}) must have mediated discrepancies in the degree of bradycardia between response types. Indeed, barostatic gain was higher in type 2 fish at all dose levels; the difference was statistically significant at 0.2 μg kg^{-1} (Table 1.2). Although type 1 fish with intact coronaries (Fig. 1.4) appear to have a greater reduction in f_H, as compared to type 1 fish with ablated coronaries (Fig. 1.3), the difference was not significant.

Maximum V_s was reached at approximately 4–6 minutes at 0.2 μg kg^{-1} and at approximately 8–10 minutes at 2.0 μg kg^{-1} (Figs. 1.3, 1.4, 1.5). In type 1 fish V_s was elevated at two minutes post-injection, at all dose levels. In type 2 fish, however, the initial response of V_s to epinephrine injection appears to be dose-dependent. Increases in V_s were observed at 2 minutes post-injection at 1.0 and 2.0 ug kg^{-1}, but not at 0.2 or 0.5 μg kg^{-1}.

Figure 1.3. Type 1 cardiovascular responses for trout with ablated coronary arteries to increasing doses of epinephrine. Each line represents a different dose: (●) 0.2 μg kg^{-1}; (■) 0.5 μg kg^{-1}; (▼) 1.0 μg kg^{-1}; (▲) 2.0 μg kg^{-1}. Vertical bars indicate the S.E. for each mean value (N = 9).

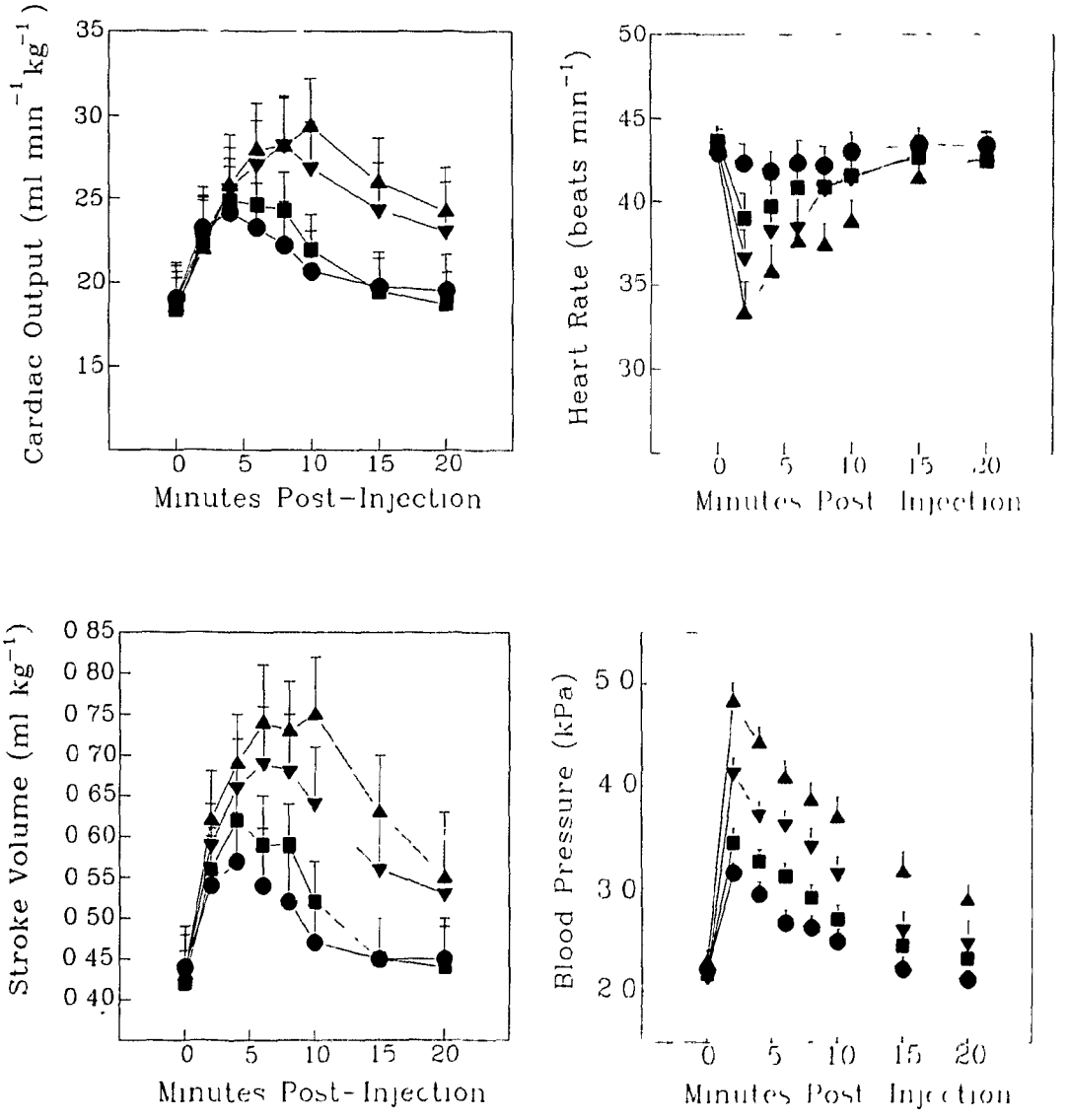
Cardiac Output (ml min^{-1} kg^{-1})
35
30
25
20
15
0 5 10 15 20
Minutes Post-Injection
Heart Rate (beats min^{-1})
50
45
40
35
30
0 5 10 15 20
Minutes Post Injection
Stroke Volume (ml kg^{-1})
0 85
0 80
0 75
0 70
0 65
0 60
0 55
0 50
0 45
0 40
0 5 10 15 20
Minutes Post-Injection
Blood Pressure (kPa)
5 0
4 0
3 0
2 0
0 5 10 15 20
Minutes Post Injection

Figure 1.3

Figure 1.4. Type 1 cardiovascular responses for trout with functional coronary arteries to increasing doses of epinephrine. Each line represents a different dose: (●) 0.2 μg kg^{-1}; (■) 0.5 μg kg^{-1}; (▼) 1.0 μg kg^{-1}; (▲) 2.0 μg kg^{-1}. Vertical bars indicate the S.E. for each mean value (N = 4). Points without vertical bars N = 3. Response curves for P_{DA} are not shown because only 2 trout gave reliable recordings at all dose levels.

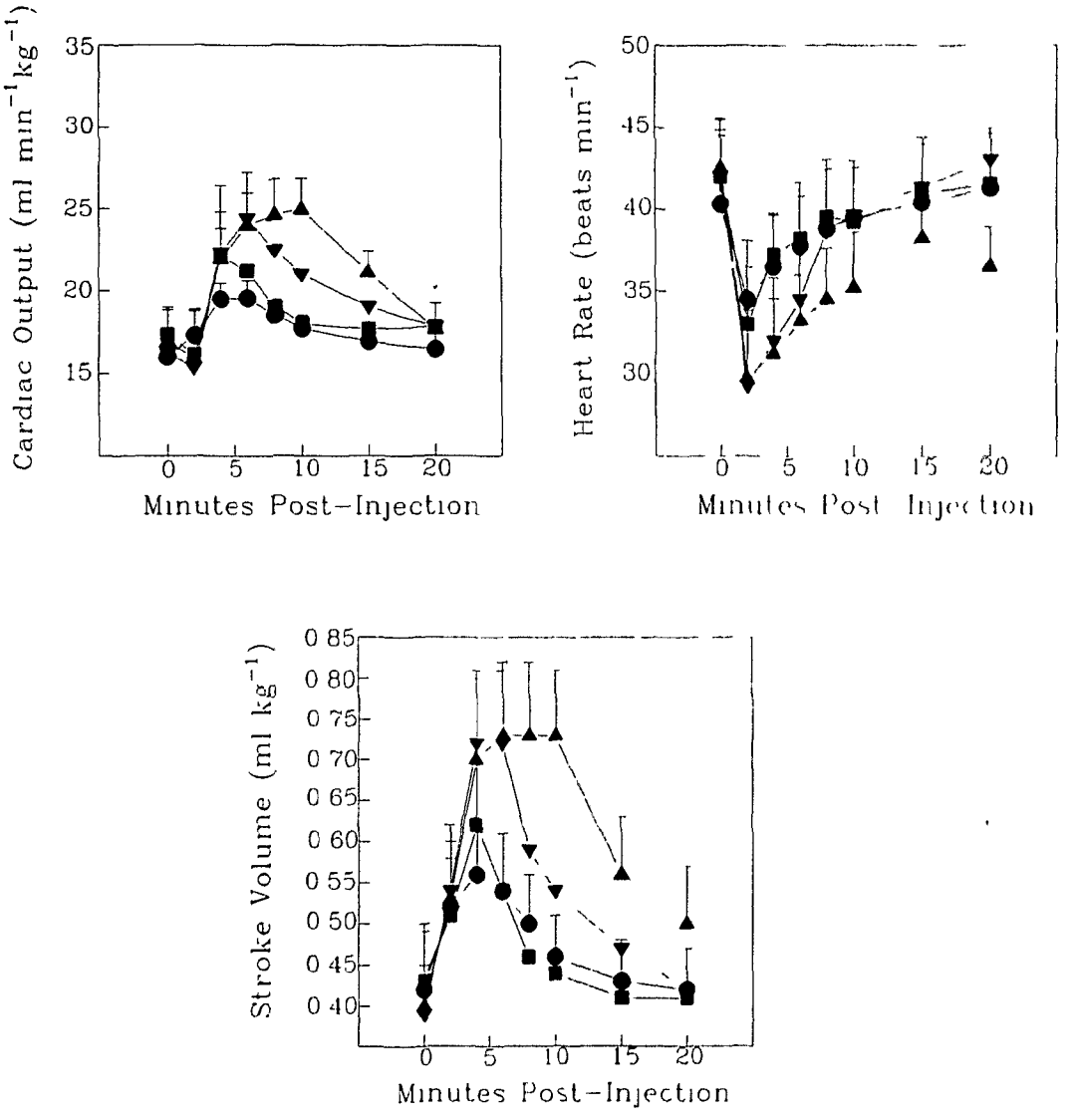
Cardiac Output (ml min^{-1}kg^{-1})
35
30
25
20
15
0
5
10
15
20
Minutes Post-Injection
Heart Rate (beats min^{-1})
50
45
40
35
30
0
5
10
15
20
Minutes Post Injection
Stroke Volume (ml kg^{-1})
0 85
0 80
0 75
0 70
0 65
0 60
0 55
0 50
0 45
0 40
0
5
10
15
20
Minutes Post-Injection

Figure 1.4

Figure 1.5. Type 2 cardiovascular responses to increasing doses of epinephrine. Each line represents a different dose: (●) 0.2 μg kg^{-1} ; (■) 0.5 μg kg^{-1}; (▼) 1.0 μg kg^{-1}; (▲) 2.0 μg kg^{-1}. Data is for trout with ablated (N = 4) and functional coronary arteries (N = 3) combined. Statistical analysis (2 x 2 factorial ANOVA, repeated measures) showed that presence or absence of the coronary artery did not affect type-dependent alterations in cardiovascular performance. Vertical bars indicate the S.E. for each mean value.

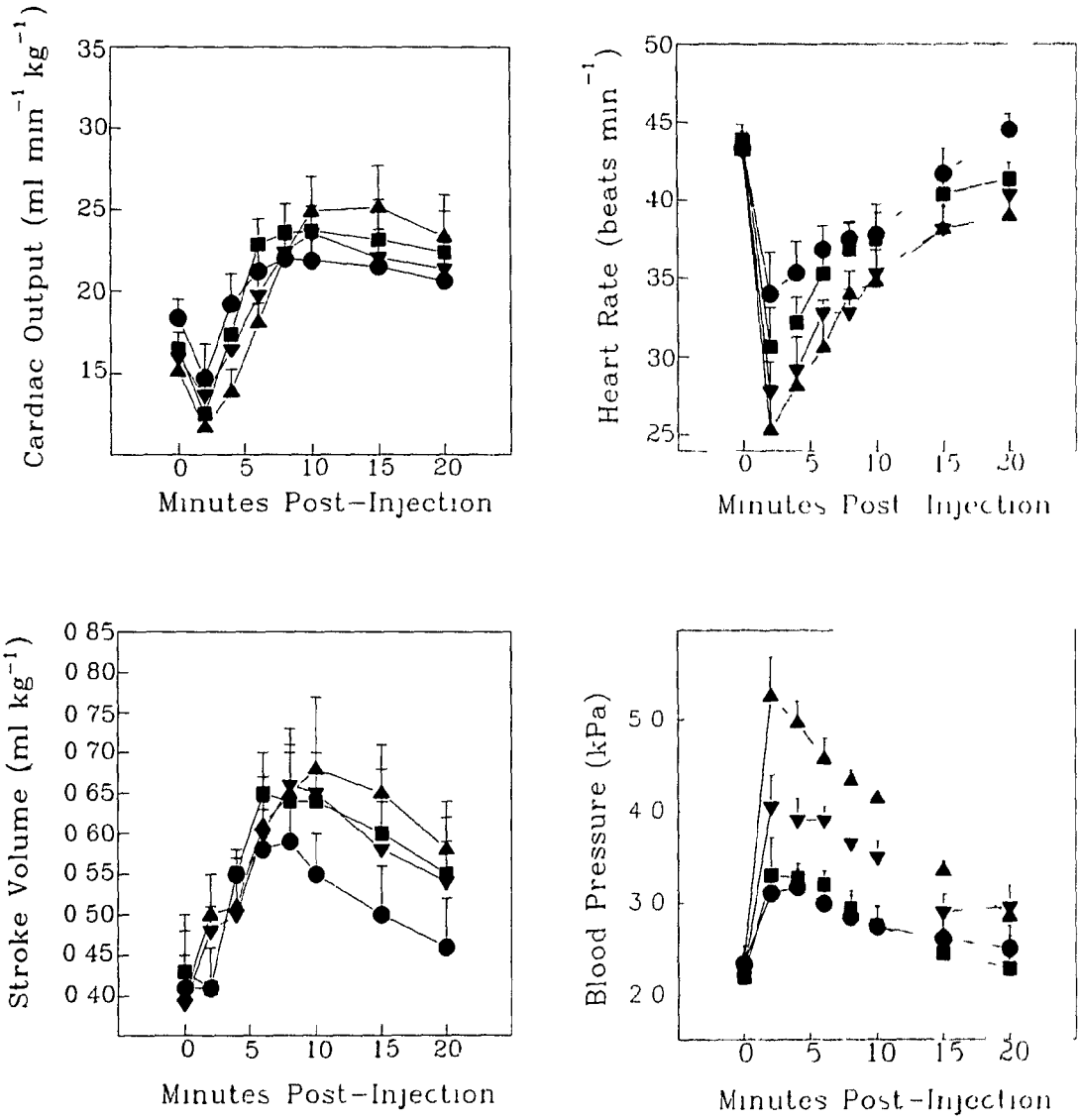
Cardiac Output (ml min^{-1} kg^{-1})
35
30
25
20
15
0 5 10 15 20
Minutes Post-Injection
Heart Rate (beats min^{-1})
50
45
40
35
30
25
0 5 10 15 20
Minutes Post Injection
Stroke Volume (ml kg^{-1})
0 85
0 80
0 75
0 70
0 65
0 60
0 55
0 50
0 45
0 40
0 5 10 15 20
Minutes Post-Injection
Blood Pressure (kPa)
5 0
4 0
3 0
2 0
0 5 10 15 20
Minutes Post-Injection

Figure 1.5

Table 1.2. Barostatic gain values (%Δ f_H kPa^{-1}) from heart rate and dorsal aortic blood pressures at pre-injection and 2 minutes post-injection. Data from trout with intact and ablated coronary arteries combined. (*) Indicates a significant difference ($p < 0.05$) between type 1 and type 2 trout.

	Dose ($\mu g\ kg^{-1}$)			
	0.2	0.5	1.0	2.0
Type 1 (N = 12)	5.9 ± 2.2*	13.5 ± 3.3	10.2 ± 2.2	9.4 ± 2.1
Type 2 (N = 7)	30.3 ± 7.1	28.0 ± 6.0	21.6 ± 4.7	15.7 ± 3.4

Maximum P_{DA} and minimum f_H were generally reached within 1–2 minutes post-injection, independent of epinephrine dose. However, the time required to reach Q_{max} increased steadily with epinephrine dose (Figs. 1.2, 1.6B). Both trout with type 1 and type 2 cardiac responses required significantly longer to reach Q_{max} at 1.0 and 2.0 $\mu g\ kg^{-1}$, as compared to 0.2 and 0.5 $\mu g\ kg^{-1}$. The presence or absence of a coronary artery had no effect on time required to reach Q_{max}. However, type 2 fish required substantially (170% to 270%) longer to reach Q_{max} when compared to type 1 fish (Fig. 1.6B).

Figure 1.6. Effect of increasing epinephrine dosage on the magnitude (A) and timing (B) of maximum cardiac output: (●) type 1 trout with ablated coronary arteries (N = 9); (○) type 1 trout with functional coronary arteries (N = 4); (■) type 2 trout, data from fish with ablated and functional coronary arteries combined (N = 7). Vertical bars indicate S.E. for each mean value. Dissimilar letters indicate significant differences ($p < 0.05$) between doses, within each group. (*) Indicates a significant difference ($p < 0.05$) between type 1 and type 2 trout within a particular dose. Statistical analysis was performed using a 2 x 2 factorial (ANOVA) with repeated measures. Comparisons across doses were made using multiple contrasts. Saline injection had negligible effects on cardiac output. Each fish acted as its own control.

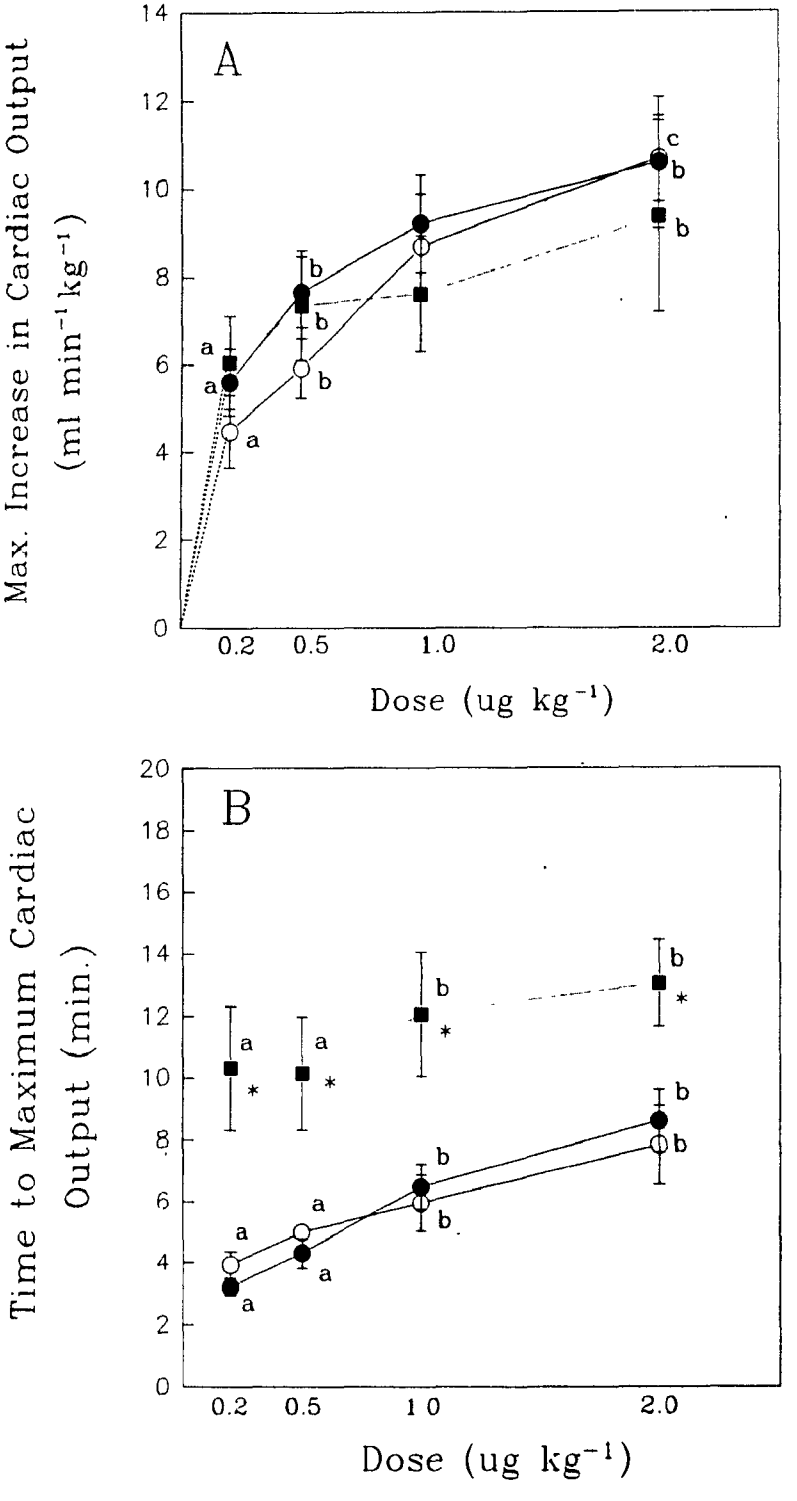
A
Max. Increase in Cardiac Output
(ml min⁻¹ kg⁻¹)
Dose (ug kg⁻¹)
0.2
0.5
1.0
2.0
B
Time to Maximum Cardiac
Output (min.)
Dose (ug kg⁻¹)

Figure 1.6

Magnitude of Cardiovascular Responses to Epinephrine Injection

A strong dose-response relationship was evident for all cardiovascular variables measured (Figs. 1.3, 1.4, 1.5). The relation was positive for Q, P_{DA} and V_s but negative for f_H. The maximum increase in Q (Q_{max}), following epinephrine injection, was approximately 5.5 ml min^{-1} kg^{-1} at 0.2 μg kg^{-1} (30% increase over rest) and 10.3 ml min^{-1} kg^{-1} at 2.0 μg kg^{-1} (57% increase over rest). Although the epinephrine-stimulated increase in Q was significantly greater at 0.5 μg kg^{-1} as compared to 0.2 μg kg^{-1}, for all groups, only type 1 trout with intact coronaries showed a significant elevation in Q_{max} between 0.5 μg kg^{-1} and 2.0 μg kg^{-1}. At all dose levels, there was no significant difference in Q_{max} between fish displaying type 1 or type 2 responses (Fig. 1.6A). The mean ED_{50} value for the relationship between epinephrine dose and Q_{max}, for all groups, was 0.22 ± 0.07 μg kg^{-1} (range 0.17 to 0.34). Although the use of chart recorders for measurement of Q and f_H precluded the determination of maximum V_s, analysis of post-injection V_s patterns indicates that elevations in V_s were also dose dependent (Figs. 1.3, 1.4, 1.5). Stroke volume increased, over pre-injection levels, by approximately 0.15 ml kg^{-1} (35%) at 0.2 μg kg^{-1} and 0.31 ml kg^{-1} (74%) at 2.0 μg kg^{-1}. Saline injection had negligible effects on any cardiovascular variable.

Post-injection elevations in P_{DA} were significantly different at all dose levels (Fig. 1.7). The mean increase for "operated" fish was 1.03 ± 0.01 kPa (48%) at 0.2 μg kg^{-1} and 2.81 ± 0.3 kPa (122%) at 2.0 μg kg^{-1} epinephrine. No significant differences in P_{DA} elevation were detected between trout displaying dissimilar response types. However, fish possessing only dorsal aortic cannulae had a greater post-injection P_{DA} increase as

Figure 1.7. Effect of increasing epinephrine dosage on peak dorsal aortic pressure (P_{DA}): (●) type 1 trout, data from fish with ablated and functional coronary arteries combined (N = 13); (■) type 2 trout, data from fish with ablated and functional coronary arteries combined (N = 7); (○) Data from fish only fitted with a dorsal aortic (DA) cannula (N = 9). Vertical bars indicate S.E. for each mean value. Dissimilar letters indicate significant differences ($p < 0.05$) between doses, within each group.(*) Indicates a significant difference ($p < 0.05$) between "operated" trout, and trout possessing only DA cannulae, within a particular dose. Statistical comparisons were performed using analysis of variance, followed by multiple contrasts. Saline injection had negligible effects on P_{DA}. Each fish acted as its own control.

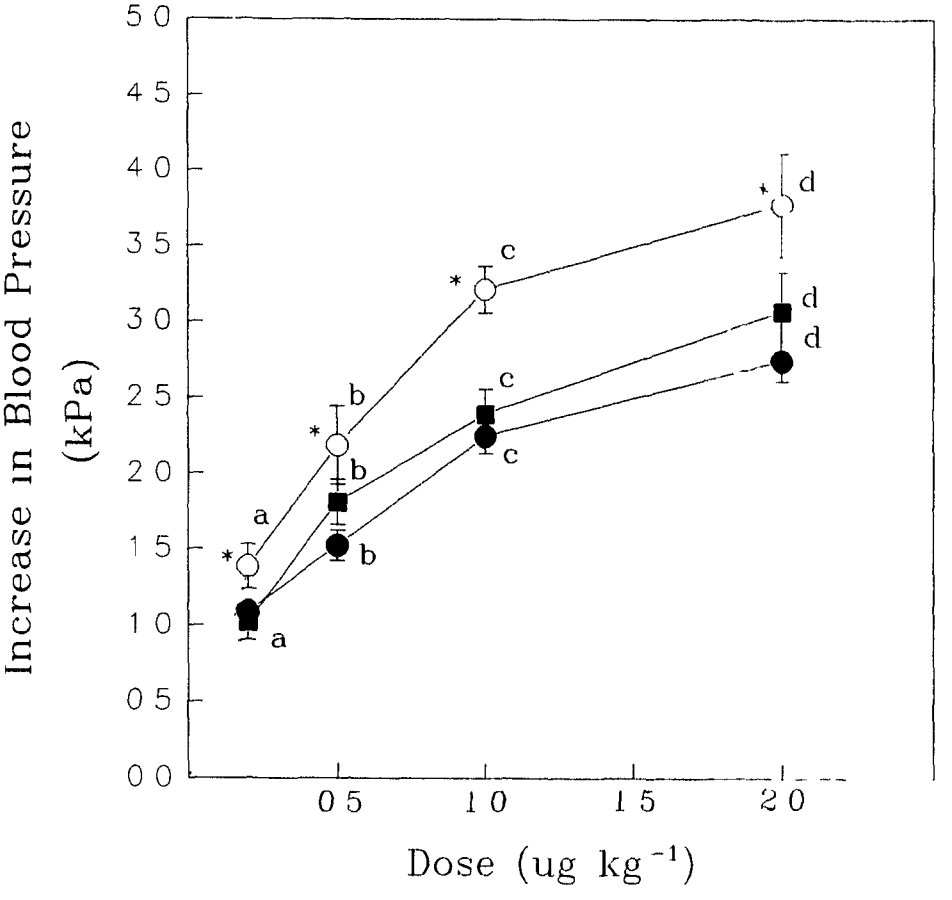
Increase in Blood Pressure
(kPa)
50
45
40
35
30
25
20
15
10
05
00
05
10
15
20
Dose (ug kg⁻¹)
a
b
c
d
*

Figure 1.7

compared to "operated" fish. The increase in P_{DA}, over "operated" fish, was approximately 30% at 0.2, 0.5 and 2.0 μg kg^{-1}, and 38% at 1.0 μg kg^{-1}. The ED_{50} values for only cannulated fish, type 1 fish, and type 2 fish, were 0.48, 0.39, and 0.56 μg kg^{-1}, respectively (mean 0.48 μg kg^{-1}).

Coronary ablation had negligible effects on epinephrine–stimulated cardiovascular performance. At all dose levels, no significant differences in P_{DA} or Q_{max} were identified between trout with intact and ablated arteries (Figs. 1.3, 1.4, 1.6A).

DISCUSSION

Cardiac Performance in Resting Trout

Although resting f_H and P_{DA} in trout with dorsal aortic cannulae (39 beats min^{-1}; 2.8 kPa) were comparable to those obtained at 5 °C (Wood, 1968; 39 beats min^{-1}), and at 12 °C (Stevens and Randall, 1967; 47 beats min^{-1}, 3.51 kPa), the f_H of my trout was 10 beats min^{-1} higher than those reported by Wood *et al.* (1979) at 5°C (29 beats min^{-1}; 2.95 kPa). In addition, while the values of resting Q (18 ml min^{-1} kg^{-1}) and V_s (0.42 ml kg^{-1}) are similar to those reported for resting trout (Kiceniuk and Jones, 1977; Cameron and Davis, 1970) and cod (Jones *et al.*, 1974; Axelsson, 1988) at 10°C, the values for Q are decidedly lower than those obtained by Wood and Shelton (1980; 36 ml min^{-1} kg^{-1}) and Neumann *et al.* (1983; 46 ml min^{-1} kg^{-1}) for trout. Differences in experimental temperature (Q_{10} for cardiovascular parameters of approximately 2.0, Farrell and Jones, 1992), in the time allowed for recovery from surgical procedures, in the method of Q measurement (Fick principle vs. direct measurement), and in the magnitude of "post–

operative stress" make comparisons of resting cardiovascular parameters between studies difficult. However, it appears that the values for resting f_H, P_{DA}, Q, and V_s in my trout are comparable to those reported by other authors.

Coronary artery ablation did not affect resting cardiovascular variables. This result is consistent with the findings of Davie and Farrell (1991) and Davie *et al.* (1992) who showed that Q is only dependent upon coronary perfusion during periods of elevated output pressure and hypoxemia. Trout exhibiting type 1 and type 2 cardiac responses to epinephrine injection had similar values for resting Q, f_H, V_s and P_{DA}. This result indicates that resting cardiovascular variables cannot be used to predict whether a trout will show a type 1 or a type 2 response upon adrenergic stimulation.

Resting "operated" trout had a significantly lower P_{DA} and a higher f_H when compared to trout fitted only with a dorsal aortic cannula. Farrell *et al.* (1988) showed that cutting the pericardium of an *in situ* perfused heart (with constant cardiac filling pressure and f_H) reduced "control" cardiac output, stroke volume, and cardiac power output by 44–46%. Rainbow trout *in vivo* can clearly compensate for this effect since cutting the pericardium does not appear to affect resting cardiac output; my values for Q are comparable to those of Kiceniuk and Jones (1977) and Cameron and Davis (1970). However, the observed alterations in f_H and P_{DA} suggest possible mechanisms by which cardiac output could be maintained. Homeometric regulation (Farrell, 1984) of preload (increase) and afterload (decrease) may have maintained Q through increased V_s, without a concomitant increase in cardiac power output (Q x afterload). In addition, a decrease in cholinergic tonus and/or an increase in adrenergic tonus could have increased f_H (Wood

et al., 1979; Axelsson, 1988), and therefore Q. Although increased levels of catecholamines due to surgery/confinement could have enhanced Q through positive inotropic and chronotropic effects (Farrell, 1984), the lower P_{DA} in "operated" fish as compared with cannulated fish is inconsistent with this hypothesis. Elevated catecholamine titers would have increased systemic vascular resistance and therefore P_{DA} through α-adrenoreceptor mediated vasoconstriction (Wood and Shelton, 1980b); these effects increase the requirement for cardiac power output.

Cardiovascular Response Types

Epinephrine injection resulted in two distinct types of cardiac response; a type 1 response characterised by a gradual increase in post-injection Q, and a type 2 response characterised by an initial fall in Q, followed by a gradual increase to a similar peak Q as for type 1 fish. Different cardiac response patterns within a fish population have been demonstrated previously. Hughes *et al.* (1988) reported that rainbow trout displayed two different *in vitro* relationships between systolic pressure and f_H, and that fish which were unable to maintain systolic pressure at high heart rates (> 50 beats min^{-1}) had reduced maximum swimming speeds. In addition, Wood and Shelton (1980a) [illegible] four patterns of cardiac response to epinephrine administration; two variations on a type 1 response, a type 2 response, and a response in which there was no post-injection elevation of Q. The results of Farrell *et al.* (1986) indicate that trout displaying type 2 responses may have "failing hearts". However, the lack of type-specific differences in resting cardiovascular parameters, and the dose-dependency of cardiac responses (Q, P_{DA},

f_H) in type 2 fish, suggest that an alternative mechanism determined the expression of response type.

Changes in Q following epinephrine injection are mediated through, an atropine sensitive reflex bradycardia, direct adrenergic stimulation of the heart (positive inotropic and chronotropic effects), and reduced systolic emptying (V_s) in the face of elevated output pressures (Helgasson and Nilsson, 1973; Wood and Shelton, 1980a; Farrell, 1984; Hipkins, 1985; Davie *et al.*, 1992). Trout exhibiting type 2 responses had a greater post-injection decrease in f_H and an increased barostatic gain, as compared with type 1 trout. These results suggest that an increased vagal cardio-inhibitory tone probably mediated the greater decrease in post-injection f_H (Q) in type 2 fish. In addition, evidence suggests that an increased barostatic gain may have reduced post-injection V_s. Holmgren (1977) showed that acetylcholine inhibits the contraction force of paced atrial strips in the cod. Because ventricular filling is dependent upon atrial contraction (Jones and Randall, 1978), an increased vagal (cholinergic) reflex may have resulted in the diminished V_s increases observed for type 2 trout. Wood and Shelton (1980a) found that atropine treatment (blockade of cholinergic input to the heart) did not affect the pattern of *in vivo* Q alterations associated with epinephrine injection. Although this result contrasts with the proposed barostatic (cholinergic) mediation of type 1 and type 2 cardiac responses, evidence suggests that the trout in Wood and Shelton (1980a) were severely stressed: 1) resting cardiac output in their trout was 36 ml min^{-1} kg^{-1}; and 2) propranolol (a β-adrenergic antagonist) severely reduced resting f_H, V_s, and Q in some fish, indicating that a considerable adrenergic tone was required to maintain cardiac function. Direct

adrenergic stimulation (chronotropic and inotropic) of the heart was evident post-injection (Figs. 1.3, 1.4, 1.5). However, it is unlikely that differences in cardiac adrenergic sensitivity resulted in the presence of two distinct cardiovascular (f_H, V_s, Q) response patterns. Farrell (1981) found that cardiovascular changes occuring in less that 100 sec following a pre-gill drug infusion are unlikely to be caused by direct agonist effects on the heart. In my study, although epinephrine was injected into the dorsal aorta, it is highly improbable that the injected epinephrine could have reached the heart in $t < 40$ sec; $t < 40$ sec was the time between epinephrine injection and the start of the fall in Q in type 2 trout (Fig. 1.1). Wood and Shelton (1980a) suggest that epinephrine-stimulated increases in blood pressure limit V_s in individuals where heart contractility is close to maximum. However the ability of type 2 fish to elevate V_s at 1.0 and 2.0 $\mu g\ kg^{-1}$ (Fig. 1.5, where peak P_{DA}, and presumably P_{VA} were highest) precludes this interpretation of my results. While an increased vagal (cholinergic) reflex is the most plausible explanation for the observed differences in V_s and f_H between type 1 and type 2 trout, other explanations cannot be ruled out. Discrepancies in the degree of damage to adrenergic nerves present in the bulbus arteriosus, in the degree of cardiac stimulation through spinal and vagal nerve sympathetic activity, and in the homeometric regulation of preload (venous pressure) may have also contributed to the presence of type specific Q response patterns.

The maximum increase in post-injection Q for type 1 and type 2 fish was not different at any dose. However, the time required to reach Q_{max} was 4-6 minutes greater at all dose levels for type 2 trout (Fig. 1.6B). Because the time course of P_{DA} and V_s did

not differ greatly between type 1 and type 2 responses (Figs. 1.3, 1.5), my data suggests that vagally (cholinergically)- mediated increases in the magnitude of f_H depression were also responsible for the delayed Q_{max} observed in type 2 fish.

Dose-Dependent Alterations in Cardiac Performance

In both response types, there was a significant relationship between epinephrine dose and time to Q_{max} (Figs. 1.2, 1.6B). Because increased afterload reduces the extent of systolic emptying (Wood and Shelton, 1980a; Davie *et al.*, 1992), the dose-dependency of P_{DA} (and presumably P_{VA}) elevation could have resulted in a delayed maximum V_s at high epinephrine doses. However, it appears that temporal alterations in V_s can only partially explain the dose-dependent increase in time to Q_{max}. In type 1 fish (Fig. 1.3) for example, it appears that maximum V_s was achieved at 4 minutes at 0.2 and 0.5 μg kg^{-1} and at 6 minutes at 1.0 and 2.0 μg kg^{-1}. This small difference in time to maximum V_s (2 minutes) only accounts for about half of the discrepancy in time required to reach Q_{max} between 0.2 and 2.0 μg kg^{-1}. Cardiac output is the product of f_H and V_s. Since the degree of f_H depression and the time required for f_H to return to pre-injection values were also dose-dependent (e.g. Fig. 1.3), the relationship between epinephrine dose and time to Q_{max} was probably determined by the interaction of changes in V_s and f_H.

From Fig. 1.6A, it appears that most of the scope for epinephrine-stimulated increases in Q_{max} occurs between 0 (saline) and 0.5 μg kg^{-1} epinephrine; injection of 0.5 μg kg^{-1} caused an increase in Q of 6-8 ml min^{-1} kg^{-1}, while increasing the dose to 1.0 or 2.0 μg kg^{-1} only increased Q a further 2-4 ml min^{-1} kg^{-1}. In contrast to Q, maximum

P_{DA} was significantly higher at all dose levels. This information on the dose-dependency of Q_{max} (ED_{50} 0.22 μg kg^{-1}) and P_{DA} (ED_{50} 0.48 μg kg^{-1}), when combined, suggests that the large increases in systemic vascular resistance (P_{DA}) and output pressure which accompany doses greater than 1.0 μg kg^{-1} limit the ability of epinephrine to increase V_s (systolic emptying). Farrell *et al.* (1986) found that epinephrine exerted its maximal effect on the perfused trout heart at 10 nM. A dose of 4.0 μg kg^{-1} epinephrine results in maximal circulating epinephrine concentrations of 107 nM (Appendix 1; Gamperl *et al.*, in press b). If I assume a 1:1 relationship between injected epinephrine dose and realised plasma levels, a dose of 0.5 μg kg^{-1} could elevate circulating epinephrine levels to 14 nM. According to Farrell *et al.* (1986) this plasma epinephrine titer would lead to near maximal stimulation of cardiac performance (V_s or cardiac power output). Therefore, the diminished ability of higher epinephrine doses (1.0 and 2.0 μg kg^{-1}) to significantly elevate Q may have also occurred because circulating epinephrine titers were above those required to achieve maximal adrenergic stimulation of the heart.

The dose-response curves for dorsal aortic pressure show that "operated" fish have a diminished ability to elevate P_{DA}, when compared with trout having only dorsal aortic cannulae. Because implantation of the Doppler flow probe is unlikely to affect α-adrenoreceptor mediated systemic constriction, the decrease in P_{DA} (approximately 30%) must have resulted from a decrease in epinephrine-stimulated Q. Farrell *et al.* (1988) showed that cutting the pericardium of *in situ* perfused trout hearts reduced maximum cardiac output and cardiac power output by 8% and 18%, respectively. These results indicate that the observed increases in Q (Fig. 1.6) may underestimate those in "intact"

fish by 10 to 30%, and provide indirect support for the hypothesis that the pericardium is important for determining maximum cardiac performance *in vivo*.

Ablation of the coronary artery in trout, under normoxic conditions, appears to have little effect on epinephrine-stimulated cardiac performance. Maximum Q, maximum P_{DA}, and time required to achieve Q_{max} were not significantly different between fish with ablated and intact coronary arteries. These results support recent *in vitro* experiments on eel (*Anguilla australis*; Davie *et al.*, 1992) and dogfish shark hearts (*Squalus acanthias*; Davie and Farrell, 1991b) which concluded that maximum cardiac output, during normoxemia, is not dependent upon coronary perfusion; i.e. the oxygen supplied by the venous (luminal) blood is sufficient to support cardiac performance in most fish. The time course for alterations in stroke volume, following epinephrine injection, were similar in trout with intact and ablated coronaries (e.g. Figs. 1.3, 1.4). This result also suggests that: 1) during normoxemia, coronary blood flow is not essential for cardiac pressure development and/or systolic emptying; and 2) that the presence of a coronary circulation is not a prerequisite for adrenergic stimulation of the compact myocardium by circulating catecholamines.

In type 1 trout, an injection of 2.0 μg kg^{-1} increased Q from 18 ml min^{-1} kg^{-1} to 28.3 ml min^{-1} kg^{-1}, P_{DA} from 2.2 kPa to 3.8 kPa (this value representing P_{DA} at Q_{max}, 10 minutes post-injection), and cardiac power output by approximately 200% (assuming that the post-injection Δ ventral aortic pressure/Δ P_{DA} was approximately 1.2; Wood and Shelton, 1980a). In most fish, cardiac power output can increase two to four-fold with exercise or adrenergic stimulation (Farrell and Jones, 1992; Table 2). Therefore, although

this study suggests that the coronary circulation is not necessary for determining epinephrine–stimulated cardiac performance, or cardiac power output during moderate swimming, it does not preclude the possibility that coronary blood flow is important for maintaining maximum cardiac performance: e.g. rainbow trout swimming at 90% of U_{crit} must increase cardiac power output, over resting levels, by 360% (Kiceniuk and Jones, 1977).

In summary, differences in epinephrine–stimulated cardiac performance between fish exhibiting type 1 and type 2 cardiovascular responses are probably mediated by disparities in the degree of vagally (cholinergically)–mediated cardiac inhibition (i.e. barostatic gain). The capacity of elevated epinephrine titers to increase Q may be limited by increases in output pressure and/or by the relatively low concentration of epinephrine (10 nM) required to cause near maximal adrenergic stimulation of the heart. Cardiac performance, during normoxic conditions, does not depend highly on coronary blood flow. Differences in cardiovascular variables between "operated" and "cannulated" trout provide indirect support for the hypothesis that the pericardium enhances cardiac performance in trout, *in vivo*.

Chapter 2

INFLUENCE OF HYPOXIA AND EPINEPHRINE STIMULATION ON CORONARY BLOOD FLOW AND CARDIAC PERFORMANCE IN TROUT

INTRODUCTION

In fish, unlike mammals, the presence of a coronary circulation is species-dependent, and closely related to activity level (Davie and Farrell, 1991a). This relationship implies that a coronary blood supply is only essential for maintaining cardiac function in fish capable of high cardiac work (i.e. high myocardial oxygen demand). Early experimental evidence for this generalization comes from the survival of coronary-ablated fish (Daxboeck, 1982; Farrell and Steffensen, 1987), and from the 35% reduction in the maximum swimming speed of coronary-ablated trout (Farrell and Steffensen, 1987).

In recent years, the role of the coronary circulation in determining fish cardiac performance has been defined further. Gamperl *et al.* (Chapter 1; in press a) have shown that epinephrine-stimulated cardiac performance in trout during normoxic conditions is not affected by coronary ablation. In addition, Davie and Farrell (1991b) and Davie *et al.* (1992) have shown that perfusion of the coronary circulation with oxygenated red cell suspensions only restored the cardiac power output of hypoxic dogfish (*Squalus acanthias*) and eel (*Anguilla dieffenbechii*) hearts to 50 - 75% of levels measured during normoxia. These experiments suggest that oxygen delivery from the luminal blood to the myocardium is not diffusion limited during normoxic conditions in fish with a small

percentage of compact myocardium, and that increases in coronary oxygen delivery are unlikely to provide for near maximal cardiac performance when venous oxygen content is diminished. Axelsson and Farrell (1993) measured coronary blood flow in the coho salmon (*Oncorhynchus mykiss*) during hypoxia, following the injection of epinephrine, and during spontaneous activity. However, while these authors were able to show that resting coronary blood flow was approximately 1% of cardiac output and that coronary blood flow increased upon exposure to all three experimental conditions, their study did not address whether elevations in coronary blood flow are capable of supporting near maximal cardiac performance during hypoxia.

Farrell (1987) using an *in vitro* perfused rainbow trout heart revealed that arterial pressure, adrenoreceptors, extravascular compression, and cardiac metabolism are involved in the regulation of coronary blood flow in fish. In addition, *in vivo* studies on coho salmon (Axelsson and Farrell, 1993) showed that α–adrenergic constriction, β–adrenergic vasodilation, dorsal aortic pressure, and hypoxic exposure can all affect coronary blood flow. However, no studies have investigated the interactive effects of these variables on coronary flow *in vivo*.

I measured *in vivo* coronary blood flow, cardiac output, and dorsal aortic pressure in trout *(Oncorhynchus mykiss)* exposed to adrenergic stimulation under conditions of normoxia and hypoxia. The goals of this study were two–fold: 1) to investigate whether cardiac performance is depressed under the combined conditions of hypoxemia and high output pressures, irrespective of alterations in coronary blood flow; and 2) to determine whether hypoxia (hypoxemia) alters adrenoreceptor mediated control of coronary blood

flow. Adrenergic stimulation is an appropriate model for studying the *in vivo* relationship between cardiac performance and coronary blood flow because epinephrine increases both cardiac output and systemic vascular resistance; the concomitant increase in cardiac power output increases oxygen demand by the myocardium (Graham and Farrell, 1990).

MATERIALS AND METHODS

Fish

Seawater-adapted rainbow trout (*Oncorhynchus mykiss*) (900-1500 g) were obtained from Merlin Fish Farms (Wentworth, Nova Scotia) and held in tanks (1 x 1 x 1.5 m) for one month prior to experimentation. Fish were fed daily, to satiation, on a diet of commercially available feed pellets, but were fasted for 48 hours prior to surgery. Photoperiod was 12 h light: 12 h dark. Experiments were conducted between May 1 and August 1, 1992.

Blood PO_2 Measurements During Graded Hypoxia

Surgical procedures

Dorsal and ventral aortic cannula were prepared from PE 50 polyethylene tubing (Clay Adams Intramedic), total length 0.8 m. Bubbles were made 5.0 cm from the tip of the dorsal cannula, and 2.5 and 5.0 cm from the tip of the ventral aortic cannula. Trout (1260 ± 102 g) were anaesthetized (0.1 g l^{-1} tricaine methane sulphonate, MS 222) and placed supine in a wetted chamois leather sling. The fish were fitted with a dorsal aortic cannula (Smith and Bell, 1964) after which intermittent retrograde irrigation with seawater

containing anaesthetic was begun. Ventral aortic cannulation was subsequently performed using a new technique. (Fig. 2.1). For ventral aortic cannulation, the trout was held upright in the sling, a hole was made at the side of the mouth with a 13 gauge steel needle and a short piece of heated flared PE 160 tubing was exteriorized through the resultant hole. A 21 gauge needle was inserted into the cartilage of the tongue (while slowly rotating), just anterior to the junction of the second gill arches at an angle of between 30° and 40°. Once resistance ceased, the needle was removed and a PE 50 cannula, with indwelling stainless steel wire, was advanced fully into the resultant hole. The steel wire was then withdrawn and the PE 50 tubing was adjusted until blood flowed freely into the cannula. After positioning the cannula to maximize blood flow a wire staple (0.75 cm long; 0.4 cm wide; fashioned from a paperclip) was used to secure the cannula to the tongue at a position just anterior to the first bubble. To finish, the remaining length of the cannula was threaded through the side of the mouth until the second bubble rested firmly against the flared PE 160 tubing, and a constricting knot was tied around the PE 160 tubing to prevent cannula movement. The main advantages of this technique are: 1) a double cannulation can be completed in 5 – 7 minutes and therefore only limited irrigation of the gills is necessary; 2) there is no leakage from the ventral aorta because the cartilaginous tissue of the tongue closes tightly around the cannula; and 3) pressure records and blood samples can be obtained for periods in excess of a week. Although blood can be withdrawn from all ventral aortic cannulae, persistent pressure records can only be obtained from approximately 50 % of fish cannulated using the above technique. Once surgery was completed, fish were placed into black perspex boxes (11 x 16 x 75 cm) to recover. Boxes were supplied with aerated seawater (8.6 ± 0.5 °C)

Figure 2.1. Photographs of the rainbow buccal cavity (A) and head (B) showing the position of dorsal and ventral aortic cannulae.

Figure 2.1

at a flow rate of 4 l min^{-1}.

Experimental Protocol

After 24 - 48 hours of recovery, to determine a point where fish were experiencing hypoxemia but not bradycardia, venous PO_2 (P_vO_2), arterial PO_2 (P_aO_2), heart rate (f_H), and dorsal aortic pressure (P_{DA}) were monitored in trout (N=7) as the seawater PO_2 (P_wO_2) was gradually reduced from 145 to 65 mm Hg. P_aO_2, P_vO_2, f_H and P_{DA} were measured in trout after every 15 mm Hg drop in P_wO_2; time between blood samples ranging from 25 to 30 minutes. Seawater oxygen content was manipulated by bubbling a controlled mixture of air and nitrogen through a gas exchange column (8 cm in diameter; 45 cm in length) and was monitored with a YSI oxygen meter (model 50). Water oxygen content (mg l^{-1}) was converted to partial pressure (P_wO_2) based on calibrations obtained with a thermostatted Radiometer O_2 electrode (model 5046-0). If fish struggled during the experiment, a 10 minute "recovery" period was allowed before any further samples were taken. Trout that struggled repeatedly were discarded.

Measurements of Cardiovascular Performance During Hypoxia and Adrenergic Stimulation

Surgical Procedures

Trout (1400 ± 47 g)(N = 9) were anaesthetized (0.1 g l^{-1} MS 222) and placed supine in a wetted chamois leather sling. The fish were quickly fitted (~45 sec) with a dorsal aortic cannula (PE 50) after which retrograde irrigation with seawater containing anaesthetic (0.05 g l^{-1} MS 222) was begun. A 3 - 4 cm incision was made through the skin and muscle at a position overlying the ventral aorta and anterior aspect of the

pericardium. After cutting through the pectoral girdle and expanding the resultant cavity with tissue spreaders, the anterior portion of the pericardium was cut to expose the ventral aorta and coronary artery. The connective tissue from the anterior portion of the ventral aorta, in addition to that adhering the coronary artery to the ventral aorta and the anterior bulbus, was subsequently removed to facilitate the placement of the Doppler flow probes. Once the flow probes were in place, the musculature and skin were closed using continuous silk sutures. The Doppler probe leads were secured to the skin at the anterior apex of the incision, and at a position just posterior to the pectoral fins. The operation generally took between one and two hours, and the bleeding was usually minimal. The integrity of the pericardium was not restored by suturing because of time constrains. Once surgery was completed, the fish were placed into black perspex boxes to recover. During recovery, and subsequent experiments, the boxes were supplied with aerated seawater (10.9 ± 0.9 °C) at a flow rate of 4 l min^{-1}.

Probe Design

Flow probes (Fig. 2.2) were constructed by implanting piezoelectric crystals with 80 cm leads (Crystal Biotech, Hopkinton MA) into a 1 cm length of Tygon® tubing (i.d. 2.2 mm), and into a 3 – 4 mm length of PE 50 or PE 60 tubing; these probes utilised for the measurement of ventral aortic flow (cardiac output, Q) and coronary flow (CF), respectively. Both sections of tubing were split to facilitate placement on their respective vessels, and had small notches to allow for crystal attachment. Piezoelectric crystals were

Figure 2.2. Schematic diagram of the Doppler flow probes used to measure ventral aortic flow (cardiac output) and coronary flow in intact rainbow trout. Arrows indicate the direction of blood flow. The drawing is 21 X actual size.

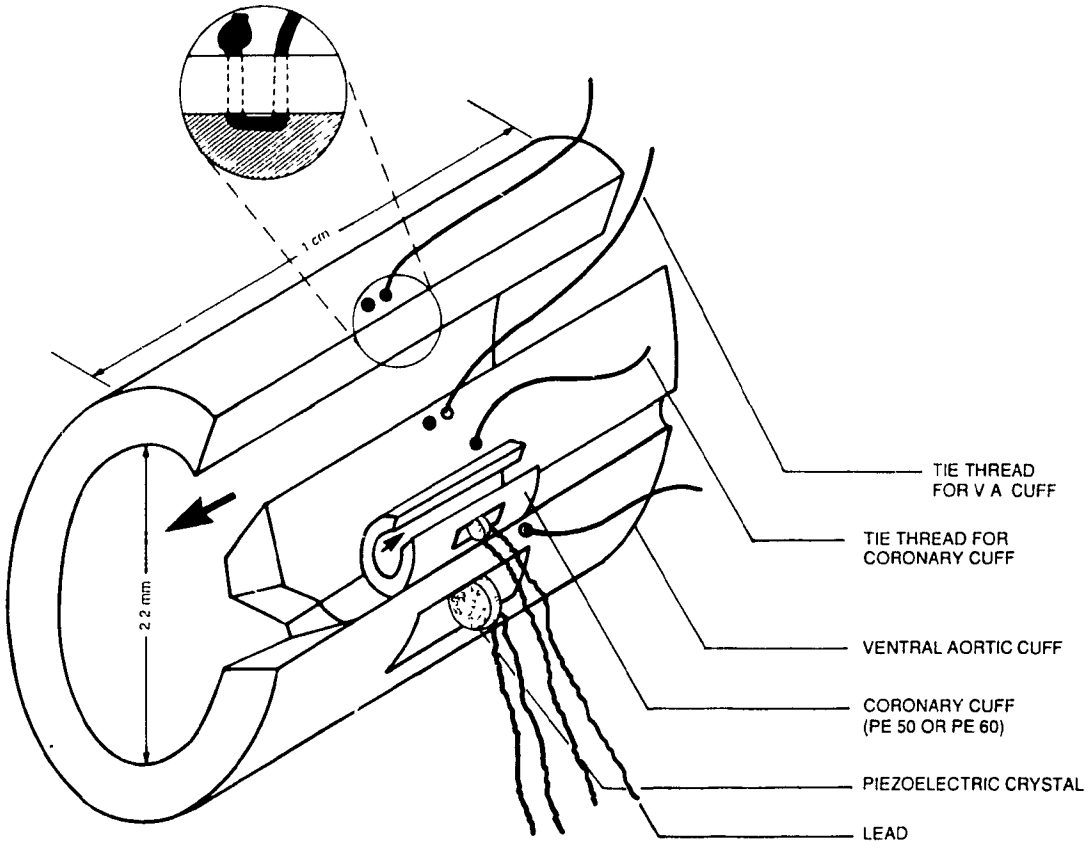
1 cm
2.2 mm
TIE THREAD
FOR V A CUFF
TIE THREAD FOR
CORONARY CUFF
VENTRAL AORTIC CUFF
CORONARY CUFF
(PE 50 OR PE 60)
PIEZOELECTRIC CRYSTAL
LEAD

Figure 2.2

secured in the notches with cyanoacrylate cement after thoroughly roughening the area with a scalpel blade. A notch at the anterior end of the ventral aortic cuff, and a shallow groove along the length of the ventral aortic cuff (for coronary cuff placement), ensured that the coronary artery was not bent or deformed as it passed over the ventral aortic cuff and through the coronary artery cuff. Probes were fitted with tie-strings to prevent tube diameter from increasing at elevated ventral aortic pressures.

Experimental Protocol

After a 24 - 48 hr recovery period, cardiovascular variables (Q, f_H, P_{DA} and CF) were measured in trout before and after the injection of 1.0 μg kg^{-1} epinephrine (Sigma Chemical Co, St. Louis, MO) under three conditions: 1) normoxia; 2) following 30 minutes of graded hypoxia (final P_wO_2 90 mm Hg); and 3) after recovery from hypoxia. Treatments were separated by 2.5 hours. During the normoxic treatments, "resting" cardiovascular variables and P_aO_2 were measured 5 minutes prior to epinephrine injection. In the hypoxic treatment, cardiovascular variables were continuously measured during the graded hypoxia, and "resting" Q, f_H, P_{DA}, CF and P_aO_2 were measured 7-10 minutes after P_wO_2 had stabilized at 90 mm Hg. In all treatments, Q, f_H, P_{DA}, and CF were recorded for 20 minutes following epinephrine injection. Epinephrine was administered through the dorsal cannula in a 0.2 - 0.4 ml carrier volume of saline. The second normoxic period was used to ensure that any hypoxia-induced differences in adenaline-stimulated cardiovascular performance were not due to repeated epinephrine injection, and to provide some preliminary information on the duration of the recovery period required to restore

the scope for adrenergically-stimulated cardiovascular performance.

Measurement Techniques

P_{DA} was measured by attaching the dorsal aortic cannula to a Gould Statham (Model P23-10) pressure transducer connected to a amplifier recorder (Asea Brown and Boveri; Model SE-120). Pressure was calibrated daily against a static water column.

Mean ventral aortic flow (Q) was measured by connecting a pulsed Doppler Flowmeter (Model 545c-4; Bioengineering, University of Iowa) to an RC-integrator, and an amplifier recorder. Coronary flow (CF) was measured by connecting the Doppler flowmeter to an amplifier recorder. In order to determine absolute flow rates (ml min^{-1}) an *in situ*, post-mortem, calibration of the ventral aortic flow probe, and an *in vitro* calibration of the coronary flow probe were performed using a peristaltic pump (Gilson, Minipuls®II) and human blood (approximate haematocrit 20 %). To calibrate the ventral aortic flow probe, the sinus venosus and atrium were removed, the ventricle was bisected laterally, and the peristaltic pump outflow tubing (PE 160) was tied into the ventricular lumen (see Gamperl *et al.*, in press a for further details). To calibrate the coronary flow probe, the coronary artery was cut and the flow probes (with coronary artery in place) were placed in a petri-dish filled with teleost saline (Hoar and Hickman, 1983). After tying a piece of 27 gauge needle, with an attached length of PE 10 tubing, into each end of the segment of coronary artery, the coronary flow probe was calibrated at flow rates between 0.05 and 0.55 ml min^{-1}.

P_aO_2, P_vO_2, and P_wO_2 were measured using a thermostatted Radiometer O_2

electrode (E 5046-0, Denmark) connected to an amplifier recorder. The PO_2 electrode was calibrated with humidified N_2 and air prior to each experiment, and the calibration was rechecked with air prior to each sample.

Analysis

f_H at rest, or at a particular time post-injection, was determined by measuring the number of systolic peaks on the P_{DA} record during a 30 second interval, the interval being 15 seconds on either side of the desired time. Mean P_{DA} was calculated as (systolic pressure + 2 (diastolic pressure)) / 3 (Burton, 1972; Wood *et al.*; 1979). Stroke volume (V_s) was calculated from Q / f_H. Coronary vascular resistance (R_{cor}) was calculated as P_{DA} / CF (Axelsson and Farrell, 1993). Statistical differences ($p < 0.05$) between treatments (Normoxia, Hypoxia; Normoxia II) for post-injection cardiovascular variables were determined using a repeated measures analysis of variance (Proc GLM; SAS Institute Inc.) with multiple contrasts. Comparisons between cardiovascular variables at normoxia (P_wO_2 145 mm Hg) and at various levels of hypoxia (P_wO_2 130 -90 mm Hg) were made using the same analysis. Tests for between subject effects (ie. type effects, see results) indicated that response type had no effect on resting or post-injection cardiovascular variables.

RESULTS

Blood PO_2 During Graded Hypoxia

As P_wO_2 was gradually lowered from 145 mm Hg to 65 mm Hg, both arterial and venous PO_2 decreased (Fig. 2.3). In all fish, P_aO_2 fell linearly ($0.99 \geq r^2 \leq 0.92$), while

Figure 2.3. The relationship between environmental oxygen tension (P_wO_2), arterial oxygen tension (P_aO_2) and venous oxygen tension (P_vO_2) in 7 seawater–acclimated rainbow trout as P_wO_2 was lowered from approximately 150 mm Hg to 60 mm Hg over a three hour period. Numbers beside the symbols (upper right corner) indicate the haematocrit of individual fish.

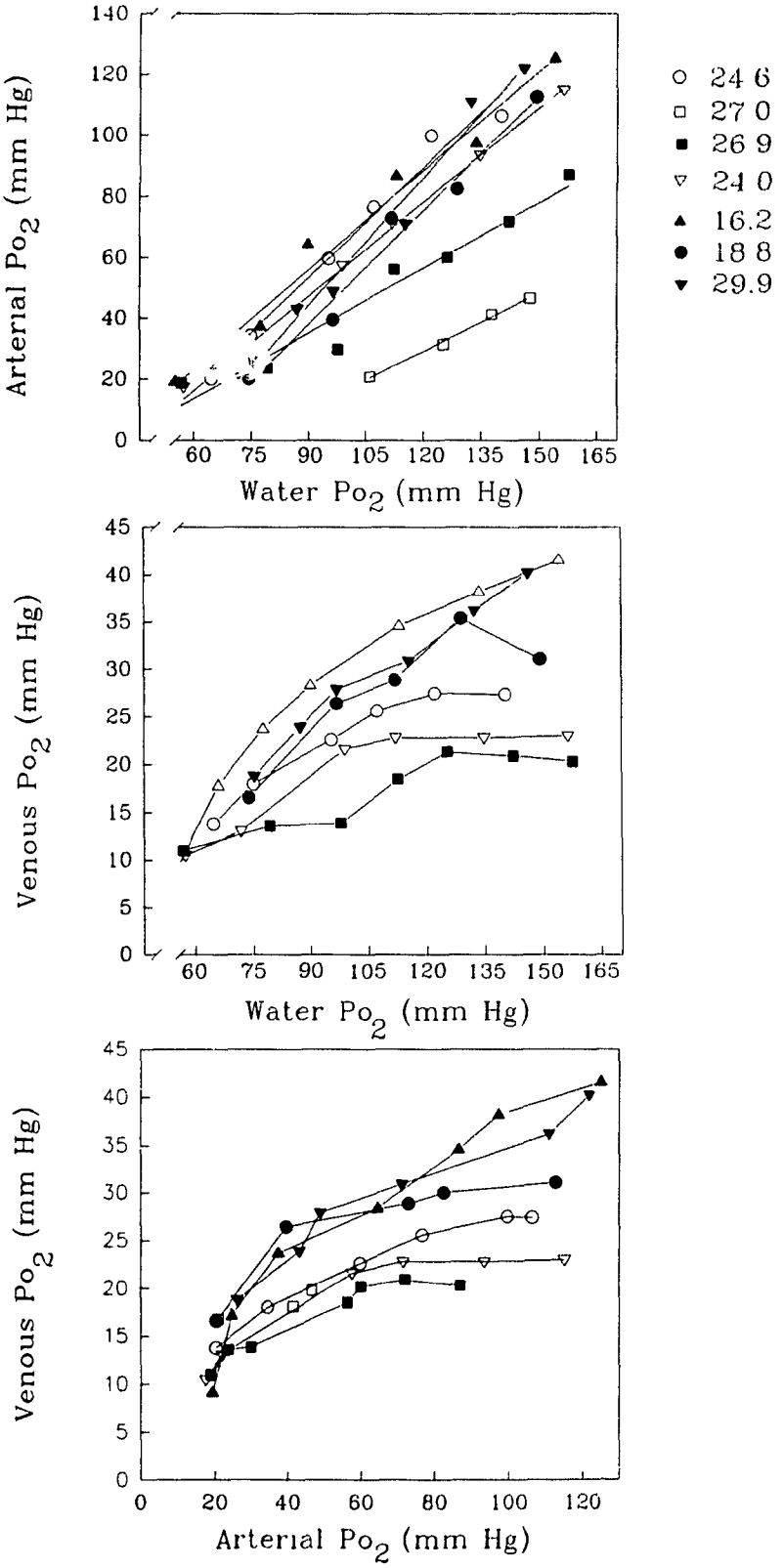
Arterial Po_2 (mm Hg)
Water Po_2 (mm Hg)
Venous Po_2 (mm Hg)
Water Po_2 (mm Hg)
Venous Po_2 (mm Hg)
Arterial Po_2 (mm Hg)
24 6
27 0
26 9
24 0
16.2
18 8
29.9

Figure 2.3

the P_vO_2 decline appeared to be curvilinear. As P_wO_2 fell, P_aO_2 decreased more (by 80 – 90 mm Hg) than did P_vO_2 (11 – 25 mm Hg). P_{DA} and f_H remained at the normoxic value until P_wO_2 was reduced below approximately 80 – 85 mm Hg. However, a severe bradycardia was seen in 5 of 9 trout, as P_wO_2 dropped below 80 mm Hg. Because severe bradycardia would have complicated the comparison of cardiovascular performance and CF between hypoxic and normoxic fish, a P_wO_2 of 90 mm Hg was chosen for subsequent experiments. At this P_wO_2, P_aO_2 and P_vO_2 were be 35 – 45 mm Hg and 15 – 25 mm Hg, respectively; levels approximately 40% – 50% of those seen during normoxia.

Cardiovascular Performance During Hypoxia and Adrenergic Stimulation

Resting Cardiovascular Variables

Cardiac output, CF, P_{DA}, f_H, V_s and R_{cor}, during the initial normoxic period averaged 18 ml min^{-1} kg^{-1}, 0.14 ml min^{-1} kg^{-1}, 2.8 kPa, 64 beats min^{-1}, 0.29 ml kg^{-1} and 22.5 kPa min kg ml^{-1}, respectively (Table 2.1). The P_wO_2 threshold for hypoxia–induced alterations in cardiovascular variables (CF, Q, f_H, V_s) appeared to be between 95 and 105 mm Hg (Fig. 2.4). After 30 minutes of exposure to graded hypoxia and at a P_wO_2 of 90 mm Hg, changes in Q, CF, V_s and R_{cor}, but not P_{DA} and f_H, were significant ($p < 0.05$)(Table 2.1). Q and V_s increased by 3.1 ml min^{-1} kg^{-1} (17 %) and 0.07 ml kg^{-1} (25 %), respectively, while R_{cor} decreased by 6.3 kPa min kg ml^{-1} (28 %). Absolute CF increased significantly (0.052 ml min^{-1} kg^{-1}; 37%) following exposure to graded hypoxia. Although the % increase in CF was greater than that for Q, the percentage of Q delivered to the coronary circulation (CF/Q X 100) was not increased significantly ($0.10 > p >$

0.05). P_aO_2 fell from 88 to 38 mm Hg during the 30 minute hypoxic exposure.

Two and a half hours after the trout had been returned to normoxia (P_wO_2 ~150 mm Hg), most resting variables (Q, CF, f_H, V_s and R_{cor}) were intermediate between those measured during the initial normoxic period and those recorded during the hypoxic

Table 2.1. Cardiovascular variables in resting rainbow trout (*Oncorhynchus mykiss*) (N = 8) under conditions of normoxia and hypoxia. Values with dissimilar letters are significantly different at $p < 0.05$ (*) and $p < 0.10$ (§).

	NORMOXIA	HYPOXIA	NORMOXIA II
Cardiac Output (ml min^{-1} kg^{-1})	18.0 ± 1.4^{a}	$21.1 \pm 1.8^{b*}$	20.0 ± 1.5^{ab}
Coronary Flow (ml min^{-1} kg^{-1})	0.143 ± 0.020^{a}	$0.195 \pm 0.019^{b*}$	0.148 ± 0.018^{a}
Coronary Flow (% Cardiac Output)	$0.837 \pm 0.13^{a§}$	0.937 ± 0.11^{b}	$0.791 \pm 0.12^{a*}$
Heart Rate (beats min^{-1})	$63.6 \pm 2.8^{a§}$	58.9 ± 2.7^{b}	62.3 ± 2.6^{ab}
P_{DA} (kPa)	$2.81 \pm 0.17^{a§}$	$3.02 \pm 0.21^{a*}$	2.64 ± 0.19^{b}
Stroke Volume (ml kg^{-1})	$0.287 \pm 0.020^{a*}$	$0.358 \pm 0.02^{b*}$	$0.322 \pm 0.02^{c*}$
P_aO_2 (mm Hg)	$87.7 \pm 8.4^{a*}$	$37.9 \pm 4.0^{b*}$	$80.4 \pm 5.3^{c*}$
Coronary Resistance (kPa kg min ml^{-1})	$22.5 \pm 3.0^{a*}$	16.2 ± 1.6^{b}	$18.9 \pm 2.1^{c§}$

Figure 2.4. A summary of cardiovascular responses to 30 minutes of graded hypoxia in the seawater adapted rainbow at 10 – 12 °C (N = 8). Asterisks indicate statistically significant differences from normoxic conditions (P_wO_2 = 150 mm Hg). Vertical bars indicate the S.E. for each mean value.

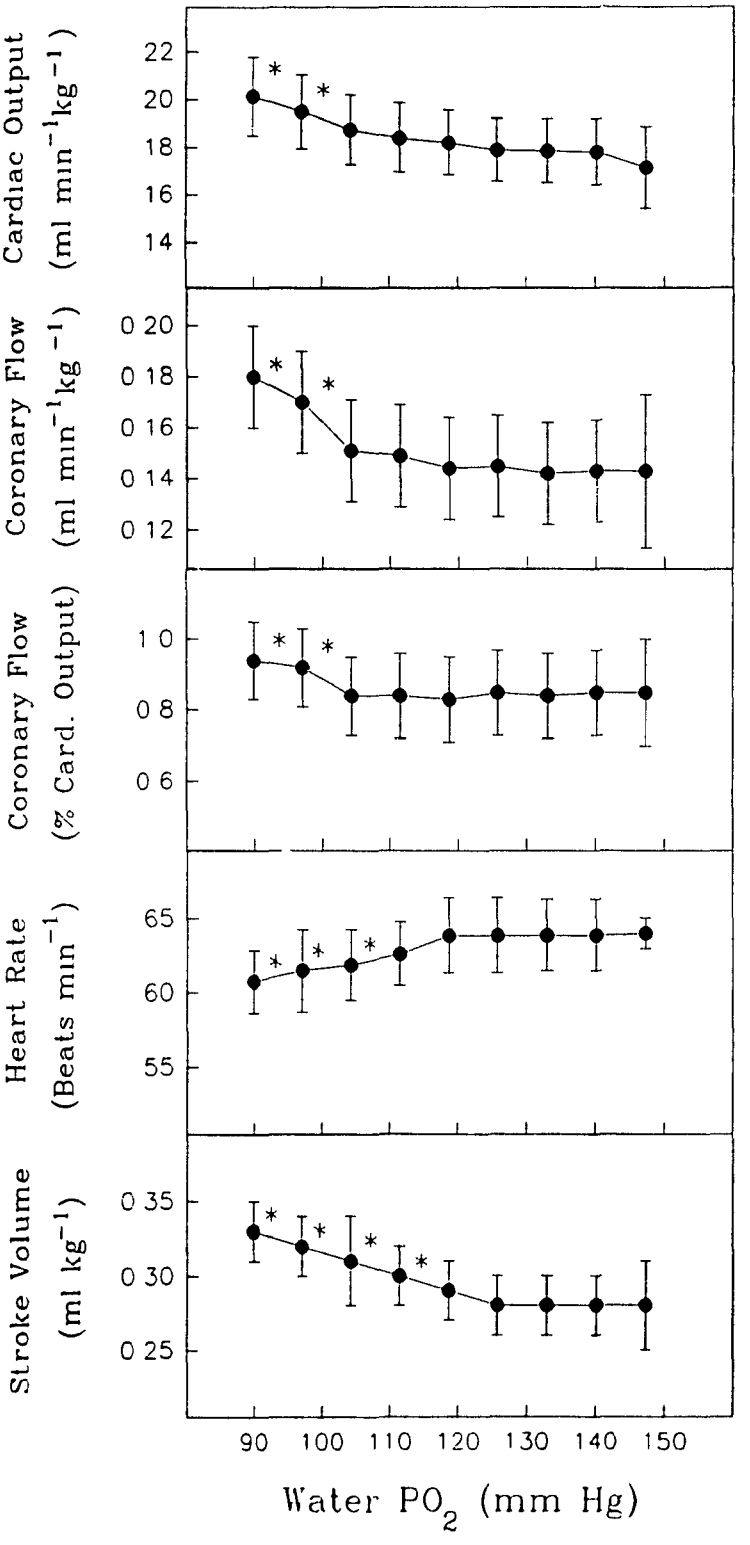
Cardiac Output (ml min^{-1}kg^{-1})
22
20
18
16
14
Coronary Flow (ml min^{-1}kg^{-1})
0 20
0 18
0 16
0 14
0 12
Coronary Flow (% Card. Output)
1 0
0 8
0 6
Heart Rate (Beats min^{-1})
65
60
55
Stroke Volume (ml kg^{-1})
0 35
0 30
0 25
90 100 110 120 130 140 150
Water PO_2 (mm Hg)

Figure 2.4

exposure (Table 2.1). A notable exception was P_{DA}, which was marginally lower than in the normoxic and hypoxic groups ($p = 0.07$). P_aO_2 during the second normoxic period was 80 mm Hg, a level significantly lower than that measured during the initial normoxic period (Table 2.1).

Variations in the Response to Epinephrine Injection

Response Type

Adrenergic stimulation during normoxia or hypoxia resulted in two types of cardiovascular responses (Figs. 2.5, 2.6). Type 1 was characterised by an intial post-injection increase in Q, Q reaching a maximum value within 4 - 6 minutes. Type 2 was characterised by an initial drop in Q, followed by a steady increase until maximum values were reached at approximately 10 - 14 minutes. As shown in Gamperl *et al.* (Chapter 1; in press a), these two types of response to epinephrine injection were mediated by type-specific differences in the temporal pattern of f_H and V_s responses. Type 2 trout showed a greater post-injection bradycardia and a 2 - 4 minute delay in the increase in V_s (Fig. 2.7).

Differences in the temporal pattern of changes in coronary flow were also evident between fish exhibiting type 1 and type 2 responses (Figs. 2.5, 2.6, 2.11). In type 1 fish CF increased immediately following epinephrine injection and reached a maximum within 6 - 8 minutes. In type 2 fish, increases in CF were delayed by approximately 4 minutes and maximum CF values were not reached until 8 - 10 minutes post-injection. In type 2 fish CF, measured as a % of Q, increased immediately following epinephrine injection

Figure 2.5. An original recording of cardiac output (-----) and coronary blood flow (——) in a rainbow trout exhibiting a type 1 response to the injection of 1.0 μg kg^{-1} epinephrine, under conditions of normoxia ($\bar{P}_wO_2$ 150 mm Hg), hypoxia ($\bar{P}_wO_2$ 90 mm Hg) and after 2.5 hrs of recovery from hypoxia (Normoxia II). Arrows indicate the point of epinephrine injection.

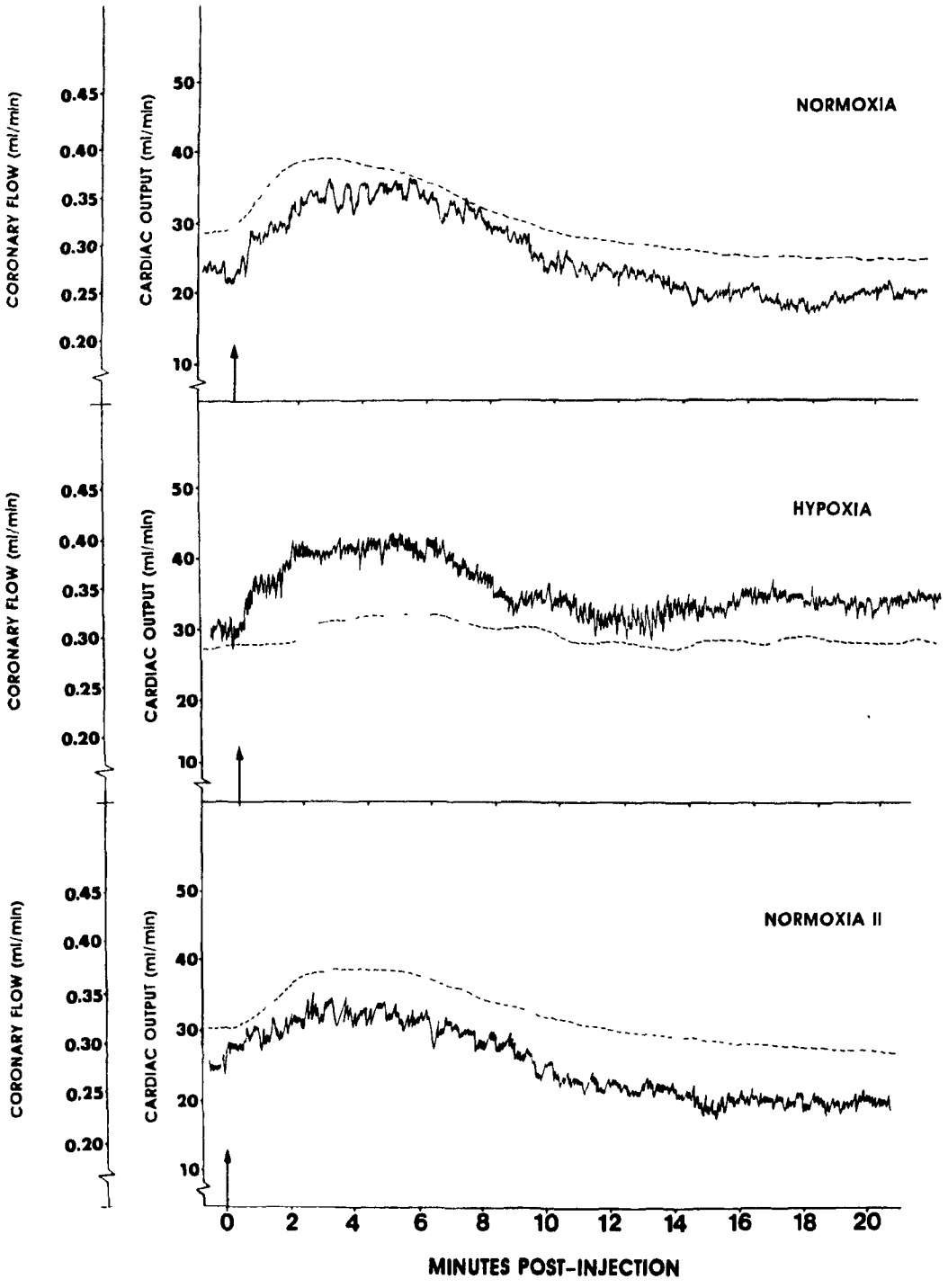
CORONARY FLOW (ml/min)
0.45
0.40
0.35
0.30
0.25
0.20
CARDIAC OUTPUT (ml/min)
50
40
30
20
10
NORMOXIA
HYPOXIA
NORMOXIA II
0
2
4
6
8
10
12
14
16
18
20
MINUTES POST-INJECTION

Figure 2.5

Figure 2.6. An original recording of cardiac output (-----) and coronary blood flow (——) in a rainbow trout exhibiting a type 2 respose to the injection of 1.0 μg kg^{-1} epinephrine, under conditions of normoxia ($\bar{P}_wO_2$ 150 mm Hg), hypoxia ($\bar{P}_wO_2$ 90 mm Hg) and after 2.5 hrs of recovery from hypoxia (Normoxia II). Arrows indicate the point of epinephrine injection.

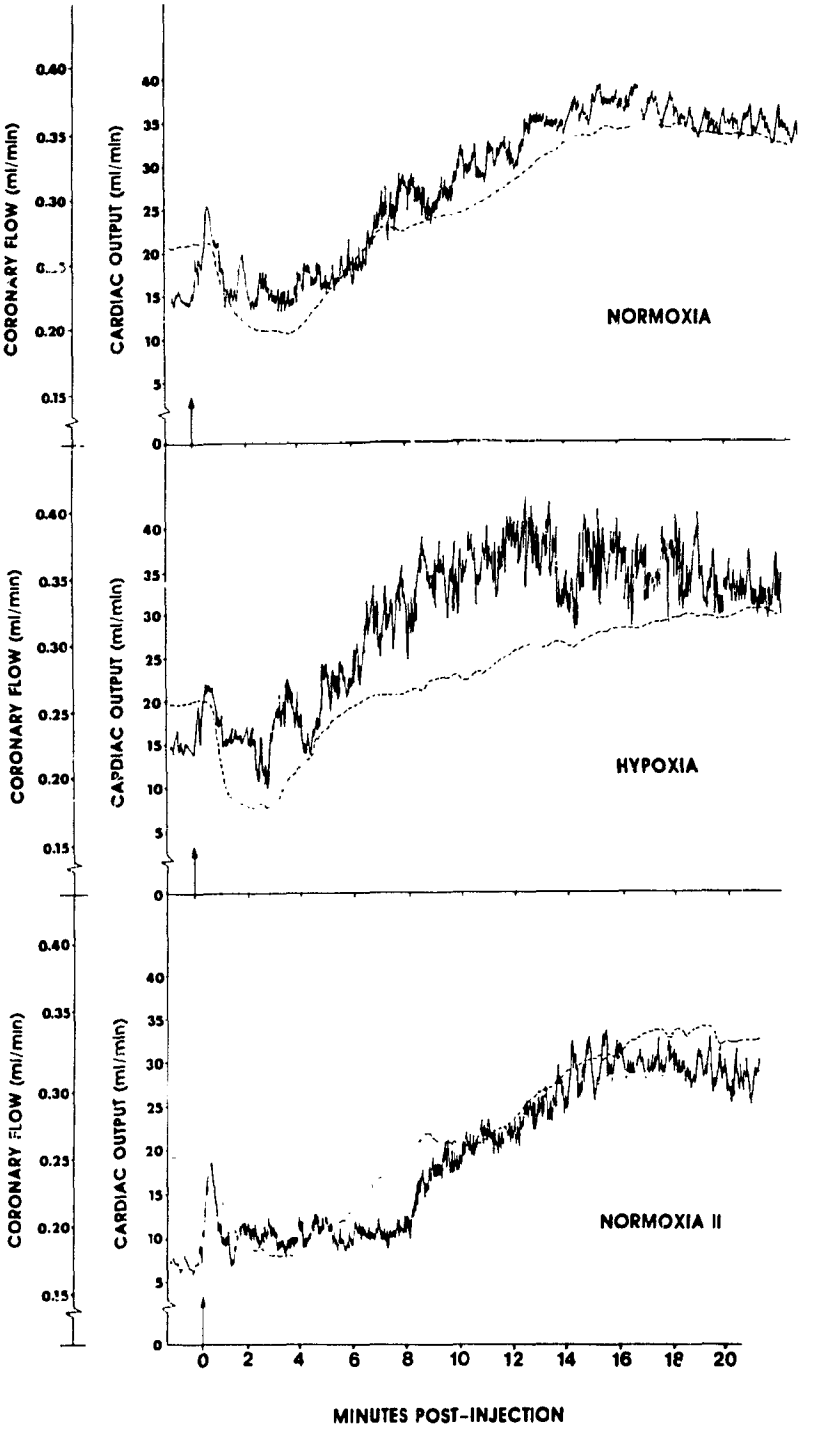
CORONARY FLOW (ml/min)
CARDIAC OUTPUT (ml/min)
NORMOXIA
HYPOXIA
NORMOXIA II
MINUTES POST-INJECTION

Figure 2.6

Figure 2.7. Effect of epinephrine injection (1.0 μg kg^{-1}) on heart rate (f_H) and stroke volume (V_s) in type 1 (○) and type 2 (●) seawater adapted rainbow trout (N = 4 per response type). Vertical bars indicate the S.E. for each mean value.

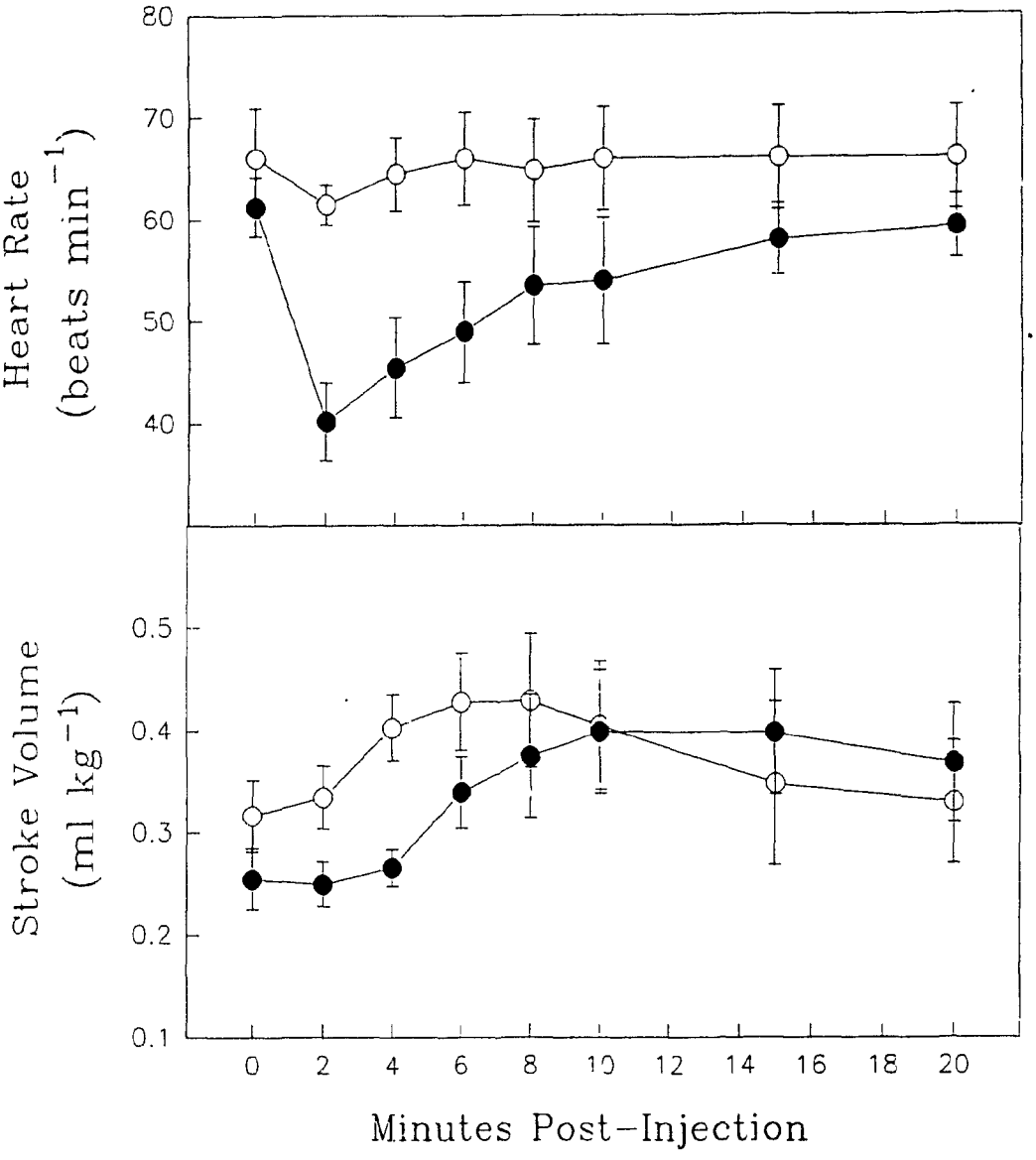
Heart Rate
(beats min^{-1})
80
70
60
50
40
Stroke Volume
(ml kg^{-1})
0.5
0.4
0.3
0.2
0.1
0
2
4
6
8
10
12
14
16
18
20
Minutes Post-Injection

Figure 2.7

and remained elevated until 6 minutes post-injection. In contrast, type 1 fish displayed no post-injection alterations in the percentage of Q delivered to the coronary circulation.

Response Magnitude

The degree to which hypoxic exposure affected epinephrine-stimulated cardiovascular variables and CF was quantitatively similar in both response types (Fig. 2.5, 2.6). Epinephrine injection into normoxic trout increased Q by 45%, CF by 66% and P_{DA} by 100% (Figs. 2.8, 2.9; Table 2.2). However, epinephrine injection into hypoxic fish resulted in a 50% smaller increase in Q, as compared to fish during the initial normoxic treatment, despite similar increases in CF and P_{DA} (Figs. 2.5, 2.6, 2.8, 2.9; Table 2.2). The smaller increase in epinephrine-stimulated Q during hypoxia, as compared to normoxia, was due to differences in both V_s and f_H. For example, in type 1 normoxic fish V_s increased by approximately 0.12 ml kg^{-1} while f_H returned to pre-injection levels by 4 - 6 minutes. However, in hypoxic fish, V_s only increased by approximately 0.08 ml kg^{-1} and f_H remained below pre-injection levels for at least 15 minutes (Fig. 2.10). Although hypoxic fish had a 50% smaller increase in Q, post-injection maximum Q in hypoxic fish (25.05 ± 1.72 ml min^{-1} kg^{-1}) was not significantly different from that measured in normoxic fish (26.17 ± 1.3 ml min^{-1} kg^{-1}) because pre-injection Q was higher in hypoxic fish.

Although maximum post-injection CF, measured as %Q or ml min^{-1} kg^{-1}, was significantly greater during hypoxia than during normoxia (Table 2.2; Fig. 2.11), this difference was related to discrepancies that existed prior to epinephrine injection. Post-

injection increases in CF were not significantly different between hypoxic and normoxic fish (Table 2.2).

Table 2.2. Cardiovascular variables in adrenergically stimulated (1.0 ug kg^{-1} epinephrine) rainbow trout (*Oncorhynchus mykiss*) during normoxia and hypoxia. Values with dissimilar letters are significantly different at $p < 0.05$ (*) and $p < 0.10$ (§).

	NORMOXIA	HYPOXIA	NORMOXIA II
(A) Maximum Increase			
Cardiac Output (ml min^{-1} kg^{-1})	$8.17 \pm 1.1^{a*}$	$3.91 \pm 0.9^{b*}$	$6.33 \pm 0.9^{c*}$
Coronary Flow (ml min^{-1} kg^{-1})	0.095 ± 0.020	0.074 ± 0.016	0.079 ± 0.015
P_{DA} (kPa)	2.84 ± 0.17	2.46 ± 0.29	2.86 ± 0.24
(B) Absolute Levels at Maximum			
Cardiac Output (ml min^{-1} kg^{-1})	26.2 ± 1.3	25.05 ± 1.7	26.30 ± 1.4
Coronary Flow (ml min^{-1} kg^{-1})	0.221 ± 0.02^{a}	$0.270 \pm 0.03^{*b}$	0.226 ± 0.03^{a}
(C) Cardiovascular Parameters at Maximum Coronary Flow			
Coronary Flow (% Cardiac Output)	0.88 ± 0.12^{a}	$1.13 \pm 0.15^{b*}$	0.88 ± 0.13^{a}
Coronary Resistance (kPa kg min ml^{-1})	21.7 ± 2.8^{a}	$17.1 \pm 2.3^{b*}$	18.5 ± 1.8^{ab}

Figure 2.8. Effect of epinephrine injection (1.0 μg kg^{-1}) on cardiac output (Q), coronary blood flow (CF), dorsal aortic pressure (P_{DA}), and coronary artery resistance (R_{cor}) in type 1 rainbow trout (N = 4) under conditions of normoxia (○; $\bar{P}_wO_2$ 150 mm Hg), hypoxia (●; $\bar{P}_wO_2$ 90 mm Hg) and after 2.5 hrs of recovery from hypoxia (□). Values are missing when N < 4. Vertical bars indicate the S.E. for each mean value.

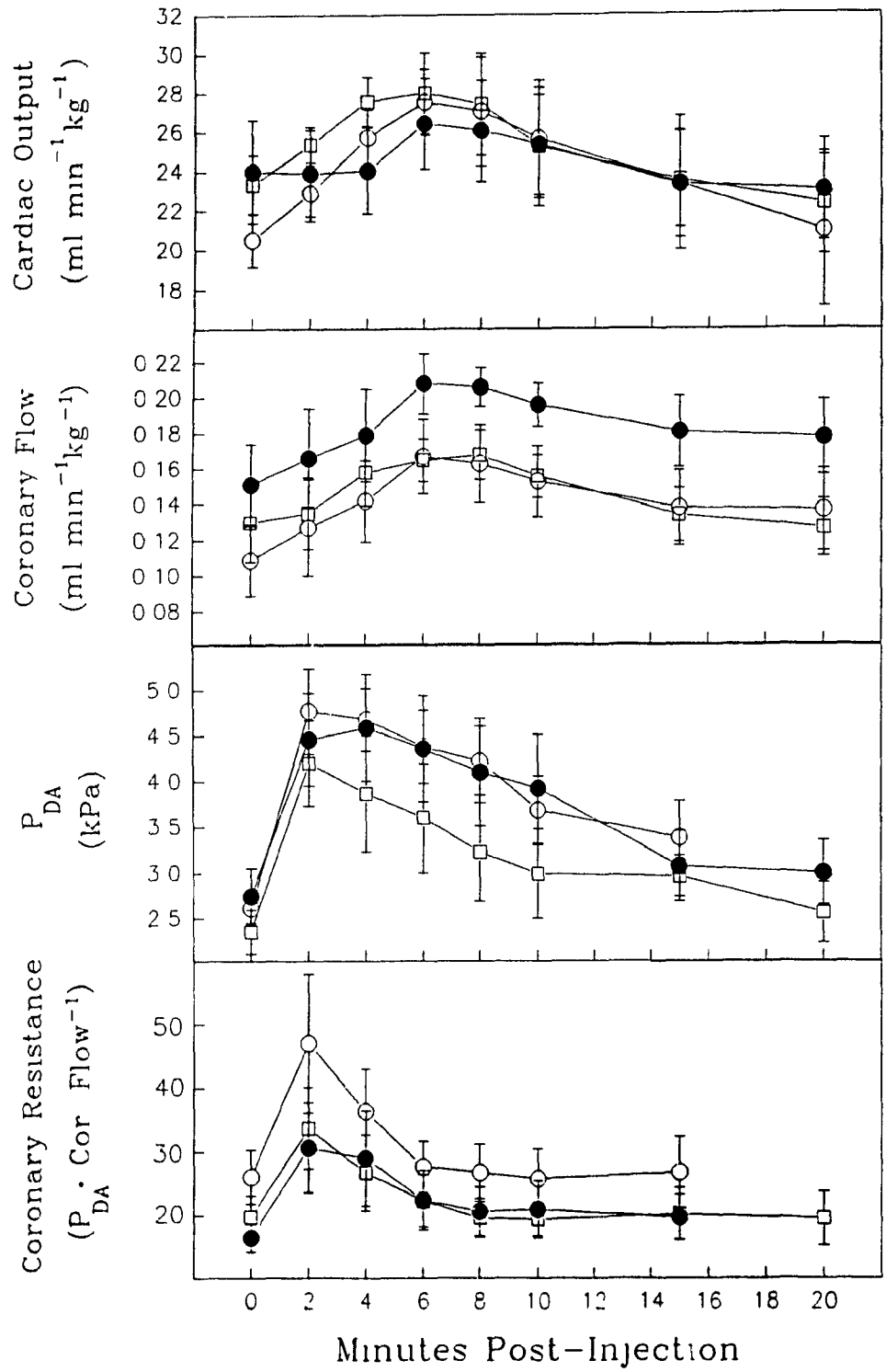
Cardiac Output
(ml min⁻¹kg⁻¹)
32
30
28
26
24
22
20
18
Coronary Flow
(ml min⁻¹kg⁻¹)
0 22
0 20
0 18
0 16
0 14
0 12
0 10
0 08
P DA
(kPa)
5 0
4 5
4 0
3 5
3 0
2 5
Coronary Resistance
(P DA · Cor Flow⁻¹)
50
40
30
20
0
2
4
6
8
10
12
14
16
18
20
Minutes Post-Injection

Figure 2.8

Figure 2.9. Effect of epinephrine injection (1.0 μg kg^{-1}) on cardiac output (Q), coronary blood flow (CF), dorsal aortic pressure (P_{DA}), and coronary artery resistance (R_{cor}) in type 2 rainbow trout (N = 4) under conditions of normoxia (○; $\bar{P}_wO_2$ 150 mm Hg), hypoxia (●; $\bar{P}_wO_2$ 90 mm Hg) and after 2.5 hrs of recovery from hypoxia (□). Values are missing when N < 4. Vertical bars indicate the S.E. for each mean value.

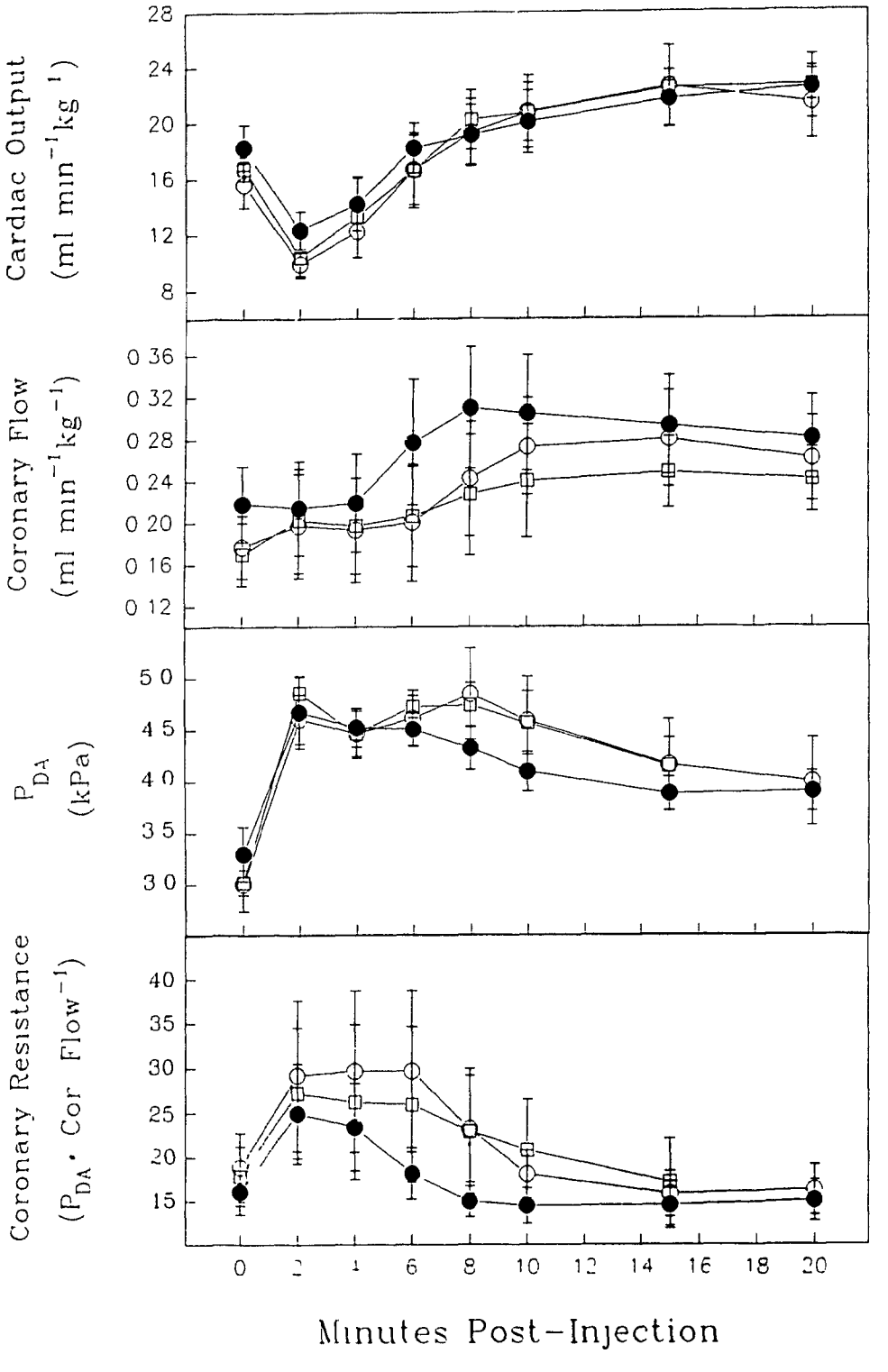
Cardiac Output
(ml min^{-1}kg^{-1})
28
24
20
16
12
8
Coronary Flow
(ml min^{-1}kg^{-1})
0 36
0 32
0 28
0 24
0 20
0 16
0 12
P_{DA}
(kPa)
5 0
4 5
4 0
3 5
3 0
Coronary Resistance
(P_{DA} · Cor Flow^{-1})
40
35
30
25
20
15
0
2
4
6
8
10
12
14
16
18
20
Minutes Post-Injection

Figure 2.9

Figure 2.10. Effect of epinephrine injection (1.0 μg kg^{-1}) on heart rate (f_H) and stroke volume (V_s) in type 1 rainbow trout (N = 4) under conditions of normoxia (○; $\bar{P}_wO_2$ 150 mm Hg) and hypoxia (●; $\bar{P}_wO_2$ 90 mm Hg). Vertical bars indicate the S.E. for each mean value.

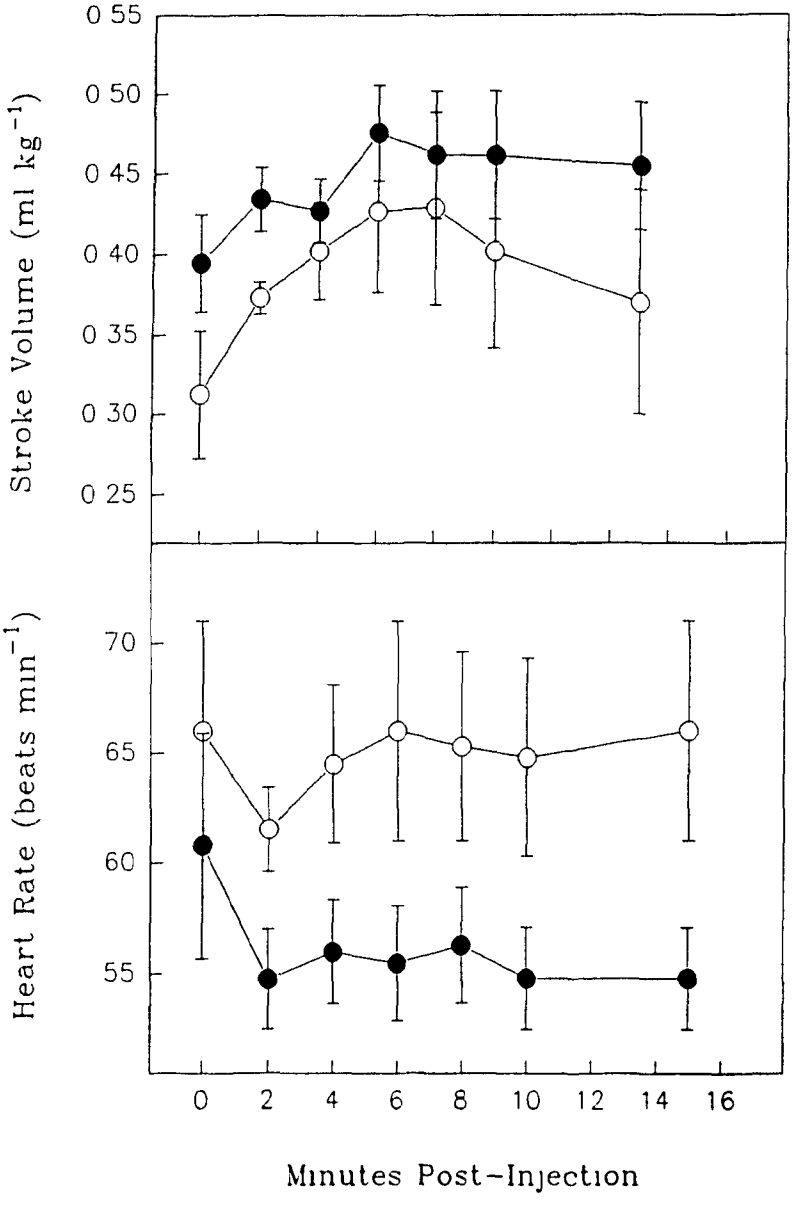
0 55
0 50
0 45
0 40
0 35
0 30
0 25
Stroke Volume (ml kg^{-1})
70
65
60
55
Heart Rate (beats min^{-1})
0
2
4
6
8
10
12
14
16
Minutes Post-Injection

Figure 2.10

Figure 2.11. Effect of epinephrine injection (1.0 μg kg^{-1}) on coronary blood flow (% Q) in type 1 (N = 4) and type 2 (N = 4) rainbow trout under conditions of normoxia (○; $\bar{P}_wO_2$ 150 mm Hg), hypoxia (●; $\bar{P}_wO_2$ 90 mm Hg) and 2.5 hrs after recovery from hypoxia (□). Vertical bars indicate the S.E. for each mean value.

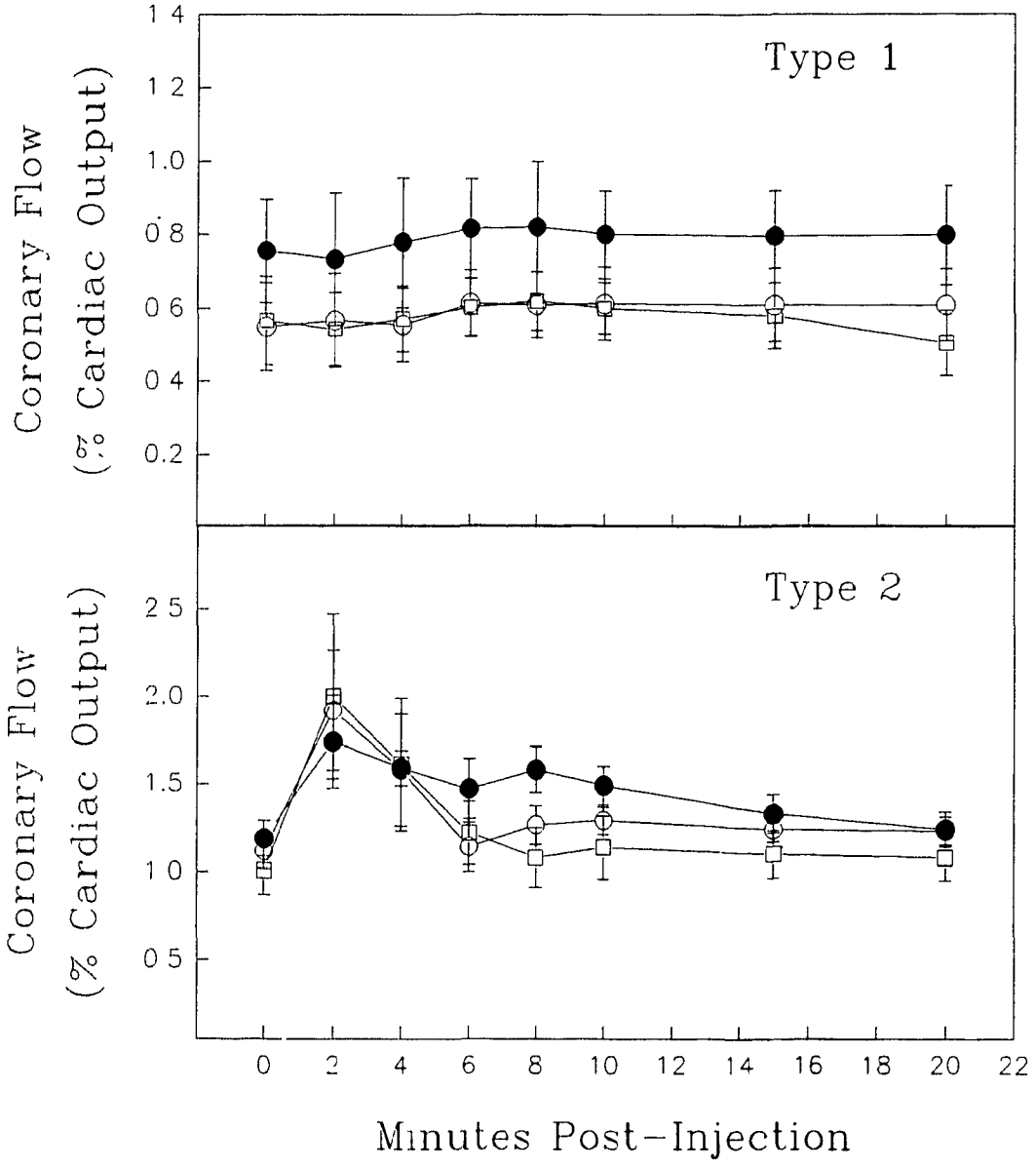
Type 1
Coronary Flow
(% Cardiac Output)
1 4
1 2
1.0
0 8
0 6
0 4
0.2
Type 2
Coronary Flow
(% Cardiac Output)
2 5
2.0
1.5
1 0
0 5
0
2
4
6
8
10
12
14
16
18
20
22
Minutes Post-Injection

Figure 2.11

Although R_{cor} increased following epinephrine injection, differences in R_{cor} between resting hypoxic and normoxic fish were maintained (Figs. 2.8 and 2.9). R_{cor} was 39% higher at rest and 27% higher after epinephrine injection in normoxic fish as compared to hypoxic fish (Table 2.1, 2.2).

Post-injection changes in CF (as % Q or ml min^{-1} kg^{-1}) and P_{DA} during the second normoxic period were comparable to those observed during the initial normoxic period (Table 2.2; Figs. 2.5, 2.6, 2.8, 2.9, 2.11). In contrast, although the maximum increase in Q (6.3 ml min^{-1} kg^{-1}) was higher than that measured during hypoxia (3.9 ml min^{-1} kg^{-1}), it was significantly lower than that recorded during the initial normoxic treatment (8.2 ml min^{-1} kg^{-1}). Post-injection maximum Q during the second normoxic period, was not significantly different from that measured in either of the other two treatments. The magnitude of changes in R_{cor}, measured during the second normoxic period, were intermediate between those obtained in the initial normoxic and the hypoxic treatment.

DISCUSSION

Blood PO_2 During Graded Hypoxia

P_aO_2 and P_vO_2 fell by 80 - 90 mm Hg and 12 - 25 mm Hg, respectively, as the P_wO_2 was decreased from 145 to 60 mm Hg (Fig. 2.3). The magnitude of these decreases is consistent with those of Holeton and Randall (1967), who recorded reductions in P_aO_2 and P_vO_2 of 80 mm Hg and 17 mm Hg in rainbow trout when P_wO_2 was decreased by a similar magnitude. In addition, the reduced arterial-venous PO_2 difference during hypoxia,

as compared to normoxia, is in agreement with previous studies on trout (Holeton and Randall, 1967) and flounder (*Pseudopleuronectes americanus*; Cech *et al.*, 1976). The threshold P_wO_2 for hypoxia-induced alterations in cardiovascular variables (V_s, Q, f_H) in my fish was approximately 95 – 105 mm Hg (P_aO_2 ~40 mm Hg)(Fig. 2.4). Again this is in agreement with previous studies on rainbow trout. Holeton and Randall (1967) and Randall and Smith (1967) were unable to demonstrate a reduction in f_H or an increase in V_s until P_wO_2 was reduced below 80 – 100 mm Hg. Based on *in vivo* dissociation curves for rainbow trout blood at a similar temperature (P_{50} 22.9 mm Hg; Perry and Reid, 1992), the oxygen content in the arterial and venous (estimated P_vO_2 20 mm Hg) blood at the threshold changes in Q (P_wO_2 95 mm Hg) was approximately 1.02 and 0.56 ml O_2 g^{-1} Hb, respectively.

Resting Cardiovascular Variables

Cardiac Performance

Resting Q in the present study (1400g trout; 18 ml min^{-1} kg^{-1}) at 10°C was identical to that observed in Gamperl *et al.* (450 – 750g trout; in press a) at 5°C. This result is surprising, given that the difference in temperature between the two studies, the Q_{10} for cardiac output in fish following temperature acclimation (2.7; Cech *et al.*, 1976: 2.6, Barron *et al.*, 1987), and the relatively constant Q (ml min^{-1} kg^{-1}) for trout between 500 and 1500g (Wood and Shelton, 1980a). f_H and V_s in my trout (64 beats min^{-1}; 0.29 ml kg^{-1}) were 53% higher and 31% lower, respectively, as compared with the trout in Gamperl *et al.* (Chapter 1; in press a). However, while the increase in f_H was expected

(Q_{10} ~ 2; Farrell and Jones, 1992), the lower V_s (and therefore comparable Q) was not anticipated. Stroke volume may have been lower in the present experiment, as compared with Gamperl *et al.* (Chapter 1; in press a), because of the effects of increased f_H on V_s. Farrell *et al.* (1989) found that V_s in *in vitro* perfused hearts, under "resting conditions", was reduced as f_H increased from 30 to 58 beats min^{-1}, and suggested that a decrease in V_s occurs at high pacing frequencies because atrial filling time is compromised. While the results of Farrell *et al.* (1989) explain the discrepancies in V_s between the present study and that of Gamperl *et al.* (Chapter 1; in press a), they offer little insight as to why a Q_{10} effect on cardiac output is present in Cech *et al.* (1976) and Barron *et al.* (1987) but not in my experiments. Measurements of cardiovascular parameters in my experiments (present study: Chapter 1; Gamperl *et al.*, in press a) and those of Farrell *et al.* (1989) were made with the pericardium absent or its integrity severely compromised. Therefore, it is possible that the lack of *vis-a-fronte* atrial filling in my trout diminished the capacity of the heart to increase Q and/or V_s at elevated levels of f_H (Farrell *et al.*, 1988). In contrast to Farrell *et al.* (1989), Graham and Farrell (1990) showed that the Q of *in situ* trout hearts under "resting conditions" was elevated at heart rates of 40 beats min^{-1} (5°C) and 60 beats min^{-1} (15°C); the result of a stable V_s. Because the main difference between the in *situ* preparation of Graham and Farrell (1990) and the *in vitro* preparation of Farrell *et al.* (1989) was the integrity of the pericardium, the discrepancy in the relationship between f_H and V_s between the two studies supports the theory that *vis-a-fronte* filling is important for maintaining *in vivo* V_s in the face of elevated f_H. Although Q in the trout of Gamperl *et al.* (Chapter 1; in press a)(18 ml min^{-1} kg^{-1}; f_H 42 beats

min^{-1}) and Barron et al. (1987; 19.8 ml min^{-1} kg^{-1}) was similar at 6°C, the Q of the trout in the present study (18 ml min^{-1} kg^{-1}; f_H 64 beats min^{-1}) at 10 - 12 °C was well below that recorded in Barron et al. (1987)(38.7 ml min^{-1} kg^{-1}) at 10°C. This result when combined with the above information on the relationship between f_H and V_s in trout without intact pericardia, provides indirect evidence that *vis-a-tergo* filling (atrial filling by venous pressure) may only be sufficient to support resting V_s (and therefore Q) at low heart rates. *Vis-a-fronte* filling (filling by negative pericardial pressure) may be essential for maintenance of resting V_s at high heart rates, because it requires only half as much time as *vis-a-tergo* filling. Information provided by Farrell *et al.* (1989) indicates that *vis-a-tergo* filling of the heart probably requires about 45% of the cardiac cycle in trout, while *vis-a-fronte* filling (which is coupled to ventricular contraction) may only last 21% of the cardiac cycle.

According to Farrell (personal communication) results from recently conducted experiments suggest that the Q_{10} for resting cardiac output *in vivo* may be closer to 1.5. If this is true, temperature compensation may partially explain the identical Q values for trout at 5°C (Chapter 1; Gamperl *et al.*, in press a) and 10 - 12 °C (present study).

Coronary Blood Flow

Resting CF, during normoxia, was 0.145 ml min^{-1} kg^{-1} or 0.84% of Q; an estimate very comparable to the value of 1.1% Q measured by Axelsson and Farrell (1993) in coho salmon (*Oncorhynchus kisutch*). The only other direct measurement of CF in fish was recently provided by Davie and Franklin (1993). Mean CF in the anaesthetized school

shark (*Galeorhinus australis*) was estimated as 0.103 ml min^{-1} kg^{-1} BM. However, because the authors were unable to calibrate the ventral aortic flow signal and therefore provide measurements of CF as %Q, and because CF was recorded in only one of the two coronary arteries, this estimate of CF must be viewed with caution. The reported CF for the school shark, 0.103 ml min^{-1} kg^{-1} BM, probably represents a minimum value. Using microspheres Cameron (1975) estimated CF in the burbot (*Lota lota*) and the sucker (*Catastomus catastomus*) at 0.56% and 0.65% of Q, respectively. Although these values are somewhat less than those obtained in the present study and that of Axelsson and Farrell (1993), the injected microspheres passed through more than one capillary bed before reaching the coronary circulation. Although this suggests that the values presented in Cameron (1975) probably underestimated coronary flow, the lower values reported for these species may also reflect species-specific differences in activity and/or lifestyle as compared with salmonids.

Effects of Hypoxia

Cardiac Performance

During the 30 minutes of graded hypoxia V_s and Q increased, while f_H decreased (Fig. 2.3). The drop in f_H with hypoxia is thought to be caused by chemoreceptor-mediated inhibitory activity in the efferent cholinergic fibers of the vagus nerve (Shelton and Randall, 1962; Randall and Smith, 1967; Wood and Shelton, 1980b; Smith and Davie, 1984; Fritsche and Nilsson, 1989). However, although an increase in V_s, which results in the maintenance or elevation of Q is often observed during hypoxic bradycardia (Holeton

and Randall, 1967; Cech *et al.* 1977; Wood and Shelton, 1980a; Fritshe and Nilsson, 1989), the mechanisms which mediate this effect are unclear. Numerous factors increase V_s *in vivo* or *in vitro* including elevations in circulating catecholamines (Wood and Shelton, 1980a; Farrell, 1984; Chapter 1, Gamperl *et al.*, in press a), increases in cardiac filling time (i.e. decreases in f_H; Farrell *et al.*, 1989) and increases in cardiac filling pressure (venous pressure)(Farrell, 1984; Farrell and Jones, 1992). In rainbow trout exposed to gradual hypoxia increases in plasma epinephrine and norepinephrine are not detected until P_wO_2 falls below 50 mm Hg (approximate P_aO_2 21 mm Hg)(Perry and Reid, 1992). Because increases in V_s were detected at P_wO_2 levels were between 90 and 100 mm Hg (approximate P_aO_2 35 – 45 mm Hg), humoral adrenergic stimulation of the heart is highly unlikely. While a decrease in f_H did occur during hypoxic exposure, the magnitude of this change was relatively minor (Fig. 2.4). At a P_wO_2 of approximately 100 mm Hg, f_H had only fallen by 2 – 3 beats min^{-1}, while V_s had increased by about 15% (0.15 ml kg^{-1} – 0.17 ml kg^{-1}). In addition, at the final P_wO_2 of 90 mm Hg, f_H had decreased by approximately 5 beats min^{-1} while V_s had increased by 25%. Although a decrease in f_H will increase the time available for atrial and ventricular filling (Farrell, 1984), it is probable that some other factor, in combination with the small change in f_H, mediated the observed change in V_s. Data from Farrell *et al.* (1982, 1984) for *in situ* perfused hearts have shown that preloads (venous pressures) of 0.3 – 0.5 cm H_2O (0.03 – 0.05 kPa) are sufficient to generate Q levels comparable to those seen in resting fish, and that small increases in preload can substantially increase V_s and Q. Hypoxic exposure in fish causes ventilation volume to increase (Holeton and Randall, 1967; Nonnette *et al.*,

1993) and results in a synchrony between f_H and breathing (Randall and Smith, 1967). Because both of these adjustments have been suggested as possible mechanisms that increase venous return (Farrell, 1984), it is feasible that a hypoxia-induced increase in venous return partially mediated the elevated V_s that was concomitant with hypoxia. Although this is an attractive hypothesis for explaining the hypoxia-induced increase in V_s, Cech *et al.* (1976) found that V_s increased in the flounder despite non-significant changes in f_H or venous pressure. It is clear that the mechanisms mediating increases in V_s during hypoxia require further investigation.

Coronary Blood Flow

CF in my trout increased as the P_wO_2 fell below 100 mm Hg and was 36% (0.052 ml min^{-1} kg^{-1}) greater in hypoxic trout, as compared to normoxic trout, prior to epinephrine injection (Table 2.1). Although an increase in CF was also observed in hypoxic coho salmon (Axelsson and Farrell, 1993), the magnitude of the increase (60%) was greater than that reported here. The difference between the two studies may be due to three factors. Firstly, Axelsson and Farrell (1993) only used two fish to investigate the effect of hypoxia on coronary flow. Secondly, the increase in P_{DA} (60%), and presumably P_{VA}, during hypoxia in the coho salmon would have increased myocardial power output and therefore oxygen demand (Graham and Farrell, 1990). In my study, no change in P_{DA} was associated with hypoxic exposure. Thirdly, the level of hypoxia used in Axelsson and Farrell (1993)(60 - 75 mm Hg) very probably lowered P_vO_2 to a greater degree than in the present study (see Fig. 2.3). Because oxygen delivered to the heart by the venous

blood appears to be limited by the partial pressure of oxygen (P_vO_2)(Davie and Farrell, 1991a), it is likely that Q and V_s in the coho salmon were more dependent upon O_2 supplied by the coronary artery. Taken together, the results of Axelsson and Farrell (1993) and the present study suggest that elevations in CF are important for determining cardiac performance during environmental hypoxia. For example, in my study a 36% increase in CF was associated with the 25% increase in myocardial power output (Q x P_{DA}) that was concomitant with hypoxic exposure.

Increases in CF during experimental hypoxia were associated with a significant decrease in coronary resistance (29%) but no significant increase in P_{DA} (Table 2.1), indicating that the observed increase in CF was mediated primarily by coronary vasodilation. This finding confirms the results of Axelsson and Farrell (1993) who found that an important component of the increase in CF was not dependent upon α-adrenergic, cholinergic or physical (arterial pressure) mechanisms. Although changes in P_{DA} were not important for determining CF in resting, hypoxic fish, it is clear that increases in P_{DA} associated with adrenergic stimulation (see below) or more severe hypoxia (Axelsson and Farrell, 1993) may mediate elevations in coronary flow. Axelsson and Farrell (1993) indicated that β-adrenergic mechanisms and/or increases in cardiac metabolism can potentially alter CF to the myocardium. However, β-adrenergic control of CF was probably not the predominant factor mediating the increased CF in hypoxic trout: 1) Perry and Reid (1992) have shown that endogenous catecholamine release does not occur in rainbow trout until P_wO_2 falls below 50 mm Hg; and 2) perfusion of the coronary circulation (coronary artery and associated arterioles) with epinephrine *in vitro*, and

injection of epinephrine *in vivo*, indicates that α–adrenoreceptor effects dominate β–adrenoreceptor effects; i.e. vasoconstriction predominates (Farrell and Graham, 1986; Farrell, 1987; Axelsson and Farrell, 1993). Results from Farrell (1987) and Axelsson and Farrell (1993) suggest that there is a small tonic α–adrenergic constriction of the coronary vasculature and that metabolism–related vasodilation of the coronary circulation, as occurs in mammals (Feigl, 1983), could override direct sympathetic vasoconstriction. Therefore, it is possible that the increase in CF and decrease in R_{cor} during hypoxia was due to a metabolically–related coronary vasodilation. Although vasodilation of the coronary vasculature due to local changes in metabolism is the most plausible explanation for the decrease in R_{cor} associated with hypoxia other mechanisms cannot be excluded. These include neural vasodilatory reserve, alterations in α–adrenergic tone, and the release of vasoactive substances from the myocardium.

Effects of Epinephrine Injection

Cardiac Performance During Normoxia

Epinephrine injection into normoxic trout resulted in two distinct types of cardiovascular response; a type 1 response characterised by a gradual increase in post–injection Q, and a type 2 response characterised by an initial fall in Q, followed by a gradual increase to a similar peak Q as for type 1 fish (see Figs. 2.5, 2.6, 2.8, 2.9). This confirms the results of Gamperl *et al.* (Chapter 1; in press a) who identified two response types in rainbow trout acclimated to 5°C. Hypoxic exposure failed to alter any individual fish's response type (see Figs. 2.5, 2.6, 2.8, 2.9), a result which suggests that response

type is not influenced by blood oxygen tension and/or content.

Epinephrine injection (1.0 μg kg^{-1}) during normoxia increased Q and P_{DA} by 45% and 100%, respectively. Because these values are comparable to those obtained by Gamperl *et al.* (Chapter 1; in press a) for coronary-ablated trout (Q 48%; P_{DA} 100%), the results of the present study provide additional evidence tnat oxygen supplied by the venous (luminal) blood is sufficient to support cardiac performance during normoxic conditions. As in Gamperl *et al.* (Chapter, 1; in press a), post-injection increases in Q were mediated by elevations in V_s that more than compensated for the effects of the pressor-stimulated bradycardia. Although the decrease in f_H associated with epinephrine injection may have increased the time available for atrial filling by *vis-a-tergo* mechanisms (Farrell *et al.* 1989), it is probable that direct adrenergic stimulation of the heart was the predominant factor mediating increases in V_s. This conclusion is based on: 1) the observation that relatively small decreases in f_H (2 - 4 beats min^{-1}, Fig. 2.7) were concordant with maximum post-injection Q (Figs. 2.8, 2.9); and 2) evidence showing that adrenergic stimulation increases myocardial contractility and the hearts sensitivity to filling pressure (preload) (Farrell, 1984; Farrell et al., 1986; Franklin and Davie, 1992).

Control of Coronary Blood Flow

Injection of epinephrine into the dorsal aorta during normoxia increased CF, P_{DA}, Q, and R_{cor}. It is difficult, in an *in vivo* model, to determine to what extent a particular cardiovascular variable contributes to the observed elevations in CF. However, the existence of two response types with different patterns of R_{cor}, Q and P_{DA} elevation (Figs.

2.8, 2.9), provides indirect evidence that increases in R_{cor} (α-vasoconstriction of the coronary circulation), P_{DA}, and myocardial oxygen demand (metabolic coronary vasodilation) can all mediate changes in CF in fish. This information compliments the results of Axelsson and Farrell (1993) who used pharmacological agents to investigate the control of CF in coho salmon *in vivo*.

In type 2 fish Q (f_H) fell by 40%, R_{cor} increased by 55%, P_{DA} increased by 55% and CF increased by 5 - 10% shortly (2 minutes) after epinephrine injection. Because the resultant cardiac power output (P_{DA} x Q) was slightly lower than the value recorded before drug injection, it is probable that the increase in CF was mediated by the increase in P_{DA} (Axelsson and Farrell, 1993) or the lowered f_H (increased diastolic blood flow)(Farrell, 1987), and not by increased myocardial oxygen demand. Based on *in vitro* studies of the perfused trout heart, Farrell (1987) estimated that an 18% increase in P_{DA} in swimming trout, would increase CF by approximately 30%. In my study, because an increase in P_{DA} of 55% only increased CF by 5-10%, it must be concluded that adrenergically-mediated vasoconstriction of the coronary vascular bed (Farrell, 1987) severely limited increases in CF associated with elevations in P_{DA} and cardiac power output. Axelsson and Farrell (1993) found that epinephrine injection (1.8 $\mu g\ kg^{-1}$) resulted in an increase in P_{DA} of 60% and an increase in CF of 60%. Although the reason for the difference between the two studies with regard to the relationship between CF and P_{DA} is not known, the results of Axelsson and Farrell (1993) support the conclusion that increases in R_{cor}, associated with constriction of the coronary circulation, can reduce the effect of P_{DA} on CF.

In type 1 fish, where myocardial power output (Q x P_{DA}) constantly increased

during the first minutes post-injection and reached a maximum value at approximately 6 minutes post-injection, elevations in CF mirrored alterations in Q, and increases in R_{cor} were short lived (< 6 min.). However, in type 2 fish, where myocardial power output was decreased initially (through a severe drop in Q) and failed to reach a maximum value until 10 - 15 minutes post-injection, initial increases in CF were minimal and the increase in R_{cor} was prolonged (8 - 10 min.)(Figs. 2.8, 2.9). These results suggest that myocardial oxygen demand, correlated with cardiac power output (Graham and Farrell, 1990), mediated changes in CF and R_{cor} following epinephrine injection. Farrell (1987) suggested that sympathetic stimulation of the heart could increase CF via metabolic-related vasodilation, and Axelsson and Farrell (1993) suggested that increases in CF associated with activity may be achieved via a metabolically-mediated vasodilation. When the results of Farrell (1987) and Axelsson and Farrell (1993) are considered, it appears that a metabolically-related dilation of the coronary vasculature (ie. decrease in R_{cor}) is the most probable explanation for the apparent relationship between myocardial power output and CF during normoxia.

Effects of Hypoxia

Maximum post-injection Q was the same in hypoxic and normoxic trout. However, hypoxic trout had a diminished capacity to increase Q over resting levels, as compared to normoxic trout (Table 2.2), despite the fact that increases in CF (ml min^{-1} kg^{-1}) were not significantly different from those measured during normoxia. One possible explanation for the failure of hypoxic hearts to increase Q above levels measured in

normoxic trout is that post-injection V_s during normoxia was already approaching a maximum limit, and that the inability to increase V_s was related a reduction in cardiac emptying in the face of elevated output pressures (P_{DA}, and possibly P_{VA}). Given that P_{VA} is generally 20 - 25% greater than P_{DA} in rainbow trout *in vivo* (Kiceniuk and Jones, 1977; Wood and Shelton, 1980a), and that P_{DA} at Q_{max} in my trout (both normoxic and hypoxic) was approximately 4.3 kPa, it can be assumed that P_{VA} at Q_{max} was between 5.2 kPa (39 mm Hg) and 5.4 kPa (40.5 mm Hg). Farrell (1991) demonstrated that resting V_s in trout hearts can be maintained until output pressures exceed 60 mm Hg, and Kiceniuk and Jones (1977) showed that swimming rainbow trout can still elevate Q and V_s at values of P_{VA} greater than 50 mm Hg. Because my estimated values of P_{VA} at Q_{max} (5.3 kPa, ~ 40 mm Hg) are less than those in Farrell (1991) or Kiceniuk and Jones (1977) it is unlikely that that the elevated blood pressures associated with epinephrine injection limited V_s in hypoxic trout. In contrast, Gamperl et al. (Chapter 1, in press a) suggested that most of the scope for epinephrine-stimulated increases in V_s (Q) occurs below 0.5 $\mu g\ kg^{-1}$ and that large increases in systemic vascular resistance (output pressure), which accompany doses greater than 1.0 $\mu g\ kg^{-1}$, limit the ability of epinephrine to increase V_s (systolic emptying).

A second explanation for the inability of hypoxic fish to increase Q above levels achieved during normoxia is that increases in CF (coronary oxygen delivery) during hypoxia were not sufficient to support further increases in myocardial power output. *In vitro*, the maximum tetanic force developed by cardiac muscle is reduced by hypoxia (Gesser *et al.*, 1982; Gesser, 1985) and this effect is seen as a reduction in the ability of

hypoxic perfused preparations to generate pressure (Farrell, 1989). In addition, while perfusion of the coronary circulation with air-saturated red cell suspensions (10% Hct.) partially restored the power output of severely hypoxic dogfish (*Squalus acanthias*; Davie and Farrell, 1991b) and eel (*Anguilla dieffenbechii*; Davie *et al.*, 1992) hearts (PO_2 8 - 12 mm Hg), the achieved levels of cardiac power output were 25 - 45% lower than those measured during normoxia.

In my trout, the coronary vasodilatory reserve (measured as [[maximum post-injection CF - resting normoxic CF] / resting normoxic CF]) was 88%. Because Axelsson and Farrell (1993) suggest that the coronary vasodilatory reserve in coho salmon is at least 200%, the results of the present study suggest that adrenergically-mediated vasoconstriction of the coronary circulation (Farrell, 1987) may have limited the ability of the heart to maximally dilate the coronary vasculature (i.e. increase myocardial oxygen delivery). In my study, myocardial oxygen delivery through the coronary artery is estimated to be 10% lower in hypoxic (P_aO_2 38 mm Hg) trout, as compared to normoxic (P_aO_2 88 mm Hg) trout, at maximum cardiac output (based on: 1) the reported CF measurements; 2) the assumption that adrenergic contraction of the spleen leads to similar post-injection levels of haematocrit in hypoxic and normoxic fish (Pearson and Stevens, 1992); and 3) the *in vivo* oxygen dissociation curves for rainbow trout blood at comparable temperatures, Perry and Reid, 1992). Although this deficit in coronary O_2 delivery seems unlikely to explain the 50% decrease in the scope for Q increases, it must be remembered that the coronary O_2 supply in salmonids supplements rather than replaces the luminal O_2 supply (Farrell and Jones, 1992). *In vitro* Farrell *et al.* (1989) found that

the threshold perfusate oxygen tension for maintaining resting and maximum cardiac output in trout hearts without coronary arteries was between 25 and 46 mm Hg. *In vivo* Steffensen and Farrell (1993) showed that coronary-ligated trout swimming at 50% of U_{crit} in hypoxic water could not develop normal ventral aortic pressures (P_{VA}) below a P_vO_2 of approximately 15 mm Hg; a result which reduced cardiac power output by an estimated 37%. Because the P_vO_2 of my hypoxic trout was between 15 and 25 mm Hg (see Fig. 2.1), and because this level of P_vO_2 approximates the above *in vivo* and *in vitro* estimates of "cardiac venous PO_2 threshold", it is likely that the inability of my trout to increase post-injection CF limited the scope for epinephrine-stimulated cardiac performance.

Although the existence of a maximum adrenergically-stimulated V_s and a limited ability of hypoxic trout to increase CF are the two most probable explanations for the failure of hypoxic trout to increase Q to the same extent as normoxic trout, other possible explanations do exist. These include constraints on V_s imposed by the lack of vis-a-fronte filling, hypoxia-related differences in the regulation of venous return to the heart, differences in adrenergic tone, and a hypoxia-induced decrease in myocardial adrenoreceptor density and/or affinity.

Trout which had been allowed to recover from hypoxia for 2.5 hours also had a maximum Q similar to normoxic trout and a diminished scope for epinephrine-stimulated increases in Q. However, because it is clear that trout during the second normoxic period had not fully recovered from the hypoxic exposure, this result does not assist in resolving why hypoxic trout had a reduced increase in epinephrine-stimulated Q. Resting trout, in

the second normoxic period, had an elevated Q (V_s), a lower P_aO_2, and a diminished P_{DA} as compared to trout during the initial normoxic period. Because the lower P_aO_2 probably indicates that P_vO_2 was also depressed during the second normoxic period, and because Steffensen and Farrell (1993) found that the homeometric lowering of P_{VA} by coronary-ablated coho salmon was concomitant with a diminished ability of the heart to maintain cardiac power output, it is probable that the power generating ability of the hearts in my trout was compromised prior to epinephrine injection.

Farrell and Steffensen (1987b) have suggested that oxygen delivered to the tissues is limited by the maximum capacity of the heart and buccal pump. Therefore, the results of the present study suggest that the maximum performance of fish could be compromised under the combined conditions of moderate environmental hypoxia (hypoxemia) and "stress" (elevated plasma catecholamines). Although the number of circumstances where moderate hypoxia and elevated plasma catecholamines occur together are probably limited in nature, it is certainly possible that situations like predator-prey interactions could involve both physiological conditions. In a predator-prey interaction, the inability of a fish to increase Q maximally could have dire consequences.

In summary, the *in vivo* estimate of resting coronary blood flow in this study (0.85% Q) compares well with that obtained by Axelsson and Farrell (1993) (1.1% Q), and indicates that coronary blood flow in salmonids is approximately 1% of resting Q. Increased CF, probably mediated by metabolically-related coronary vasodilation, is associated with hypoxia-induced alterations in resting Q. Maximal dilation of the coronary vasculature (i.e. increased myocardial oxygen delivery) may be limited under

conditions where an adrenergically-mediated vasoconstriction is also present. Exposure of fish to moderate environmental hypoxia reduces the scope for adrenergically-mediated increases in Q by approximately 50%. A recovery period greater than 2.5 hours is required to restore the cardiovascular performance of trout to levels seen during normoxia. This information may prove useful in predicting the impact of environmental hypoxia on behaviours which require maximal cardiac performance (ie. predator-prey interactions).

Chapter 3

β-ADRENORECEPTORS IN THE TROUT HEART: CHARACTERIZATION, QUANTIFICATION, AND EFFECTS OF REPEATED CATECHOLAMINE EXPOSURE

INTRODUCTION

Numerous studies have used selective receptor agonists and antagonists to identify which adrenoreceptor types mediate the inotropic and chronotropic effects of catecholamines on the fish heart (Ask *et al.*, 1980, 1981; Ask, 1983). While these studies strongly suggest that catecholamine effects on cardiac contractility are mediated entirely by β_2-adrenoreceptors in most fish, only one study has directly quantified cardiac β-adrenoreceptors in fish using receptor binding techniques. Keen et al. (1993) used radioligand binding techniques to show that the trout heart was populated by a single β-adrenoreceptor type and that increases in cardiac adrenergic sensitivity following low temperature acclimation were related to changes in the density and binding affinity of ventricular β-adrenoreceptors. Although these results complement the findings from experiments on fish erythrocytes (Reid *et al.*, 1991; Reid *et al.*, 1993) and hepatocytes (Reid *et al.*, 1992) which found that changes in cell-surface β-adrenoreceptor density were directly related to the magnitude of catecholamine-stimulated physiological effects, more studies are needed to identify factors which influence the cardiac β-adrenergic

system in fish.

In mammals, chronic *in vivo* catecholamine exposure results in the desensitization of tissues through the downregulation or uncoupling of β–adrenoreceptors (Mahon *et al.*, 1987; Elfellah *et al.*, *1988*; Deighton *et al.*, 1989; Vatner *et al.*, 1989). Given the apparent relationship between cardiac β–adrenoreceptor density and adrenergic sensitivity (Keen *et al.*, 1993), and the strong relationship between cardiovascular performance and epinephrine levels in fish (Franklin and Davie, 1992; Keen *et al.*, 1993; Chapter 1, Gamperl *et al.*, in press a), the results from mammalian experiments suggest that prolonged or repeated catecholamine exposure could reduce adrenergically–mediated cardiovascular performance in fish. Such an effect would undermine the ability of circulating catecholamines to maintain tonic levels of cardiac stimulation, to protect cardiac performance during periods of stress, and to maximally stimulate cardiac performance. To my knowledge, no experiments have examined whether fish cardiac β–adrenoreceptors are affected by previous exposure to elevated *in vivo* catecholamine levels.

Keen *et al.* (1993) measured the density and binding affinity of trout ventricular β–adrenoreceptors on tissue homogenates and isolated sarcolemmal fractions using hydrophobic β–antagonists. Although these authors were able to demonstrate that cold acclimated fish possessed a greater density of cardiac β–adrenoreceptors, questions remain about the usefulness of these techniques for adrenoreceptor studies in fish. In mammals, evidence indicates that there are qualitative and quantitative differences between adrenoreceptor binding characteristics in intact cells as compared to broken cell

preparations (Meurs *et al.*, 1982; Porzig, 1982; Middeke *et al.*, 1983; Sladeczek *et al.*, 1983; Voisin *et al.*, 1987; Mauz and Pelzer, 1990). In addition, Reid and Perry (1991) have shown that handling, centrifugation and sonication can reduce the density and binding affinity of trout erythrocyte β-adrenoreceptors. Watson-Wright *et al.* (1989) and Wilkinson *et al.* (1991) have developed a myocardial slice/punch preparation which offers a rapid means of measuring β-adrenergic binding in cardiac tissue that has undergone minimal disruption. This technique, which utilises the hydrophilic ligand [^{3}H] CGP-12177, has proven successful for the quantification of cell-surface (functional) β-adrenoreceptors in the cardiac muscle of the dog (Haddad *et al.*, 1986) and for the detection of receptor changes which occurred following *in vivo* myocardial ischemia in the guinea pig (Watson-Wright *et al.*, 1989). Adaptation of this technique for use in fish would allow rapid and inexpensive quantification of cell-surface β-adrenoreceptors in tissues that have undergone minimal disruption and that can be maintained in a metabolically active state.

It was the objective of this study to characterise and quantify the surface (functional) β-adrenoreceptors of the trout heart using tissue punch techniques and to investigate whether repeated *in vivo* catecholamine exposure affects cardiac β-adrenoreceptor density or binding affinity. In these experiments repeated bolus injections instead of prolonged infusion were used to elevate plasma catecholamines because: 1) the effects of catecholamines seem to depend on spurt-like increases rather than blood titer (Epple and Nibbio, 1985; Fievet *et al.*, 1990) and 2) the time course for alterations in plasma catecholamines following acute stress (handling; Tang and Boutilier, 1988) and

bolus injections (Appendix 1; Gamperl *et al.*, in press b) are similar.

MATERIALS AND METHODS

Fish

Rainbow trout were obtained from Sugarloaf Fish Farms (Oxford, Nova Scotia) and held in fibreglass tanks (1 x 1 x 1.5 m) for several months. Thereafter, the trout were gradually transferred to seawater over a one month period, and were maintained in seawater for several months prior to experimentation. Fish were fed daily, to satiation, on a diet of commercially prepared feed pellets, but were fasted for 48 hours prior to sampling or surgery. Photoperiod was 12h light: 12h dark. Experiments to characterise and quantify β–adrenoreceptors in the trout ventricle were carried out between December 2nd and February 10th, 1991. Studies on the effects of repeated catecholamine exposure on ventricular β–adrenoreceptors were conducted between May 25th and June 28th, 1993.

Characterization and Quantification of β–Adrenoreceptors

Trout (890 ± 43 g) held at 7 – 9°C were quickly removed from the tank and stunned by a blow to the head. The heart was quickly removed (< 30 sec), allowed to beat for approximately 1 minute in cold (0 – 2°C) saltwater teleost saline (Hoar and Hickman, 1983) to remove erythrocytes from the ventricular lumen, sealed in a plastic bag, and frozen in liquid nitrogen. Hearts were stored at –70°C for no more than 1 week prior to receptor binding.

Following removal of the atrium, sinus venosus and bulbus arteriosus, cross–

sectional slices of frozen ventricle were cut on a McIlwain Tissue Chopper (Brinkmann, Rexdale, Ontario, Canada). The slices were then placed in cold (0°C) Dulbecco's phosphate-buffered saline (DPBS) (composition in g/l: $CaCl_2$, 0.1; KCl 0.2; KH_2PO_4, 0.2; $MgCl_2 \cdot 6H_2O$, 0.1; NaCl, 8.0; $NaH_2PO_4 \cdot 7H_2O$, 2.16; pH 7.4), and tissue punches (2 mm in diameter) were obtained using a sample corer (Fine Science Tools, North Vancouver, BC, Canada) and Sylgard® (Dow Corning Corp.) coated tissue culture dishes placed on ice. For all trout, a concerted effort was made to ensure that ventricular punches contained approximately equal proportions of compact and spongy myocardium. Single punches were placed in separate wells of a tissue culture plate, each well containing 500 µl of DPBS. For the subsequent β-adrenoreceptor binding procedures the tissue culture plates were kept on ice (i.e. at 0°C).

β-adrenergic binding was measured by incubating the punches in varying concentrations of the hydrophilic β-antagonist, [^{3}H] CGP-12177 (CGP) (specific activity 37 Ci/mmol) for 2 hours (see results). Non-specific binding (NSB) was measured in the presence of 10^{-5} M (±) timolol and was subtracted from total counts to yield specific binding. Normally six wells were used for specific binding and three wells were used for NSB. Both labelled and unlabelled drugs were added in 20 µl aliquots giving a final assay volume of 540 µl. For the determination of CGP binding specificity, punches were incubated with 1.5 nM CGP and various concentrations (10^{-4} - 10^{-9} M) of synthetic (alprenolol, timolol, ICI 118551, isoproterenol, atenolol) and naturally occurring (epinephrine, norepinephrine) agonists and antagonists. During the incubation period all tissue culture plates were covered with aluminum foil to prevent the photo-degradation

of CGP and its competitors.

Following incubation, aliquots of buffer (50 µl) were removed to determine the free (equilibrium) concentration of ligand. After removal of the remaining buffer, the tissue punches were given two 5 minute washes in cold (4 °C) buffer. The wells were then drained and the tissue punches were placed into 2 ml scintillation vials containing 0.8 ml of Ecolite (ICN Canada Inc., Mississauga, Ontario, Canada). Removal of punches was facilitated by touching each one to a small (7 mm diameter) piece of glass fibre filter (Whatman GF/B). All scintillation vials were shaken and allowed to sit for approximately 18 hrs prior to counting. Results (not shown) indicated that this time period was required to ensure that the scintillation cocktail completely penetrated the tissue punch. Radioactivity was quantified in an LKB Wallac Rackbeta Liquid Scintillation counter at an efficiency of 37 - 41% for [^{3}H]. Average punch weights were in the order of 1.0 - 1.2 mg per punch. Because variations in punch weight were negligible between animals, a mean weight of 1.03 mg was used in all calculations.

Effects of Freezing

Because of the large proportion of spongy myocardium found in the trout heart (60 - 70%; Farrell *et al.*, 1990), I was unsuccessful in obtaining suitable tissue slices from fresh (unfrozen) hearts using the McIlwain Tissue Chopper or a Krumdieck Tissue Slicer (Alabama Research and Development Corp., Mumford, Al). However, experiments were conducted to ensure that the freezing of hearts prior to sectioning, and the storage of hearts at -70°C did not affect β-adrenergic binding. To investigate whether the storage

of hearts at −70°C affected β−adrenoreceptor binding characteristics, CGP binding in 2 trout ventricles that were frozen just prior to sectioning was compared with that for 2 ventricles which were sectioned after 1 week of −70°C storage. To determine whether CGP binding to the myocardium was affected by the use of frozen tissue, crude 1 mm thick sections were obtained from the fresh and frozen (1.5 hours at −20°C) halves of an individual trout ventricle using a razor blade and a Jacobowitz rat brain slicer (Zivic−Miller Laboratories Inc., PA), and single punches (mean weight 2.5 mg) were incubated at 0.4 nM, 0.8 nM, and 1.5 nM CGP for three hours. For the latter experiment, 8 punches were used to determine specific binding and 4 punches were used for the measurement of NSB. The extra hour of incubation time allowed for CGP penetration into the thicker (1 mm) tissue punches.

Effect of Repeated Catecholamine Exposure

To assess the effect of repeated catecholamine exposure on cardiac β−adrenergic binding, β−adrenoreceptor density and binding affinity were measured in: 1) free−swimming trout (850 ± 19.5 g, N = 7); 2) cannulated trout (925 ± 50 g, N = 8) that received 5 injections of saline; and 3) cannulated trout (942 ± 50 g, N = 7) that received 5 injections of catecholamines (4.0 μg kg^{-1} epinephrine; 2.0 μg kg^{-1} norepinephrine). The comparison of free−swimming trout to saline injected trout allowed for the determination of cannulation and/or confinement affects on β−adrenergic binding.

Trout utilised for saline and catecholamine injection were anaesthetized in 0.1 g l^{-1} MS 222, cannulated via the dorsal aorta (Smith and Bell, 1964), and held in

individual black-boxes (45 x 15 x 8 cm) supplied with aerated seawater at a flow of 1 l min^{-1}. Seawater temperature averaged 7.5 ± 0.2 °C over the study. Forty-eight hours after surgery, trout were given 5 injections of catecholamines or saline; injections separated by 1.5 hours. Catecholamines (epinephrine and norepinephrine) were slowly (over approximately 15 sec) injected into the dorsal aortic cannulae in a concentrated form using a 0.2 – 0.4 ml carrier volume of saltwater teleost saline. Drug injections were followed immediately by a further 0.3 ml of saline to ensure that all of the drugs were delivered to the fish. Trout in the saline group were given a 0.5 – 0.8 ml bolus of saline at each injection period. One hour after the last saline or catecholamine injection, the trout hearts were removed and frozen in liquid nitrogen for later analysis of β-adrenergic binding. Free-swimming trout were sampled as above.

Materials

[^{3}H] CGP-12177 was obtained from Amersham Corp. (Oakville, Ontario, Canada). (±) Timolol and other adrenergic drugs were commercially available. Dulbecco's phosphate buffered saline was from Gibco (Burlington, Ontario, Canada). Tissue culture plates (Linbro, 24 wells) were supplied by Flow Laboratories (Mclean, VA).

Analysis

The protein content of ventricular punches was determined by a modification of the method of Lowry *et al.* (1951), using bovine serum albumin as a standard. Saturation binding curves were analyzed and binding parameters (K_d and B_{max}) were determined

using the method of Zivin and Waud (1982), which is based on an Eadie-Hofstee plot. Competition curves were fitted and IC_{50} values (concentration of ligand that reduces CGP binding by 50%) were determined using the ALLFIT program obtained from the Biomedical Computer Technology Information Centre, Vanderbilt University, Nashville. Both these programs were run on an Apple IIe.

B_{max} and K_d values were compared between groups using a one-way analysis of variance (Proc GLM). Log transformed data were used to satisfy assumptions of homogeneity of variance, although non-transformed data are shown in the text. All statistical analysis was performed using the SAS statistical package (SAS Institute, Inc.). The fiducial limit of significance was chosen as 5%.

RESULTS

CGP Binding to Myocardial Punches

CGP binding to ventricular punches reached equilibrium within 90 minutes at 0°C and remained stable until at least 4 hours (Fig. 3.1). NSB (non-specific binding) was typically 30-35% after two five minute washes. In preliminary experiments, it was found that NSB approached 50% at CGP concentrations above 2.5 nM and that using more than two 5-minute washes did not reduce NSB further (data not shown). An incubation time of 2 hours and two five minute washes were routinely used in subsequent assays. In all experiments, CGP concentrations ≤ 2 nM were used because high NSB (> 50%)

Figure 3.1. Time course for [^{3}H] CGP–12177 binding to ventricular tissue punches (2mm in diameter; 350 μm in thickness) of the rainbow trout. Values are mean ± S.E. of 6 total and 4 non–specific samples. The [^{3}H] CGP concentration was 1.5 nM. (±) Timolol (10^{-5}) was used as the competitor.

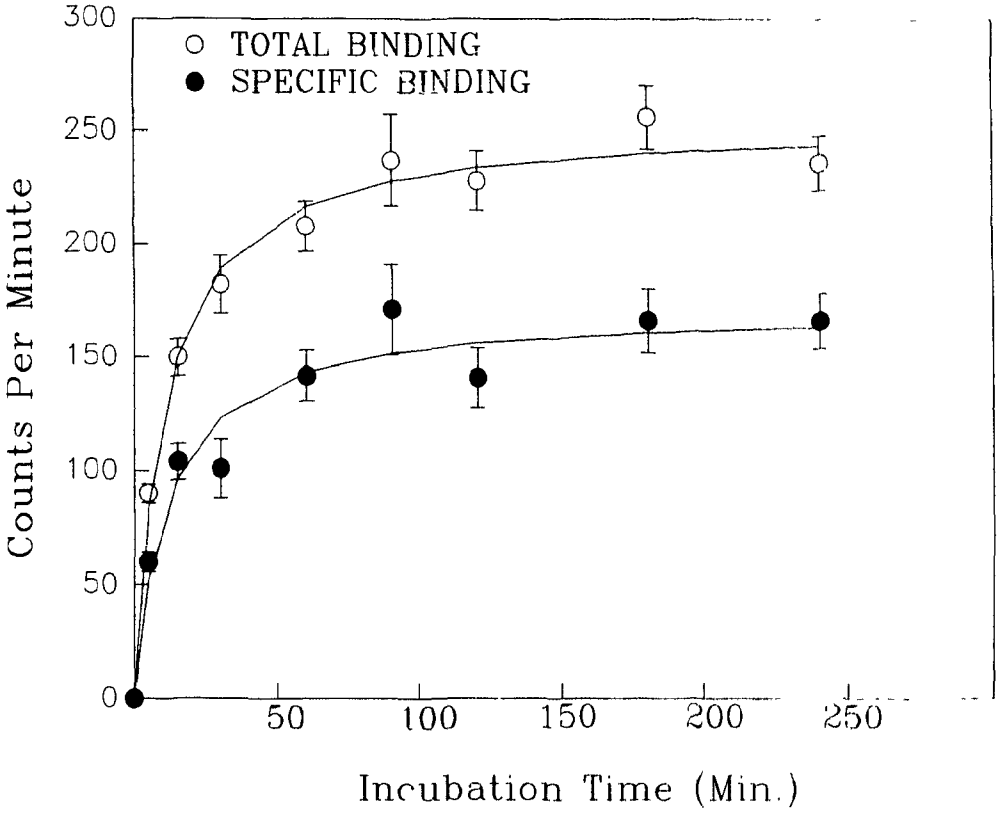
300
250
200
150
100
50
0
TOTAL BINDING
SPECIFIC BINDING
Counts Per Minute
50
100
150
200
250
Incubation Time (Min.)

Figure 3.1

precludes the accurate estimation of binding constants (Dunn et al., 1988).

Specific CGP binding to ventricular punches was directly proportional to the amount of tissue over the range of 1–4 punches (Fig. 3.2). This indicates that CGP was not being degraded during the incubation period (Burt, 1985).

Effects of Freezing

Total and NSB binding to myocardial punches was higher in the frozen ventricle as compared with the fresh ventricle. However, freezing did not affect the specific binding of CGP to ventricular punches (Fig 3.3A). Specific CGP binding for ventricles that were frozen in liquid nitrogen and stored at −70°C was comparable to that for ventricles which were frozen just prior to sectioning (Fig. 3.3B). In these two groups, the B_{max} and K_d were approximately 0.025 fmol μg protein^{-1} and 0.17 nM, respectively.

Saturation Binding

Figure 3.4 illustrates the saturability of [^{3}H] CGP–12177 specific binding to ventricular punches from rainbow trout. Incubation of tissue punches with CGP (0.05 – 2.0 nM) showed that the saturation of β–adrenoreceptors occurred at a concentration above 1.0 nM. Maximum binding was approximately 0.31 fmol μg protein^{-1} and the K_d was approximately 0.2 nM. Neither the B_{max} nor the K_d for CGP binding to ventricular punches was different between mature male and female trout.

Figure 3.2. Linearity of binding with increasing number of ventricular punches. For further details see Figure 3.1.

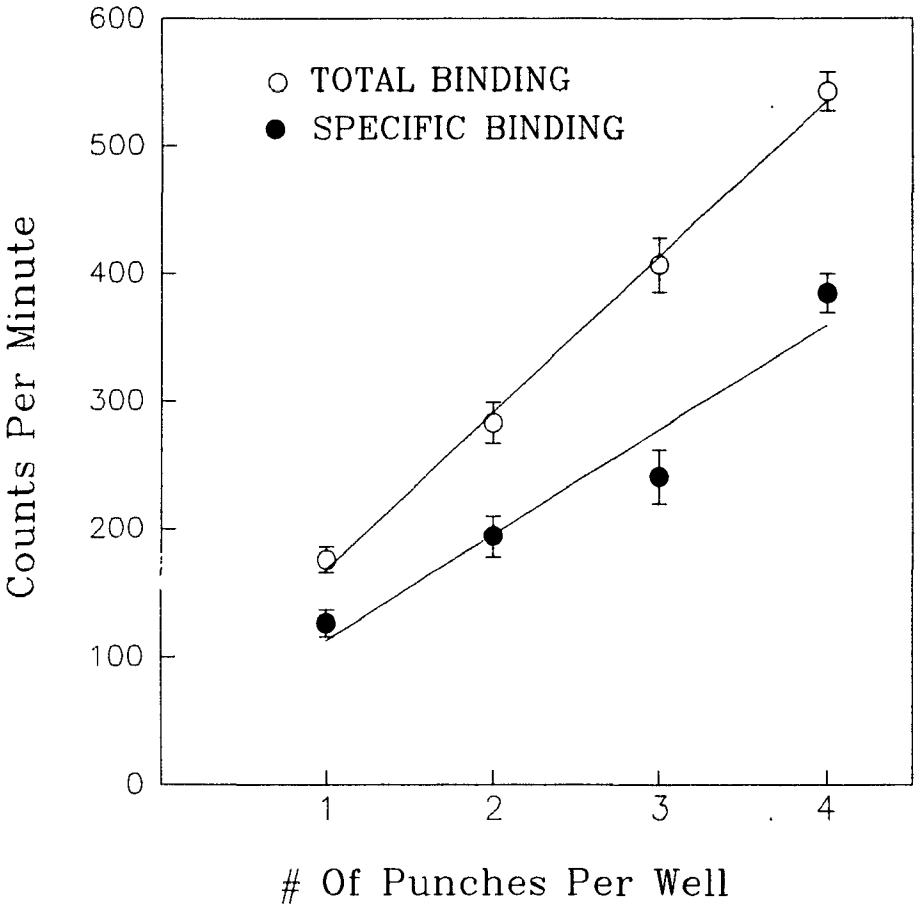
600
500
400
300
200
100
0
Counts Per Minute
TOTAL BINDING
SPECIFIC BINDING
1
2
3
4
Of Punches Per Well

Figure 3.2

Figure 3.3. Specific binding of [^{3}H] CGP–12177 to ventricular β–adrenergic receptors in the rainbow trout. (A) CGP binding to fresh (○) versus frozen (●) trout myocardial punches. The dotted line represents a theoretical binding curve with a K_d of 0.25 nM and a B_{max} of 0.031 fmol μg protein^{-1} (see Figure 3.4.). Values are mean ± S.E. of 8 total and 4 non–specific samples per point. (B) CGP binding in hearts frozen just prior to sectioning (K_d 0.18 ± 0.03 nM; B_{max} 0.025 ± 0.001 fmol μg protein^{-1}) and in hearts stored at –70°C for 1 week before sectioning (K_d 0.16 ± 0.03 nM; B_{max} 0.026 ± 0.001 fmol μg protein^{-1}). Values are mean ± S.E. of 6 total and 4 non–specific samples per point from two separate assays.

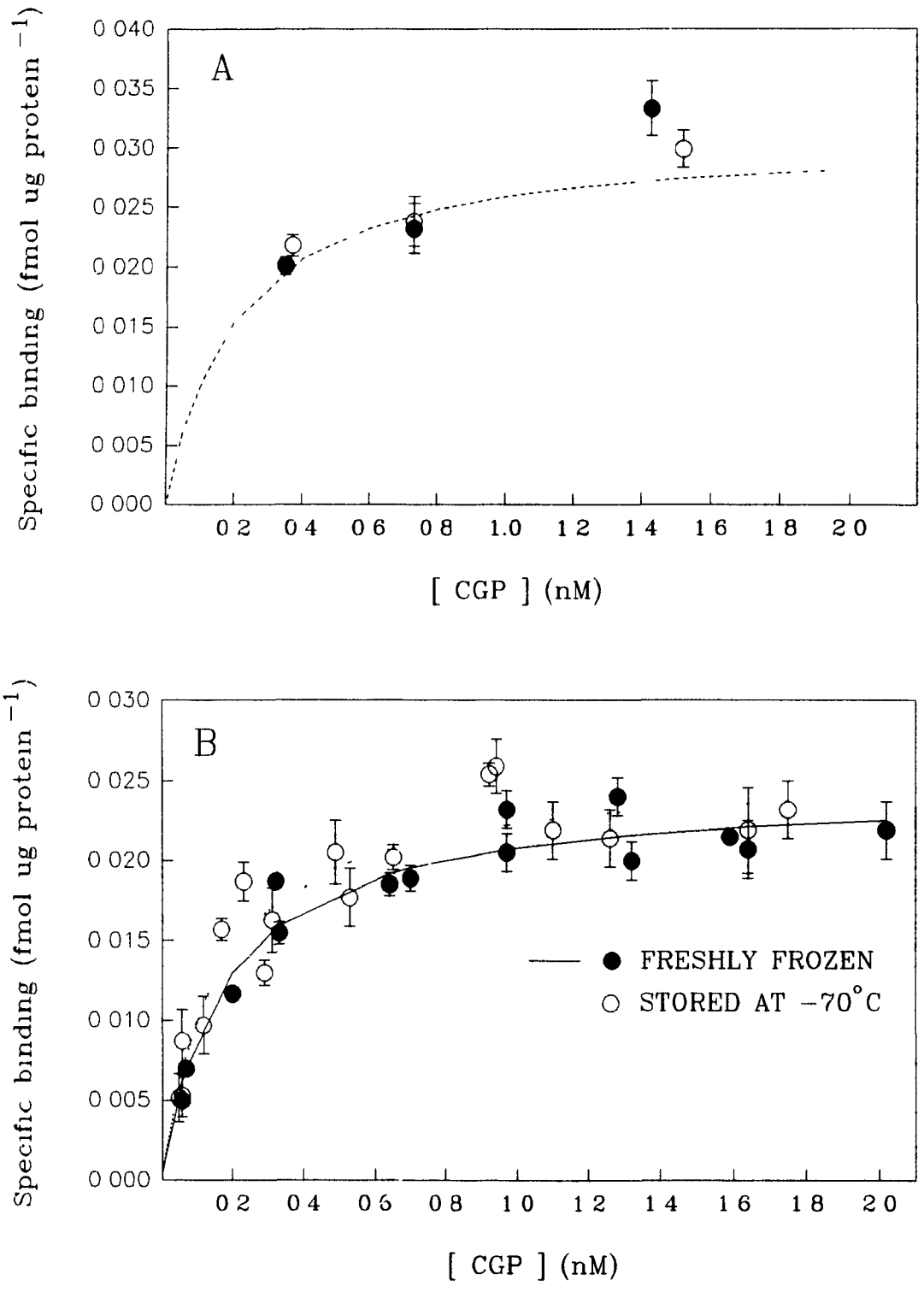
A
Specific binding (fmol ug protein $^{-1}$)
0 040
0 035
0 030
0 025
0 020
0 015
0 010
0 005
0 000
0 2 0 4 0 6 0 8 1.0 1 2 1 4 1 6 1 8 2 0
[CGP] (nM)
B
Specific binding (fmol ug protein $^{-1}$)
0 030
0 025
0 020
0 015
0 010
0 005
0 000
FRESHLY FROZEN
STORED AT −70°C
0 2 0 4 0 6 0 8 1 0 1 2 1 4 1 6 1 8 2 0
[CGP] (nM)

Figure 3.3

Figure 3.4. Representative curves illustrating the saturable binding of [^{3}H] CGP-12177 to ventricular punches from mature male (K_d 0.22 ± 0.02 nM; B_{max} 0.031 ± 0.02 fmol μg protein^{-1}) and female (K_d 0.18 ± 0.03 nM; B_{max} 0.031 ± 0.002 fmol μg protein^{-1}) rainbow trout. Values are mean ± S.E. of 6 total and 4 non-specific samples per point.

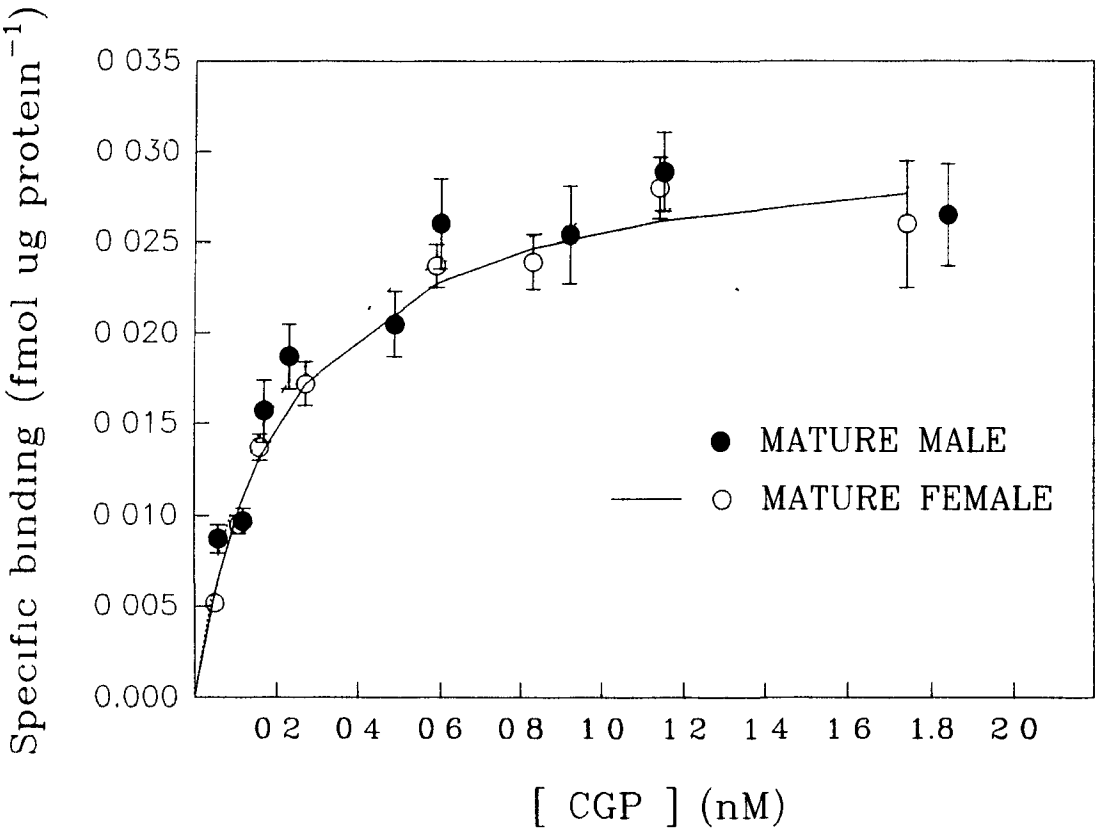
Specific binding (fmol ug protein^{-1})
0 035
0 030
0 025
0 020
0 015
0 010
0 005
0.000
0 2 0 4 0 6 0 8 1 0 1 2 1 4 1 6 1.8 2 0
[CGP] (nM)
MATURE MALE
MATURE FEMALE

Figure 3.4

Specificity of CGP Binding

Competition for CGP binding by the β_2–adrenergic antagonist ICI 118551 and the lack of competition by the β_1–adrenergic antagonist atenolol indicates that the β–adrenoreceptors in trout ventricle are exclusively of the β_2–type (Figure 3.5A). ICI 118551 displaced CGP from binding sites over the range of concentrations used (10^{-9} – 10^{-4}) and had an IC_{50} value of 2.9 x 10^{-6} M. Atenolol (±) failed to displace CGP from binding sites at any concentration, and its more potent isoform (– atenolol) only resulted in a 30% reduction in binding at 10^{-4} M. Phentolamine, an α–adrenergic agonist, failed to displace CGP at concentrations up to 10^{-4} M. This result indicates that [^{3}H] CGP–12177 only binds to β–adrenoreceptors in the trout ventricle.

The stereospecificity of CGP binding to ventricular β–adrenoreceptors was confirmed by the competitive binding curves for (–) alprenolol and (+) alprenolol. The IC_{50} value for (–) alprenolol was 100 times greater than that for (+) alprenolol (Fig. 3.5B). Timolol (±) was the most potent displacer of CGP, with an IC_{50} of 1.54 x 10^{-8} M. The β–adrenergic agonist isoproterenol had an IC_{50} value of 2.98 x 10^{-7} M.

The IC_{50} values for the synthetic β–adrenergic agonist isoproterenol and the physiologically active β–agonists epinephrine and norepinephrine were 2.98 x 10^{-7} M , 8.17 x 10^{-7} M, and 1.82 x 10^{-5} M, respectively (Fig. 3.6). Based on the criterion for β_2–adrenoreceptors in mammalian tissues (Lefkowitz, 1978), the order of agonist affinity (isoproterenol > epinephrine >> norepinephrine) also suggests that β–adrenoreceptors in the trout ventricle are of the β_2–type.

Figure 3.5. Characteristics of [^{3}H] CGP–12177 binding to ventricular tissue punches. (A) Competition for binding by type (α, β_1, β_2) specific agonists. (B) Competition for binding by various β agonists and antagonists. IC_{50} values were ICI 118551 (2.9 x 10^{-6} M), (-) alprenolol (3.73 x 10^{-8} M), (+) alprenolol (3.85 x 10^{-6} M), isoproterenol (2.98 x 10^{-7} M) and (±) timolol (1.54 x 10^{-8} M).

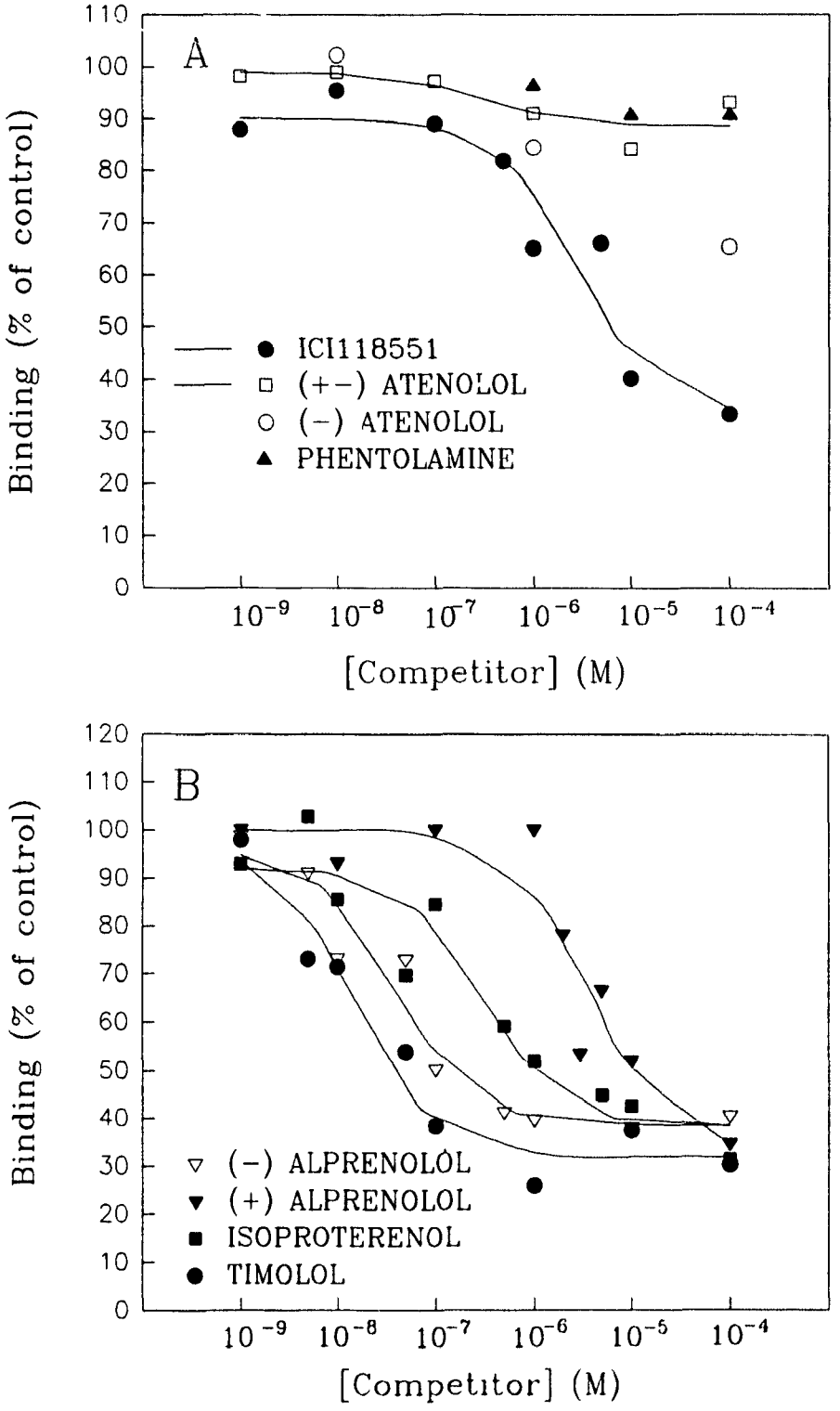
A
Binding (% of control)
110
100
90
80
70
60
50
40
30
20
10
0
ICI118551
(+−) ATENOLOL
(−) ATENOLOL
PHENTOLAMINE
10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4}
[Competitor] (M)
B
Binding (% of control)
120
110
100
90
80
70
60
50
40
30
20
10
0
(−) ALPRENOLOL
(+) ALPRENOLOL
ISOPROTERENOL
TIMOLOL
10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4}
[Competitor] (M)

Figure 3.5

Figure 3.6. Comparison of the ability of isoproterenol and two physiologically active agonists (epinephrine and norepinephrine) to displace [^{3}H] CGP–12177 from ventricular β–adrenoreceptors. IC_{50} values were isoproterenol (2.98 x 10–7 M), epinephrine (8.17 x 10^{-7} M), and norepinephrine (1.82 x 10^{-5} M).

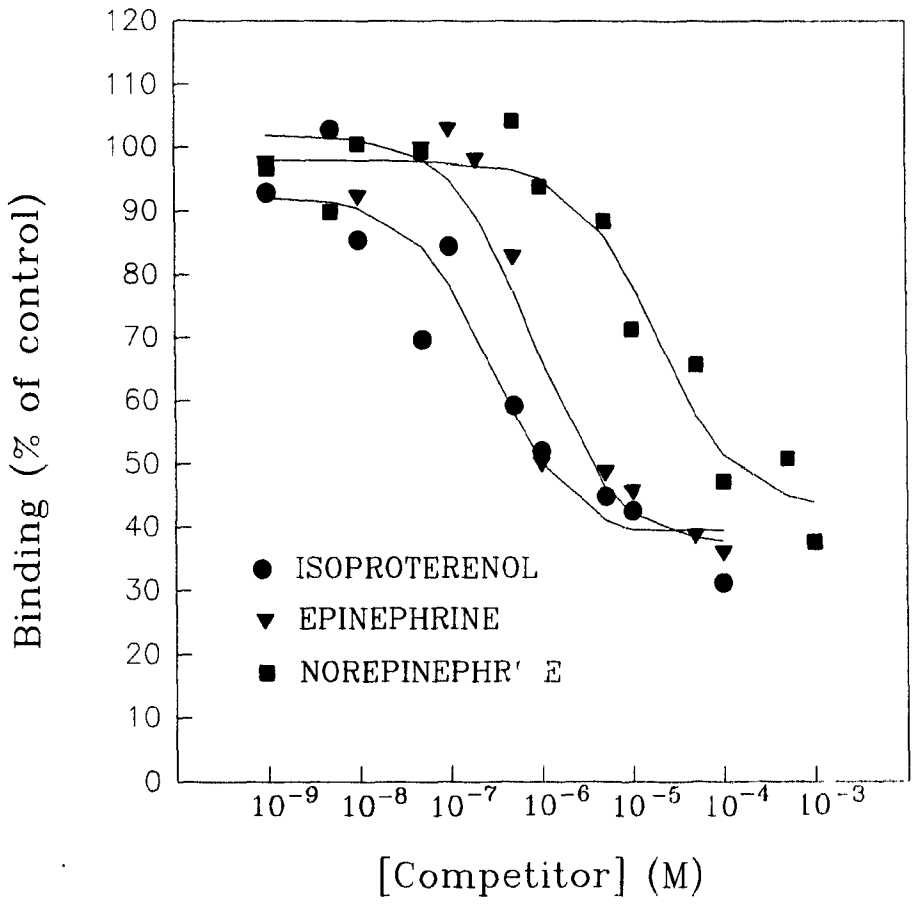
Binding (% of control)
120
110
100
90
80
70
60
50
40
30
20
10
0
ISOPROTERENOL
EPINEPHRINE
NOREPINEPHRINE
10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4} 10^{-3}
[Competitor] (M)

Figure 3.6

Effect of Repeated Catecholamine Injection on Ventricular β–Adrenoreceptors

The mean receptor number in trout ventricular punches was not affected by cannulation/confinement or repeated epinephrine administration (i.e. g. 3.7). B_{max} values were 0.041 ± 0.001 fmol μg protein^{-1} for free–swimming trout, 0.038 ± 0.004 fmol μg protein^{-1} for saline injected trout, and 0.040 ± 0.002 fmol μg protein^{-1} for catecholamine injected trout. However, saline injected trout had a significantly greater variation in B_{max} values as compared to the other two groups ($p < 0.05$, Proc DISCRIM, SAS). Neither the mean values for K_d nor the degree of variation in K_d values was significantly different between groups.

DISCUSSION

CGP Binding to Myocardial Punches

In the past, studies of β–adrenoreceptor properties were performed on tissue homogenates or fragmented membranes because the use of intact cells made discriminating between stereospecific low capacity binding to receptors and nonstereospecific binding to membranes and intracellular structures difficult (Porzig, 1982). However, the development of the highly specific, hydrophilic, β–adrenergic antagonist (±)[^{3}H] CGP–12177 has enabled researchers to effectively use intact cells for studies on β–adrenoreceptors (Staehelin and Hertel, 1983). In this study, the tissue punch technique of Wilkinson *et al.,* (1991) was adapted for use in studies on the β–adrenergic system of the fish heart. Using this technique, which allows for the quantification of cell–surface (functional) receptors on intact cells, I have demonstrated that CGP binding to

Figure 3.7. Trout ventricular β–adrenoreceptor density (B_{max}) and [^{3}H] CGP–12177 dissociation constants (K_d) as affected by the repeated injection of saline and catecholamines (4.0 μg kg^{-1} epinephrine; 2.0 μg kg^{-1} norepinephrine). Control refers to values obtained in free–swimming trout. Both catecholamine and saline injected trout were cannulated and confined prior to experimentation. Bars represent the group mean for each treatment. Symbols represent the values obtained for individual fish.

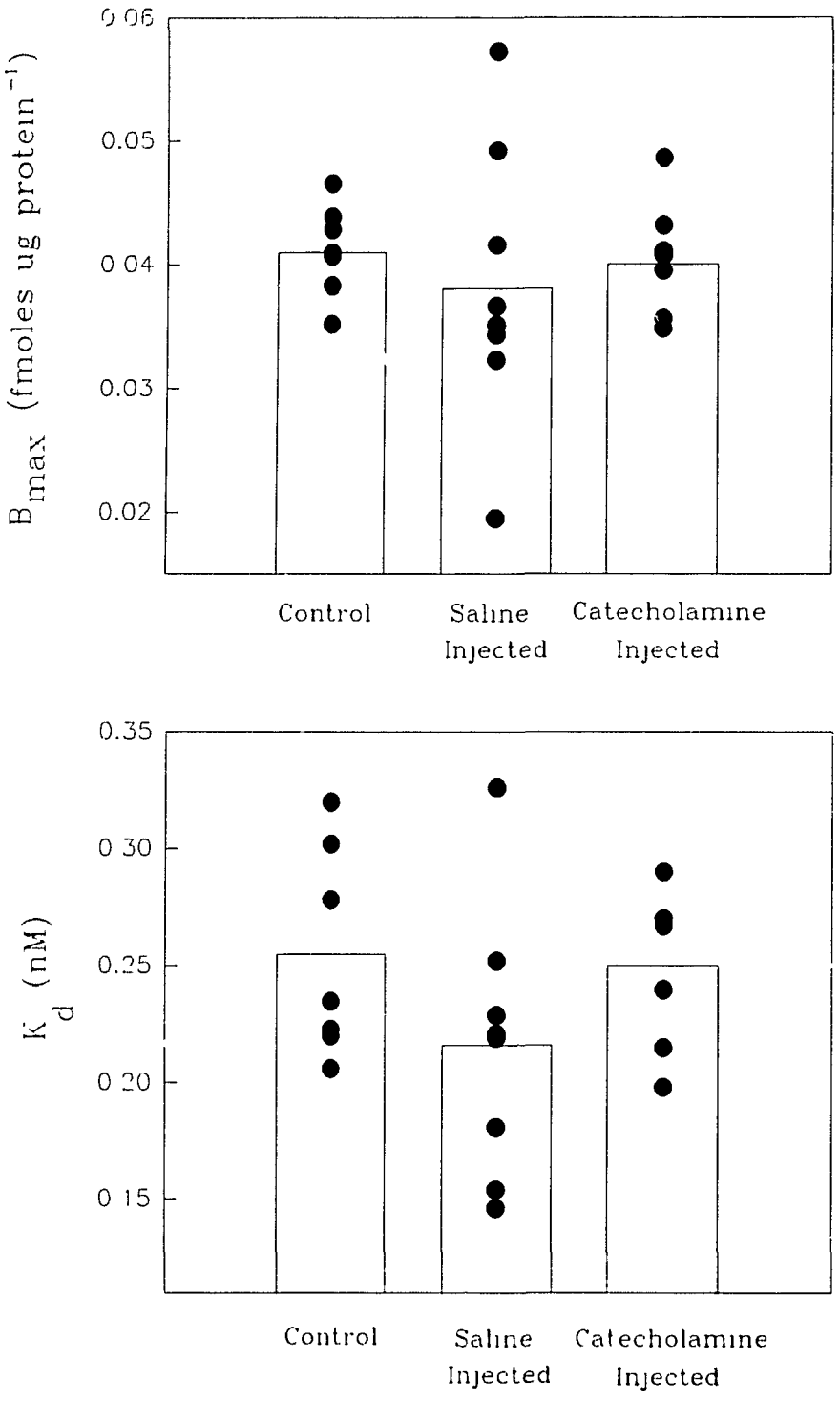
0.06
0.05
0.04
0.03
0.02
B_{max} (fmoles ug protein^{-1})
Control
Saline Injected
Catecholamine Injected
0.35
0.30
0.25
0.20
0.15
K_d (nM)
Control
Saline Injected
Catecholamine Injected

Figure 3.7

trout ventricular punches is stereospecific, saturable, of high affinity, and displaceable by appropriate β–adrenergic agonists and antagonists. These binding characteristics indicate that the use of CGP in conjunction with tissue punches may prove a valuable tool for quantifying β–adrenoreceptors in the fish heart and other tissues.

Although tissue homogenates, fragmented membranes, and intact cells obtained through the use of enzymatic (collagenase) dispersion have all been utilised for the quantification of β–adrenoreceptors in fish tissues (Reid *et al.*, 1992; Keen *et al.*, 1993), the micropunch method for β–adrenergic binding has numerous advantages as compared with these methods. First, the micropunch technique quantifies cell–surface β–adrenoreceptors in tissue that has undergone minimal disruption. The preparation of homogenates and fragmented membranes forcibly removes β–adrenoreceptors from their cellular environment, and several authors have expressed concerns as to whether the examination of receptors in homogenates or fragmented membranes provides physiologically significant information about the β–adrenergic system in living cells (Meurs *et al.*, 1982; Porzig, 1982; Mauz and Pelzer, 1990). Although limited information is available on the effect of collagenase dispersion on cell–surface β–adrenergic receptor properties, other receptor systems appear to be susceptible to degradation by enzymatic treatment (Ascoli and Segaloff, 1986). In addition, the numerous washing and resuspension procedures involved in the isolation of intact cells (Moon *et al.*, 1985; Mauz and Pelzer, 1990) probably result in an impairment of apparent radioligand affinity and receptor density (Reid *et al.*, 1991).

Second, the micropunch method of β–adrenoreceptor quantification can be

completed on fish tissue samples as small as 0.5 – 0.7 g (present study), indicating that binding studies are generally feasible on individual fish. For the determination of myocardial β–adrenoreceptor density and binding affinity using homogenates and fragmented membranes, Keen *et al.* (1993) required the ventricles from 13 – 15 (290 – 670 g) trout. Their study suggests that investigations which utilise tissue homogenates or membrane fractions to study fish β–adrenoreceptors would be difficult to perform on individual fish or small groups of fish.

Third, the preparation of micropunches for β–adrenoreceptor studies can be completed in approximately 1 hour and requires relatively inexpensive equipment. This is in contrast to studies which utilise membrane fragments to determine β–adrenergic binding. The preparation of membrane fragments requires 18 – 24 hours to complete, is extremely labour intensive, and requires a high speed centrifuge (Keen *et al.*, 1993).

Two possible shortcomings with the use of tissue punches are: 1) that the cell damage associated with cutting and punching would affect CGP binding; and 2) that CGP would bind only to the punch exterior and not penetrate to the interior. However, data suggests that neither of these concerns are valid. The similar binding in 1 mm and 350 μm thick punches (present study; Figs. 3.3 and 3.4) suggests that binding is not restricted to the superficial layer of damaged cells and that the micropunches retain a sufficient number of intact cells to permit accurate estimates of cell–surface β–adrenoreceptor density. In addition, autoradiographic data from skeletal muscle slices (1 mm thick) revealed that CGP binding (at 1 nM) was identical in the interior and exterior of the tissue slice (Watson–Wright *et al.*, 1989).

Freezing trout ventricles prior to CGP binding had no effect on the density of β–adrenergic receptors (Fig. 3.3). This result is in agreement with studies on the dog (Haddad *et al.*, 1987), but in contrast to results obtained with the hamster myocardium (Watson–Wright *et al.*, 1989) where freezing reduced ventricular β–adrenoreceptor binding by 50%. Because Watson–Wright *et al.* (1989) indicate that the loss of β–receptors is not related to factors such as the rate of freezing, these findings emphasize the need to assess the effects of freezing on CGP binding for each species and/or tissue if frozen sections are to be used for myocardial β–adrenergic binding studies. In the present study, the lack of an effect of freezing on ventricular β–adrenoreceptor density or binding affinity indicates that frozen tissue is suitable for the determination of trout myocardial β–adrenergic binding. The use of frozen tissue may prove advantageous since the performance of binding assays on freshly isolated tissue is not always feasible.

β–Adrenoreceptor Density and Binding Affinity

Based on a B_{max} value of 0.40 fmol μg protein^{-1}, a protein content of 91.8 μg protein mg tissue^{-1} (wet wt.)(present study), and a ventricular myocyte mass of 5.3 ng and surface area of 3550 μm^2 (Farrell *et al.*, 1988; Tibbits *et al.*, 1990), I estimate the cell–surface β–adrenoreceptor density in trout ventricular myocytes as 3.38 sites μm^{-2} (12,000 sites per cell). These values are approximately three times the values obtained by Keen *et al.* (1993) using the hydrophobic antagonist (–)–[^{125}I] iodocyanopindolol (ICYP), and sarcolemmal fractions prepared from freshwater trout acclimated to 8°C (1.29 ± 0.23 sites μm^{-2}). Although the higher β–adrenoreceptor density measured in my study could be

related to seawater vs. freshwater acclimation or to intra-specific differences in β-adrenoreceptor populations, it is more likely that Keen *et al.* (1993) were unable to recover sarcolemmal fragments from the ventricle without denaturing a significant proportion of the receptors. Voisin *et al.* (1987) measured β-adrenoreceptor density in intact cerebellar astrocytes using CGP, and in membrane fractions using both CGP and ICYP. These authors found that β-adrenoreceptor density (fmol 10^6 $cells^{-1}$) in the membrane fractions was 30-50% lower than that measured in intact cells, regardless of the ligand used for membrane β-adrenoreceptor quantification.

The estimated number of cell-surface β-adrenoreceptors in trout ventricular myocytes (12,000 sites per cell, present study) is approximately 7.5 x that reported for trout erythrocytes (1460 sites per cell, Reid *et al.*, 1991) and trout hepatocytes (1700 sites per cell, Reid *et al.*, 1992). However, although this comparison suggests that there is considerable variability in cell-surface β-adrenoreceptor density between tissues, differences in cell size and the concentrations of CGP used for β-adrenoreceptor quantification must be taken into account. Based on values for the surface area of fish erythrocytes (approximately 700 μm^2: calculated using a cell length of 14 μm and a width of 8 μm, Conroy, 1972; and surface area = $2(\pi \times (\text{length} \times \text{width}))$, I estimate the density of cell-surface β-adrenoreceptors in trout erythrocytes to be approximately 2.08 sites per μm^{-2}. Although the similarity between this value and that obtained in my study for the trout ventricle (3.38 sites per μm^{-2}) suggests that the density of cell-surface β-adrenoreceptors is comparable between tissues, care must be taken when comparing my results to those of Reid *et al.* (1991) or Reid *et al.* (1992). In my study, I identified a

population of β–adrenoreceptors that showed saturable binding to CGP at very low ligand concentrations (~1.0 nM, K_d 0.25 nM). In contrast, Reid *et al.* (1991) and (1992), who used CGP concentrations between 2 and 40 nM, were unable to achieve saturable binding until the CGP concentration exceeded 20 – 25 nM; the K_d values for these studies were 6.49 nM and 55 nM, respectively. In Reid *et al.* (1991, 1992), it is probable that CGP was binding to both the "high affinity" receptor identified in my study, and a receptor which possessed a lower affinity for CGP. Although fish tissues may possess a population of cell–surface "low" affinity β–adrenoreceptors and a population of "high" affinity β–adrenoreceptors, the high NSB observed in Reid *et al.* (1991) at saturating concentrations of CGP (approximately 175%) makes any conclusions about the presence of a "low" affinity β–adrenoreceptor tenuous.

My values for trout β–adrenoreceptor density (B_{max} = 0.04 fmol μg protein^{-1}; 3.76 fmol mg tissue^{-1} wet wt.) and binding affinity (K_d = 0.25 nM), measured using ventricular micropunches, are comparable to values obtained for the hamster (B_{max} = 3.28 ± 0.15 fmol mg^{-1}; K_d = 0.21 ± 0.02 nM) and guinea pig (B_{max} = 5.00 ± 0.62 fmol mg^{-1}; K_d = 0.38 nM) (Watson–Wright *et al.*, 1989), but slightly lower than those obtained for the dog (B_{max} = 10.3 ± 1.0 fmol mg^{-1}; K_d = 1.4 ± 0.2 nM)(Haddad *et al.*, 1987). These results indicate that the density and binding affinity of cell–surface β–adrenoreceptors in the myocardium does not vary much between species when β–adrenergic binding is performed using comparable methods.

In the present study, I have shown that the β–adrenoreceptors of the trout ventricle are exclusively of the β_2–type. This result agrees with the findings of Keen *et al.* (1993)

and Ask *et al.* (1980, 1981). Keen *et al.* (1993) showed that the Scatchard plot correlation coefficients for trout myocardial membranes were always greater than 0.90, indicating the presence of a single β-adrenoreceptor population. Ask *et al.* (1980, 1981), using various selective adrenergic agonists and antagonists, characterised the humoral and the 'innervated' adrenoreceptors mediating adrenaline-stimulated inotropic and chronotropic effects in the trout atrium as β_2. In trout, the presence of a single β-adrenoreceptor type does not appear to be unique to cardiac myocytes. Reid *et al.* (1992) showed that trout hepatocytes had a single population of relatively high-affinity receptors that appeared to be of the β_2-subtype, and Tetens *et al.* (1988) provided indirect evidence that trout erythrocyte β-adrenoreceptors were of the β_1-type. Although strong evidence exists to suggest that the trout myocardium is only populated by β_2-adrenoreceptors, it is clear from mammalian studies that the trout should not be used as a model for all fish. In the cat and guinea pig only β_1-adrenoreceptors are found in ventricular myocytes (Hedberg *et al.*, 1980; Molenaar *et al.*, 1985). However, in the human and the dog, the ratio of ventricular β_1-adrenoreceptors to β_2-adrenoreceptors is 75:25 and 85:15, respectively (Manalan *et al.*, 1981)

Effect of Repeated Catecholamine Injection on Ventricular *β*-Adrenoreceptors

In my trout, the repeated administration of catecholamines had no effect on the mean density or binding affinity of cell-surface β-adrenoreceptors (Fig. 3.7). However, it is unlikely that these variables were unaltered because the levels of circulating catecholamines following injection were not representative of those seen in seawater-

acclimated trout after exposure to a severe stressor. Based on the results of Gamperl *et al.* (Appeindix 1; in press b), the doses of epinephrine (4.0 μg kg^{-1}) and norepinephrine (2.0 μg kg^{-1}) used in this study would have resulted in maximal post-injection epinephrine and norepinephrine titers of approximately 110 and 50 nM, respectively. Both these hormone levels are greater than those observed in seawater-acclimated rainbow trout following a 10 minute violent chase (epinephrine, 88 nM; norepinephrine, 20 nM) (Tang and Boutilier, 1988). Instead, it is likely that there was no loss of β-adrenoreceptors because downregulation of the myocardial β-adrenergic system only occurs following prolonged exposure to supraphysiological or pharmacological catecholamine concentrations. Horackova and Wilkinson (1992) concluded that short-term (< 2 hour) down-regulation of cardiac β-adrenoreceptors does not occur at physiological catecholamine concentrations. This conclusion was based on: 1) the small (10 - 20%) reduction in β-adrenoreceptor density following the brief (5 - 60 minute) incubation of adult rat ventricular myocytes with 1μm isoproterenol; (2) the finding that isoproterenol was approximately 10 times more effective than norepinephrine in reducing cell-surface myocyte β-adrenoreceptor density: and (3) the lack of a significant reduction in cell-surface β-adrenoreceptor density when myocytes were exposed to 10^{-9} M - 10^{-6} M norepinephrine for 2 hours.

An interesting finding was the increased variation in β-adrenergic binding in saline-injected (confined) trout as compared to free-swimming or epinephrine-injected trout. One possible explanation for this result is that variations in plasma cortisol and/or catecholamine levels during confinement (Appendix 1; Gamperl *et al.*, in press b) resulted

in the large variability in individual ventricular β–adrenergic binding characteristics, and that the repeated injection of catecholamines returned the degree of individual variability to pre–confinement levels. Because catecholamine levels associated with confinement (generally < 15 nM: Appendix 1; Gamperl *et al.*, in press b) are unlikely to affect β–adrenoreceptor binding characteristics, it is probable that cortisol levels in individual trout during confinement affected β–adrenoreceptor density. Exposure of trout hepatocytes to cortisol can increase the number of CGP detectable β–adrenoreceptors (Reid *et al.*, 1992).

In conclusion, the myocardial punch technique offers an accurate, reliable and relatively inexpensive means of measuring β–adrenoreceptor density and binding affinity in fish tissues. The characterization and quantification of β–adrenoreceptors in the trout ventricle using [^{3}H] CGP–12177 revealed a myocyte cell–surface population of β_2–adrenoreceptors with a density of 12,000 sites per cell (3.38 sites per μm^2 of sarcolemma) and a K_d of 0.25 nM. Results from experiments on the effect of repeated catecholamine administration on β–adrenergic binding characteristics indicate that the exposure of fish to acute stressors is unlikely to affect ventricular β–adrenoreceptor density or binding affinity.

Chapter 4

EFFECT OF ACUTE AND CHRONIC EPINEPHRINE ADMINISTRATION ON CLEARANCE AND METABOLISM OF [^{3}H]-EPINEPHRINE IN TROUT

INTRODUCTION

Catecholamines (CAs) released from the chromaffin tissue and sympathetic nerves are important regulators of teleost respiratory, circulatory, metabolic and acid-base status (Nilsson, 1983; Randall and Perry, 1992). In addition, the clearance of these amines from the plasma, and their subsequent metabolism (see Fig. 4.1) is essential for maintaining low concentrations of catecholamines in the plasma of resting fish and for restoring catecholamine levels after removal of a stressful stimulus. In fish, *in vivo*, researchers have determined the clearance rate of exogenous radiolabeled epinephrine and norepinephrine (Nekvasil and Olson, 1986a), and have identified those tissues which are potential sites of catecholamine metabolism (Busacker and Chavin, 1977; Nekvasil and Olson, 1986a, b). However, I are unaware of any studies which have investigated the effect of repeated catecholamine exposure on *in vivo* CA clearance and metabolism in fish. The repeated exposure of fish to elevated plasma CA levels ("stress") could diminish the fish's ability to clear and metabolise circulating CAs, thus prolonging the adrenergic stimulation of its tissues. Alternatively, this repeated exposure may induce alterations within the CA clearance and metabolic systems which enhance CA removal, and thereby

Figure 4.1. Metabolic degradation of epinephrine and norepinephrine in teleost fish. Abbreviations: AD, adrenaline (epinephrine); NA, noradrenaline (norepinephrine); MAO, monoamine oxidase; COMT, catechol-O-methyl transferase; DOMA, 3,4-dihydroxy-mandelic acid; DOPEG, 3,4-dihydroxyphenol glycol; MN, metanephrine; NM, normetanephrine; MOPEG, 3-methyoxy-4-hydroxyphenyl glycol; VMA, vanillylmandelic acid. ● indicates compounds measured during this study. Taken from Randall and Perry (1992).

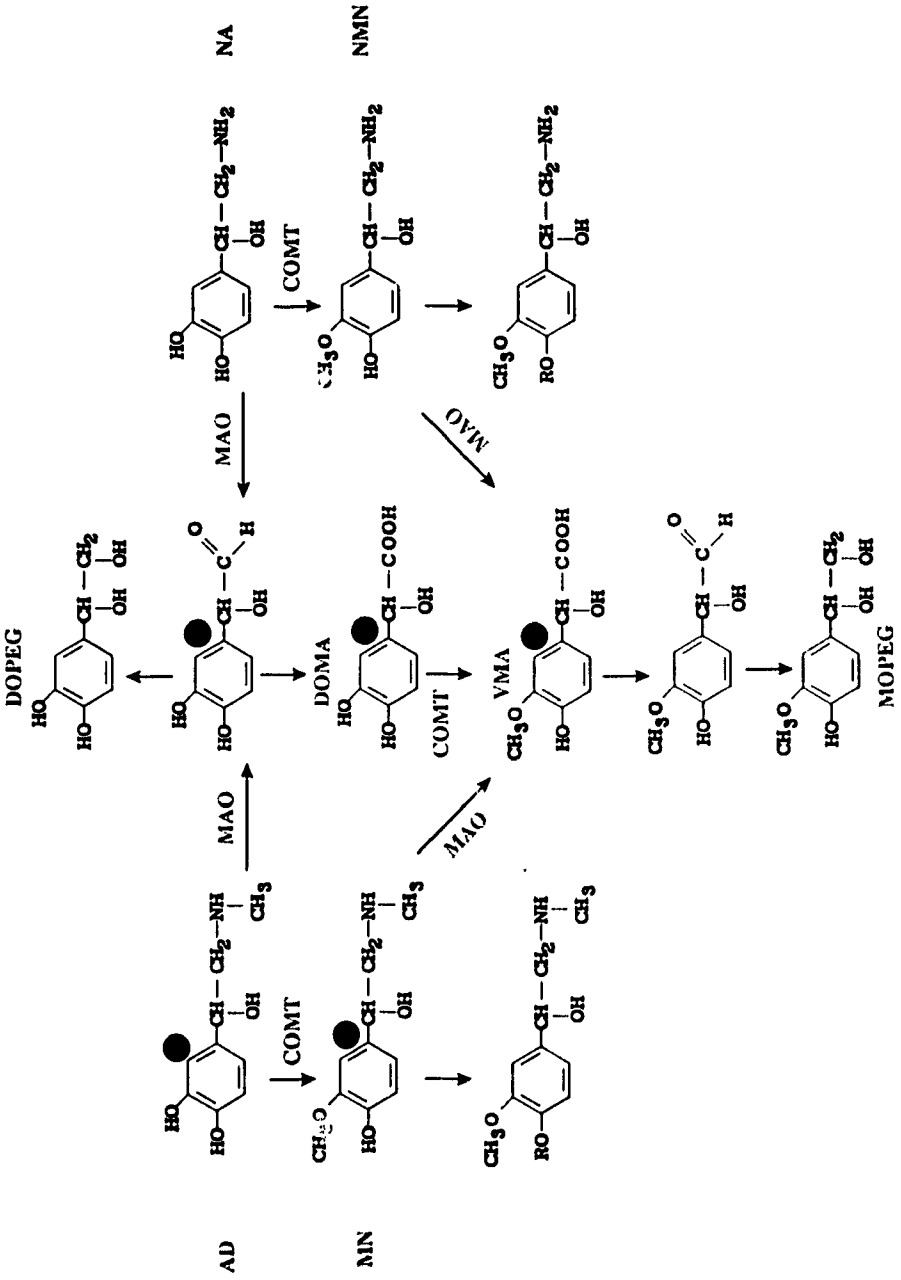

Figure 4.1

reduce the duration of tissue exposure to elevated catecholamines. The present study investigates whether repeated administration of epinephrine affects [^{3}H]-epinephrine clearance and metabolism in rainbow trout, *Oncorhynchus mykiss*. Repeated bolus injections, instead of prolonged infusion, were chosen as the method of epinephrine administration because: 1) the effects of catecholamines seem to depend on spurt-like increases rather than blood titre (Epple and Nibbio, 1985; Fievet *et al.*, 1990) 5); and 2) the time course for alterations in plasma catecholamines following acute stress (handling; Tang and Boutilier, 1988) and bolus injections are similar (Appendix 1; Gamperl *et al.*, in press b).

Colletti and Olson (1988) found that the extraction of catecholamines by the gills *in vitro* was greater when exposed to 10^{-6} M CA as compared with 10^{-8} M CA, and suggested that amine removal (clearance and metabolism) may be enhanced during high CA challenge. To investigate whether CA removal from the plasma is dependent upon the degree of catecholamine release, [^{3}H]-epinephrine clearance and metabolism was measured in trout injected with radiolabel alone, and radiolabel plus 4.0 $\mu g\ kg^{-1}$ of unlabelled epinephrine.

MATERIALS AND METHODS

Animals

Rainbow trout were obtained from Sugarloaf Fish Farms (Oxford, Nova Scotia) and held in fibreglass tanks (1 x 1 x 1.5m) for several months. Thereafter, fish were gradually transferred to seawater over a one month period, and were maintained in

seawater for an extended duration (months) prior to experimentation. Fish were fed daily, to satiation, on a diet of commercially prepared feed pellets, but were fasted for 48 hours prior to surgery. Photoperiod was 12h light : 12h dark.

Trout (450 – 1000g; mean 630 ± 18 g) were anaesthetized in 0.1g l^{-1} MS 222 (tricaine methane sulphonate), cannulated via the dorsal aorta (Smith and Bell, 1964), and held in individual black Perspex® boxes (45 x 15 x 8 cm) supplied with aerated seawater at a flow of 1 l min^{-1}. Seawater temperature averaged 11.9 ± 0.6 °C over the study. All experiments were conducted between July 20th and October 30th, 1991. Trout were allowed to recover for 48 hours before experiments commenced. Fish were not fed during the experimental period.

Experimental Protocols

1. Acute Catecholamine Challenge

Trout were given 5 intra-arterial injections of 4.0 μg kg^{-1} epinephrine (N = 9) or saline (N = 8), separated by 1.5 hours, in a single day. One hour after the final epinephrine injection, trout were injected with [^{3}H]-epinephrine and [^{14}C]-sucrose.

2. Chronic Catecholamine Challenge

Trout were injected with 4.0 μg kg^{-1} epinephrine (N = 7) or saline (N = 6) five times per day for four days. Eighteen hours following the final injection trout were injected with [^{3}H]-epinephrine and [^{14}C]-sucrose.

3. Saturation

Trout (N = 7) were simultaneously injected with [^{3}H]-epinephrine, [^{14}C]-sucrose and 4.0 μg kg^{-1} of unlabelled epinephrine. In this experiment, the saline group from the "acute" protocol served as a control.

An epinephrine dose of 4.0 μg kg^{-1} was used in the above experiments for two reasons. Firstly, the injection of 4.0 μg kg^{-1} into seawater-acclimated trout results in maximum epinephrine titers (approx. 110 nM: Appendix 1; Gamperl *et al.*, in press b) that are comparable to those observed in seawater trout following a severe handling stress (90 nM; Tang and Boutilier, 1988). Secondly, the repeated injection of higher epinephrine doses (e.g. 7.0 μg kg^{-1}) can result in violent struggles and/or tissue haemorrhage (Gamperl *et al.*, in press c).

Blood Sampling

At 2, 4, 6, 8, 10, 15, 20, 40 and 60 minutes after the injection of radiolabels, blood samples (0.8 ml) were collected from the dorsal aortic cannula and centrifuged at 10,000 xg for 30 seconds. Radioactivity (DPM, disintegrations per minute) in one of the two plasma aliquots (250 μl) was measured without further processing using a Beckman LS 5000E scintillation counter, to determine the rate of [^{3}H]-epinephrine and [^{14}C]-sucrose extraction from the plasma. The other plasma aliquot was subjected to ion-exchange chromatography (see below) to quantify the amount of O-methylated, deaminated, and unmetabolised [^{3}H]-epinephrine. Comparison of the DPM in the first aliquot to the total eluted DPM allowed for the estimation of chromatographic recovery. Quench curves for

the measurement of [^{3}H]-epinephrine and [^{14}C]-sucrose DPM were constructed using commercially available standards (Amersham Inc., Oakville, Ontario). Approximately 0.6 ml of saline was reinjected into the fish after each blood collection to partially restore blood volume.

Drugs

Epinephrine (Sigma Chemical Co., St. Louis, MO), and/or [^{3}H]-epinephrine and [^{14}C]-sucrose (NEN® Research Products, Boston, MA) were injected slowly (over approximately 15 sec) into the dorsal aortic cannulae in a concentrated form using a 0.2 - 0.4 ml carrier volume of saline (Hoar and Hickman 1983). Drug injections were followed immediately by a further 0.3 ml of saline to ensure that all of the drug was delivered to the fish. Control fish were given a 0.5 ml - 0.8 ml bolus of saline at each injection period. For the determination of [^{3}H]-epinephrine clearance and metabolism, each fish was injected with ~8.6 μCi kg^{-1} of [^{14}C]-sucrose (4.4 mCi mM^{-1}) and 35 μCi kg^{-1} of [^{3}H]-epinephrine (67 Ci mM^{-1}). [^{14}C]-sucrose served as an inert volume marker to distinguish between the simple dilution of amines in plasma and CA-specific extraction.

Identification of Metabolites

Unmetabolised [^{3}H]-epinephrine was separated from its O-methylated and deaminated metabolites using the methods of Minard and Grant (1972) and Roth (1982), and the cation-exchange resin Biorex 70 (100 - 200 mesh sodium form, Bio-rad Laboratories, Richmond, CA). A 250 ul plasma sample was added to the resin columns

and sequentially eluted with 6 ml of water, 6 ml of boric acid (2%) and 6 ml of HCl (0.2 N). One ml samples of the three fractions were assayed (in triplicate) for [^{3}H]-epinephrine DPM; these counts representing deaminated metabolites, unmetabolised [^{3}H]-epinephrine, and O-methylated metabolites, respectively. Stock samples of [^{3}H]-epinephrine were chromatographed with each group to determine the background levels of O-methylated and deaminated products. These were then subtracted from levels measured in samples from experimental fish. Recovery of radioactivity from the columns was between 85 and 95%. Ion-exchange chromatography was performed at 10 °C to ensure that daily temperature fluctuations would not affect [^{3}H]-epinephrine and metabolite recovery.

Calculations

Rate constants for [^{3}H]-epinephrine and [^{14}C]-sucrose clearance from the plasma were calculated from a semilog plot of radioactivity vs. time (Fig. 4.2) using a commercially available graphics program (Sigmaplot® 5.0, Jandel Scientific). The disappearance of both [^{3}H]-epinephrine and [^{14}C]-sucrose was described by a two-component system (Lassen and Perl, 1979):

$$A_t = A_{eq} + A_{1t}\, e^{-k1*t} + A_{2t}\, e^{-k2*t}$$

where A_t is the plasma radioactivity (^{3}H or ^{14}C) at time t, A_{eq} is plasma radioactivity at equilibrium, A_{1t} and A_{2t} are plasma radioactivity due to components 1 and 2 at time t, respectively, k_1 and k_2 are rate constants for components 1 and 2, and t is the time elapsed since radiolabel injection.

The normalized ratio of [^{3}H]-epinephrine to [^{14}C]-sucrose radioactivity (^{3}H DPM : ^{14}C DPM) at each sampling period was calculated for each fish. Data were normalized to the values obtained at 60 minutes post-injection to account for individual differences in injected ^{3}H and ^{14}C DPM, circulation time (cardiac output) and haematocrit (plasma volume).

Statistical Analysis

Rate constants (k_1 and k_2) for [^{3}H]-epinephrine and [^{14}C]-sucrose disappearance were compared between groups using a one-way ANOVA followed by Duncan's multiple range test. ^{3}H : ^{14}C ratios, and the proportions of unmetabolised, O-methylated, and deaminated [^{3}H]-epinephrine were compared between groups, at each sampling time, using an ANOVA with repeated measures followed by multiple contrasts. All statistical analysis was performed using the SAS statistical package (SAS Institute, Inc.). 5% was chosen as the fiducial limit of significance. Values are expressed as means ± S.E.

RESULTS

Epinephrine Clearance

Decreases in plasma [^{3}H]-epinephrine and [^{14}C]-sucrose were biphasic (Fig. 4.2). In the initial 8 minutes post-injection both ^{3}H and ^{14}C DPM fell rapidly. Thereafter, although ^{3}H and ^{14}C DPM continued to fall, the rate of radiolabel loss was considerably slower. In addition, the rate and amount of [^{3}H]-epinephrine loss from the plasma was greater than that observed for [^{14}C]-sucrose. These observations were confirmed when

comparing the k_1 and k_2 constants for the disappearance of [^{3}H]–epinephrine and [^{14}C]–sucrose from the plasma (Table 4.1). The k_1 constants for both radiolabels were greater than the k_2 constants, and the k_1 and k_2 constants for [^{3}H]–epinephrine were higher than those for [^{14}C]–sucrose.

Table 4.1. Rate constants (min^{-1}) for the loss of [^{3}H]–epinephrine and [^{14}C]–sucrose from the plasma of rainbow trout. (*) indicates a significant difference between the rate constants for ^{3}H and ^{14}C loss ($p < 0.05$). Values are means ± 1 S.E.

	^{3}H		^{14}C	
	k_1	k_2	k_1	k_2
Acute				
Control	0.244 ± 0.03	0.0016 ± 0.0001 *	0.212 ± 0.04	0.00059 ± 0.0001
Experimental	0.245 ± 0.01*	0.0017 ± 0.0001*	0.192 ± 0.02	0.00050 ± 0.0001
Chronic				
Control	0.218 ± 0.02	0.0016 ± 0.0001*	0.163 ± 0.02	0.00067 ± 0.0001
Experimental	0.199 ± 0.01	0.0015 ± 0.0001*	0.178 ± 0.02	0.00063 ± 0.0001
Saturation	0.241 ± 0.01*	0.0016 ± 0.0001*	0.165 ± 0.01	0.00049 ± 0.0001

No differences in the k_1 and k_2 constants for [^{3}H]–epinephrine and [^{14}C]–sucrose were evident between experimental groups (Table 4.1). This result indicates that the rate of [^{3}H]–epinephrine clearance from the plasma was not affected by any of the

Figure 4.2. Plasma radiactivity following the injection of [^{3}H]-epinephrinc and [^{14}C]-sucrose into the dorsal aorta of an individual trout.

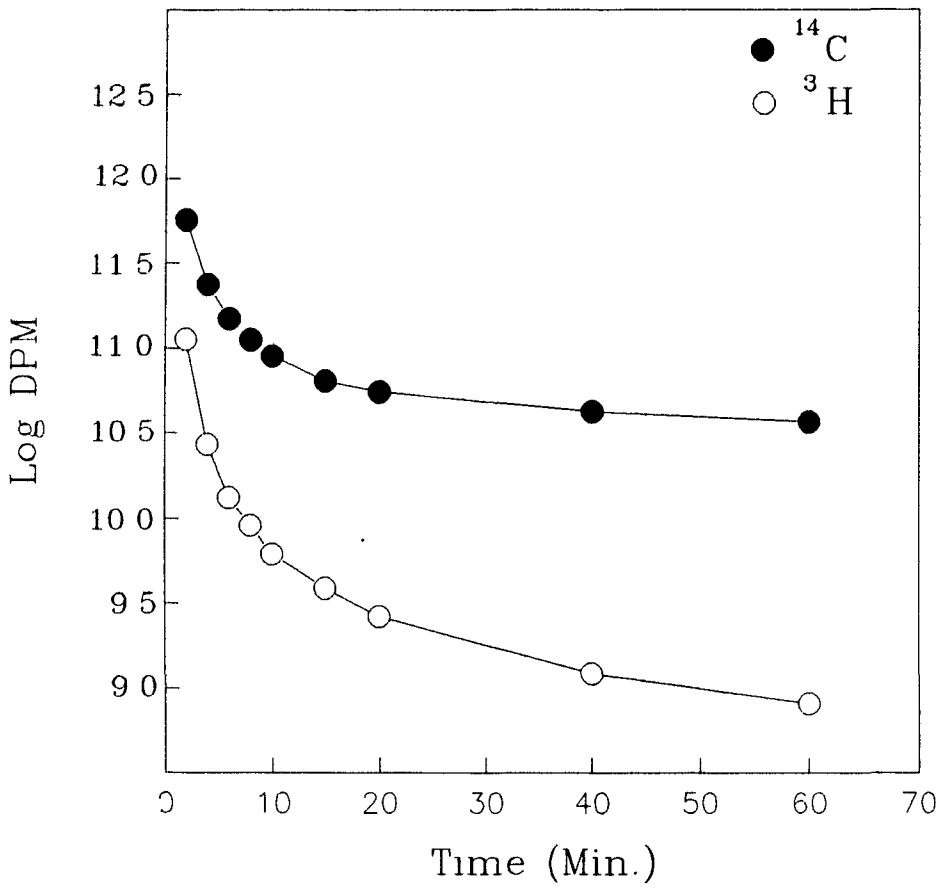
^{14}C
^{3}H
12 5
12 0
11 5
11 0
10 5
10 0
9 5
9 0
Log DPM
0
10
20
30
40
50
60
70
Time (Min.)

Figure 4.2

experimental protocols.

The normalised ^{3}H : ^{14}C ratios for the experimentally injected acute and chronic fish were not different from those of control (saline injected) fish at any time post-injection. In these groups the ^{3}H : ^{14}C ratio fell from 2.3 to 1.5 within ten minutes post-injection and only declined a further 0.5 over the next 50 minutes (Fig. 4.3). Although the fish injected with radiolabels and 4.0 $\mu g\ kg^{-1}$ of unlabelled epinephrine (saturation group) did not have different k_1 and k_2 constants than the acute or chronic fish, they had significantly greater ^{3}H : ^{14}C ratios at 2 and 4 minutes post-injection. These ratios were approximately 34% and 28% higher, respectively.

Epinephrine Metabolism

At two minutes post-injection the percentages of unmetabolised [^{3}H]-epinephrine, deaminated [^{3}H]-epinephrine, and O-methylated [^{3}H]-epinephrine averaged 25-41%, 27-40% and 25-43%, respectively. In all groups, the proportions of unmetabolised [^{3}H]-epinephrine and O-methylated [^{3}H]-epinephrine decreased from 2 to 60 minutes post-injection. In contrast, the percentage of deaminated [^{3}H]-epinephrine metabolites increased during the post-injection period (Fig. 4.4). At 60 minutes post-injection the proportions of unmetabolised [^{3}H]-epinephrine, deaminated [^{3}H]-epinephrine, and O-methylated [^{3}H]-epinephrine were approximately 15%, 75% and 10%, respectively.

In both the acute and chronic treatments, no significant differences in the proportions of unmetabolised [^{3}H]-epinephrine, deaminated [^{3}H]-epinephrine and O-methylated [^{3}H]-epinephrine were identified between saline and epinephrine injected fish

Table 4.2. Statistical analysis for the effect of epinephrine administration on the metabolism of [^{3}H]-epinephrine (Fig. 4.3). Groups with dissimilar letters were significantly different at $p < 0.05$. Significant differences were only observed for the sampling periods shown below.

	% Deaminated		% Unmetabolised		%Omethylated		
	2 min.	4 min.	2 min.	4 min.	2 min.	4 min.	6 min.
Acute Control	AB	AB	A	A	A	A	AB
Acute Experimental	AB	AB	A	A	A	A	B
Chronic Control	B	B	B	AB	C	A	AB
Chronic Experimental	B	B	B	B	C	A	B
Saturation	A	A	AB	AB	B	B	A

(Table 4.2, Fig. 4.4). This indicates that neither acute nor chronic epinephrine administration altered the rate of epinephrine metabolism. At 2 minutes post-injection, chronically treated trout had a 50% smaller proportion of unmetabolised [^{3}H]-epinephrine and a 30% greater proportion of O-methylated [^{3}H]-epinephrine as compared to acutely injected fish. Because this difference was evident for both saline and epinephrine injected fish (Table 4.2, Fig. 4.4), the results suggest that some factor unrelated to epinephrine injection affected [^{3}H]-epinephrine metabolism.

At 2-4 minutes post-injection the saturation group had a significantly greater proportion of O-methylated [^{3}H]-epinephrine as compared with the chronic and acute fish, and a smaller proportion of deaminated [^{3}H]-epinephrine as compared to chronic fish (Table 4.2, Fig. 4.4). Although the proportion of unmetabolised [^{3}H]-epinephrine in saturated fish at two minutes post-injection was less than that found in the acute group this difference was not significant ($0.10 > p > 0.05$).

DISCUSSION

The rapid loss of [^{3}H]-epinephrine from the plasma of trout suggests that the turnover of endogenously released CAs is extremely fast. In my study > 50% of the injected [^{3}H]-epinephrine had been removed from the plasma by six minutes post-injection, and > 80% had been removed by 20 minutes post-injection (Fig. 4.2). These results are very comparable to those of Ungell and Nilsson (1979) for the cod (*Gadus morhua*) and Nekvasil and Olson (1986a) for the rainbow trout. In both these studies, > 90% of the injected [^{3}H]-epinephrine was removed from the circulation within 16-20

Figure 4.3. Ratio of [^{3}H]–epinephrine DPM to [^{14}C]–sucrose DPM in trout plasma at timed intervals after dorsal aortic injection. Ratios were adjusted a ^{3}H/^{14}C ratio of one at 60 minutes post–injection. Vertical bars represent ± 1 S.E.

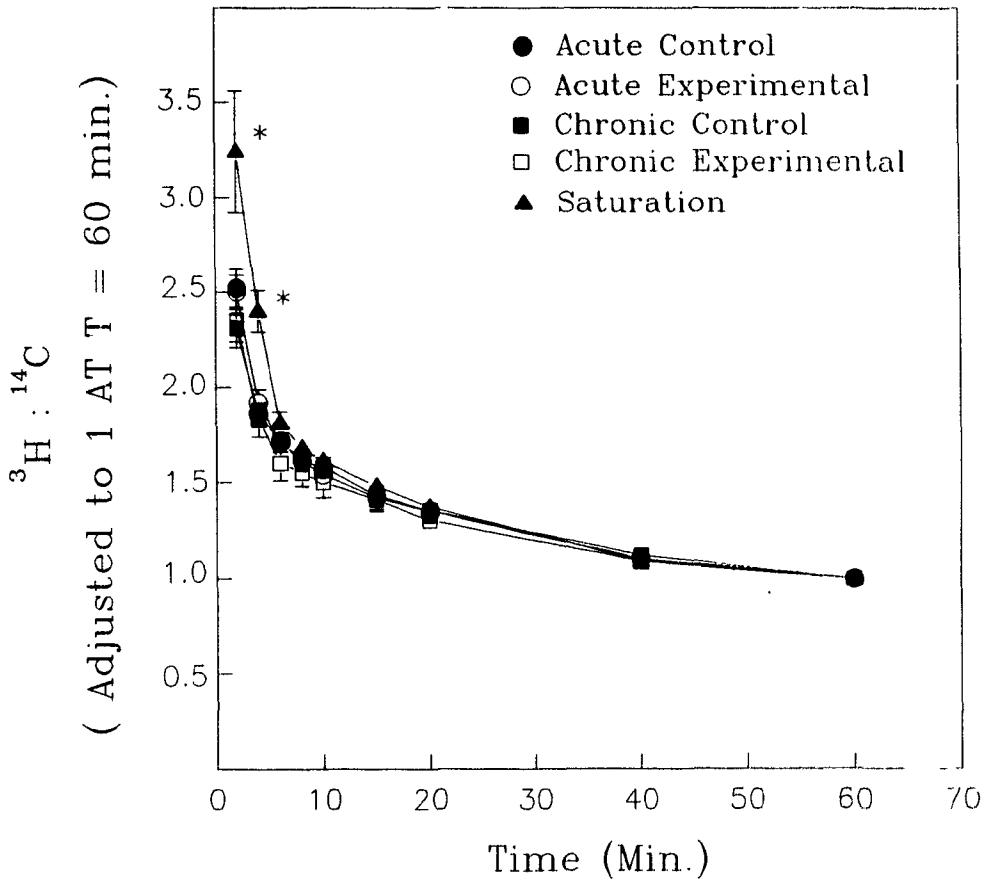
Acute Control
Acute Experimental
Chronic Control
Chronic Experimental
Saturation
(Adjusted to 1 AT T = 60 min.)
$^{3}H : ^{14}C$
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
10
20
30
40
50
60
70
Time (Min.)
*
*

Figure 4.3

Figure 4.4. The proportion of total [^{3}H]–epinephrine radioactivity in the plasma of rainbow trout that comprised unmetabolised [^{3}H]–epinephrine, O–methylated [^{3}H]–epinephrine products, and deaminated [^{3}H]–epinephrine products. Details of statistical analysis can be seen in Table 4.2. Vertical bars are ± 1 S.E.

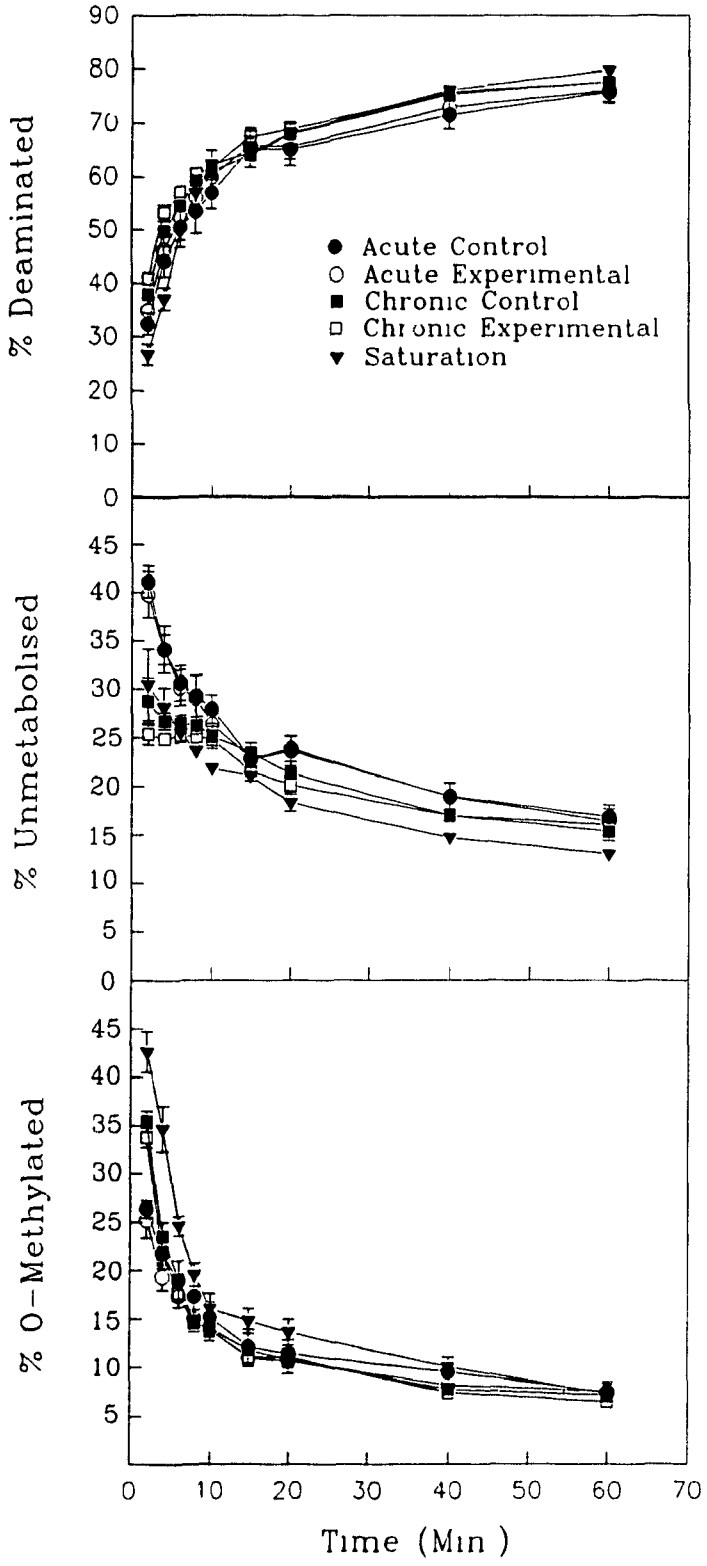
% Deaminated
% Unmetabolised
% O-Methylated
Acute Control
Acute Experimental
Chronic Control
Chronic Experimental
Saturation
Time (Min)

Figure 4.4

minutes post-injection. The rapid clearance of catecholamines ensures that the effects of these hormones are transient, not prolonged, in nature. Indeed, measurements of plasma CAs following exhaustive exercise (Tang and Boutilier, 1988) and acute hypoxia (Fievet *et al.*, 1990) suggest that catecholamines are rapidly cleared from the circulation after removal of the stressor.

The biphasic disappearance of the radiolabels from the plasma, with an initial rapid fall followed by a slow decline, is similar to that described from experiments on mammals (Axelrod *et al.*, 1959; Whitby *et al.*, 1961) and fish (Nekvasil and Olson, 1986a). Although the loss of [^{3}H]-epinephrine from the plasma during the initial phase may reflect uptake into neural and extraneural tissues, catabolism and excretion (Axelrod *et al.*, 1959; Ungell and Nilsson, 1979; Ungell and Nilsson, 1983; Nekvasil and Olson, 1986a; Tendelenburg, 1988), it is clear from [^{14}C]-sucrose disappearance that much of the early decline in [^{3}H]-epinephrine levels is attributable to dilution of the radiolabels in the plasma and extracellular fluid. Indeed, the apparent transition in radiolabel loss from first to second component-dependent processes between 10 and 20 minutes post-injection (Fig. 4.2), and the extremely small k_2 constants for [^{14}C]-sucrose loss from the plasma (0.0005 - 0.0007 min^{-1}), suggest that the injected radiolabels were almost completely distributed within the plasma and extracellular fluid within 20 minutes post-injection. Although radiolabel dilution can explain much of the [^{3}H]-epinephrine decline, the higher k_1 and k_2 constants for [^{3}H]-epinephrine, as compared to [^{14}C]-sucrose (Table 4.2), indicate that tissue uptake, metabolism and excretion play a significant role in [^{3}H]-epinephrine removal. These processes appear to be particularly important during the slow

phase of [^{3}H]-epinephrine decline; the k_2 constants for [^{3}H]-epinephrine were approximately 2.8X greater than for [^{14}C]-sucrose.

[^{3}H]-epinephrine metabolism was extremely rapid. By two minutes post-injection only 25-40% of the injected [^{3}H]-epinephrine was still active (unmetabolised)(Fig. 4.4). This indicates that CA metabolism is also an important mechanism for removing CAs from the circulation. In my study, O-methylated [^{3}H]-epinephrine metabolites appeared in the circulation before deaminated [^{3}H]-epinephrine metabolites. This result supports the findings of (Nekvasil and Olson, 1986a), and indicates that COMT acts before MAO. Given that COMT is a cytoplasmic enzyme and that MAO is a mitochondrial enzyme (Goodmann Gillmann *et al.* 1990), the appearance of O-methylated [^{3}H]-epinephrine metabolites before deaminated [^{3}H]-epinephrine metabolites is not surprising. Deaminated [^{3}H]-epinephrine products accumulated in the plasma during the post-injection period, whereas O-methylated [^{3}H]-epinephrine products declined (Fig. 4.4). However, this result is not inconsistent with the theory that O-methylation plays a major role in catecholamine degradation in fish: 1) the O-methylated product metanephrine is the first metabolite to appear in the urine of cod and shark (*Squalus acanthias*) following [^{3}H]-epinephrine injection (Ungell and Nilsson, 1979; Ungell and Nilsson, 1983); and 2) MOPEG and VMA, metabolites formed by the combined processes of O-methylation and deamination, are probably coeluted with the deaminated metabolites (Nekvasil and Olson, 1986a).

Exposure of trout to repeated injections of epinephrine, either acute or chronic, had no effect on [^{3}H]-epinephrine clearance (as measured by decay constants or ^{3}H : ^{14}C) or metabolism. This result indicates that the ability of the CA metabolism and clearance

systems to remove CAs from the circulation is unlikely to be compromised by repeated exposure to acute stressors. In addition, this finding suggests that the duration of CA-stimulated tissue responses is not altered by acute or chronic exposure to elevated CA levels.

Acutely treated fish (both epinephrine and saline injected) had a significantly greater amount of active (unmetabolised) [^{3}H]-epinephrine in their plasma at 2-4 minutes post-injection as compared to chronically treated fish (Fig. 4.4). Because a major difference between the acute and chronic fish was the length of confinement (2 days vs. 7 days), some facet related to confinement itself may have mediated the difference in [^{3}H]-epinephrine metabolism. Gamperl *et al.* (Appendix 1; in press b) have measured plasma epinephrine, norepinephrine, and cortisol levels in SW rainbow trout from 1-7 days post-confinement. Although levels of epinephrine and norepinephrine were generally less than 10 nM during confinement, and were therefore unlikely to affect [^{3}H]-epinephrine metabolism, levels of cortisol varied widely. Plasma cortisol levels were 104 ng ml^{-1} at 1 day post-confinement, ~60 ng ml^{-1} at 2 days post-confinement, and had returned to pre-confinement levels (~8 ng ml^{-1}) by 7 days post-confinement. In mammals, corticosteroids are known to impede the uptake of CAs into extraneural tissues (uptake$_2$) (Grohmann and Trendelenburg, 1984; Trendelenburg, 1988), where metabolism occurs. Because cortisol levels in trout confined for 2 days were 7 fold higher than in those confined for 7 days, it is probable that the elevated levels of unmetabolised [^{3}H]-epinephrine and the reduced levels of O-methylated [^{3}H]-epinephrine observed at 2-4 minutes post-injection were the result of a cortisol-mediated inhibition of [^{3}H]-

epinephrine extraneural uptake.

The simultaneous injection of radiolabels and 4.0 μg kg^{-1} epinephrine resulted in elevated post-injection epinephrine-to-sucrose ratios, increased proportions of O-methylated [^{3}H]-epinephrine and reduced proportions of unmetabolised [^{3}H]-epinephrine, as compared to acute control fish (Figs. 4.3, 4.4): the net result being that the absolute amount of unmetabolised [^{3}H]-epinephrine at 2-4 minutes post-injection was similar in both groups. Assuming that [^{3}H]-epinephrine and unlabelled epinephrine were bound to receptors, excreted, and taken into extraneural tissues with equal affinity, this result indicates that [^{3}H]-epinephrine extraction and/or metabolism was enhanced greatly in the saturation group. This finding is consistant with Colletti and Olson (1988) who showed that gill extraction of CAs during 10^{-6} M CA perfusion was greater than during 10^{-8} CA perfusion and that the proportion of [^{3}H]-epinephrine metabolites was 11% higher in the arterioarterial effluent of gills perfused with 10^{-6} [^{3}H]-epinephrine. Colletti and Olson (1988) ascribed the increase in gill CA extraction/metabolism during 10^{-6} M CA perfusion to three possibilities: 1) increased diffusive loss of CAs across the gill epithelium; 2) an inability of the HPLC to detect all of the CA metabolites; and 3) enhanced CA extraction during high dose challenge. However, I believe that the enhanced extraction and metabolism of [^{3}H]-epinephrine at high epinephrine doses, observed in the present study, was the result of physiological processes. Although the injection of [^{3}H]-epinephrine alone likely resulted in minimal cardiovascular changes, the administration 4.0 μg kg^{-1} of unlabelled epinephrine would have increased cardiac output by at least 60% (Chapter 1; Gamperl *et al.*, in press a). This elevated rate of tissue perfusion (cardiac output) may

have increased the rate of [^{3}H]-epinephrine extraction from the plasma. In addition, Grohmann and Trendelenburg (1984) have shown that the K_m (IC_{50}) values for uptake$_2$ in the rat heart, determined for unlabelled epinephrine, range between 87 (- isomer) and 172 (+ isomer) μmol. If I assume that the substrate specificity of fish tissues for epinephrine uptake is of a similar magnitude to that observed for the rat heart, the results of Grohmann and Trendelenburg (1984) suggest that extraneural tissues involved in CA metabolism (liver, kidneys, gills, skeletal muscle, etc.: Nekvasil and Olson, 1986a, b; Colletti and Olson, 1988) have a large capacity to extract CAs from the plasma. The injection of 4.0 μg kg^{-1} epinephrine would have resulted in circulating epinephrine levels, at 2 minutes post-injection, of approximately 110 nM (Appendix 1; Gamperl *et al.*, in press b). Because this hormone concentration is approximately 1000 fold less than the proposed K_m of the fish's extraneural uptake mechanism (uptake$_2$), it is not unreasonable to expect that a large proportion of the injected epinephrine would have been extracted and metabolised by the extraneural tissues.

The present study indicates that fish have a marked capacity to rapidly (minutes) metabolise and inactivate catecholamines that are released in response to stressful stimuli and that the exposure of fish to repeated elevations in plasma catecholamines does not alter catecholamine metabolism and/or clearance. The latter result indicates that the adrenergic stimulation of tissues is unlikely to be prolonged by the repeated exposure of fish to acute stressors.

GENERAL CONCLUSIONS

I investigated: 1) whether the exposure of fish to repeated stress (elevations in plasma catecholamines) would affect the responsiveness of the fish's adrenergic systems to subsequent catecholamine exposure; and 2) to what extent trout cardiovascular performance is dependant upon the presence of a coronary blood supply.

Using the heart as a model, I have shown that there is a significant relationship between tissue responsiveness and epinephrine dose, and that small increases in circulating catecholamine levels (small epinephrine doses) can significantly stimulate the β-adrenergic system of fish (Chapter 1). This indicates that tissues may be maximally stimulated at plasma catecholamine concentrations below those observed in fish following a severe stress, and that changes in β-receptor number/affinity or in the rates of catecholamine clearance/metabolism could affect adrenergically-mediated tissue responses. Alterations in β-receptor number and/or affinity would affect the magnitude of a tissue's response to adrenergic stimulation, while enhancement or retardation of catecholamine clearance/metabolism would alter the duration of tissue stimulation.

Neither acute nor chronic exposure to repeated elevations in plasma catecholamines reduced the number or affinity of ventricular β-receptors (Chapter 3), or the rate of [^{3}H]-epinephrine clearance/metabolism (Chapter 4), however. This indicates that the magnitude and duration of catecholamine-mediated tissue responses are unlikely to be affected by repeated exposure to physiological levels of catecholamines. Although these results contrast with many studies conducted on mammals, the results of Horachova

and Wilkinson (1992) support my conclusion, and suggest that β–adrenoreceptor down–regulation only occurs in animals when they are exposed to supra–physiological or pharmacological concentrations of catecholamines for extended periods. Catecholamine clearance and metabolism is extremely rapid in fish, with < 10 percent of the injected [^{3}H]–epinephrine being active after 10 – 15 minutes post–injection (Chapter 4). The temporal pattern of catecholamine elevation in fish closely resembles that seen in trout following catecholamine injection (Gamperl *et al.*, in press c). Therefore, the inability of *in vivo* catecholamine exposure to alter β–receptor density/affinity is probably due to the short exposure time. Although it is improbable that *in vivo* catecholamine exposure results in the loss of cell–surface β–receptors, this does not preclude the possibility that β–receptor binding affinity is diminished during the first few minutes post–stress. Phosphorylation of the β–receptor or the guanine–nucleotide–binding–protein can significantly reduce binding affinity (Lefkowitz *et al.*, 1984) and phosphorylation is likely to occur in a much shorter time–frame than β–receptor downregulation (loss of β–receptors from the cell surface). This possibility, however, requires further investigation.

In the rainbow trout ventricle, the density of cell–surface β–receptors was 3.38 sites per μm^2, a value very close to that measured in various mammals (Chapter 3). This result is surprising given the relationship between acclimation temperature (body temperature) and ventricular β–receptor density (Keen *et al.*, 1993) in fish. However, the results of Perry and Reid (1992) suggest possible mechanisms by which different taxonomic groups may regulate the sensitivity of the β–adrenergic system independent of changes in β–receptor density. Perry and Reid (1992) found that although eels

(*Anguilla rostrata*) had 50% fewer β-receptors, post-stimulation levels of intracellular cyclic AMP exceeded those measured in rainbow trout, and that the responsiveness of the eel erythrocyte Na^+/H^+ exchanger to cyclic AMP levels was reduced. These results indicate that alterations in the sensitivity of the β-receptor-adenylate-cyclase complex to adrenergic stimulation or in the sensitivity of cells to elevations in cyclic AMP may be important factors determining adrenergic sensitivity in different species.

Although it is unlikely that the β-adrenergic system of fish is affected by exposure to physiological levels of catecholamines, the results of chapters 3 and 4 (Gamperl *et al.*, in press a, b) indicate that some factor related to cannulation and/or confinement can alter catecholamine clearance or metabolism and β-adrenergic binding. Recent experiments suggest that circulating cortisol levels may be mediating these confinement effects. Cortisol levels are elevated for up to 5 days post-confinement (Appendix 1; Gamperl *et al.*, in press b). In addition, cortisol has been shown to affect β-adrenoreceptor density in fish (Reid *et al.*, 1991, 1992), and catecholamine metabolism in mammals (Trendelenburg, 1988). Therefore, it is probable that cortisol levels during confinement can modify adrenergically-mediated physiological processes. These results indicate that: (1) researchers must consider the potential effects of cortisol when designing or interpreting experiments which elucidate the physiological role of catecholamines in fish; and (2) inhibition of cortisol (see Gamperl *et al.*, in press c for a review) may prove advantageous in studies which utilise cannulated/confined fish to investigate the physiological effects of elevated catecholamine levels.

I have shown that cardiac performance in trout during normoxia does not depend

highly on the presence of a patent coronary circulation (Chapter 1). This result indicates that the diffusion of oxygen from the ventricular lumen to the compact myocardium is not limited during normoxemia. Although these results may be applicable to other fish with a relatively small proportion of compact myocardium (< 30 - 40%), it is clear that they do not apply to fish whose hearts are comprised of a large proportion of compact myocardium, or in fish during increased activity. Farrell *et al.* (unpublished observations; cited in Davie and Farrell, 1991a) have shown that the tuna (*Katsuwanis pelamis*) heart (~70% compact myocardium) is obligately dependent upon its coronary circulation for resting cardiac performance. In addition, Steffensen and Farrell (1993) have shown that although salmon with intact and ablated coronary–ablated coronaries stopped swimming at the same environmental PO_2, arterial pressure and therefore myocardial oxygen demand was reduced in the coronary–ablated fish. This suggests that the coronary circulation in fish (and other taxonomic groups) may have developed to enable the heart to generate the elevated cardiac work that is concomitant with increased activity. Additional evidence supporting this conclusion comes from the proposed relationship between activity level and coronary development in fish. Davie and Farrell (1991a) indicate that there is a positive relationship between the degree of myocardial coronary vascularization and fish activity level.

Although the coronary circulation does not appear to be important for resting cardiac performance during normoxia (Chapter 1), I have provided indirect evidence that the coronary blood supply is important for determining "resting" and epinephrine–stimulated cardiac performance during hypoxia (Chapter 2). However, it must be

remembered that the method for increasing cardiac work that I used (epinephrine injection) caused constriction of the coronary circulation and elevations in cardiac output pressure (i.e. cardiac power output). Because Steffensen and Farrell (1993) have shown that coronary-ablated salmon can maintain cardiac performance during hypoxia through the homeometric lowering of arterial pressures and therefore cardiac power output, my results indicate that adrenergic stimulation of the heart during hypoxia may have detrimental effects on cardiac performance.

My results support and extend the conclusions of Farrell (1987) and Axelsson and Farrell (1993) who found that cardiac metabolism, arterial pressure, hypoxemia and adrenoreceptors are involved in the regulation of coronary blood flow in fish. However, my results do not provide any further insight into what mechanisms mediate the apparent "metabolic vasodilation" that is associated with hypoxia or increased myocardial power output. The mechanisms which regulate this process await further investigation.

My studies indicate that repeated stress is unlikely to compromise the ability of the β-adrenergic system to respond to subsequent stressors, and indicate that the relationship between catecholamine exposure and β-receptor down-regulation in mammals may require reinvestigation. In addition, my experiments have provided a significant amount of information about the dependence of cardiac performance on coronary blood flow, and have furnished further evidence to support the theory that the presence of a coronary artery is functionally coupled to the ability of a fish's heart to develop pressure.

Appendix 1

EPINEPHRINE, NOREPINEPHRINE, AND CORTISOL CONCENTRATIONS IN TROUT FOLLOWING BLACK-BOX CONFINEMENT AND EPINEPHRINE INJECTION

ABSTRACT

The present study investigates the effect of cannulation and chronic "black-box" confinement, as well as epinephrine administration (4.0 μg kg^{-1}), on the degree and time-course of alterations in trout *(Oncorhynchus mykiss)* catecholamine and cortisol concentrations. Plasma cortisol concentrations in seawater trout acclimated to 3 - 6°C reached 110 ng ml^{-1} one day after cannulation/confinement and remained elevated above resting levels (8 ng ml^{-1}) until 6 days post-confinement. Although plasma epinephrine and norepinephrine generally declined over the period of confinement (day 1 approx. 12 nM; day 7 approx. 6 nM), norepinephrine titers were usually higher and more variable. Epinephrine injection caused elevations in plasma epinephrine levels but not in norepinephrine levels; epinephrine titers reaching 107 ± 26 nM (range 65 -238 nM) at 2 minutes post-injection and returning to pre-injection levels by 30 minutes post-injection. Plasma cortisol increased by 20 ng ml^{-1} following epinephrine administration. Based on the time-course of post-confinement alterations in plasma cortisol, it appears that up to a week may be required before cannulated fish are completely acclimated to

"black-box" confinement. The findings suggest that meaningful results from experiments utilizing epinephrine injection and "black-box" confinement are contingent upon: 1) knowledge of circulating epinephrine levels shortly after injection (i.e. within 2 minutes post-injection; and 2) an experimental design that takes into account the elevated cortisol titers that are inherent with cannulation/confinement and epinephrine injection.

INTRODUCTION

In recent years, numerous studies have examined the *in vivo* relationship between circulating levels of catecholamines and alterations in teleost acid-base (Boutilier *et al.*, 1986; Jensen, 1987; Tang and Boutilier, 1988; Vermette and Perry, 1988), respiratory (Boutilier *et al.*, 1988; Perry and Kinkead, 1989; Perry *et al.*, 1989; Fievet *et al.*, 1990), metabolic (Wright *et al.*, 1989) and circulatory (Wood and Shelton, 1980a, 1980b; Hipkins, 1985) status. To facilitate the injection of various agents and repeated blood acquisition, these fish were fitted with cannulae and individually confined within darkened perspex boxes ("black-boxes"). However, no experiments have been conducted to ascertain the effect of cannulation/confinement on the degree and time-course of alterations in both circulating catecholamines and cortisol. In this study, I have measured cortisol, epinephrine, and norepinephrine levels in SW trout (*Oncorhynchus mykiss*) for 1-7 days post-confinement to assess the physiological implications of using cannulated/confined fish in studies on adrenergically mediated physiological processes. Gamperl and Boutilier (Chapter 4, submitted a) have found that the duration of confinement can affect the rate of [^{3}H]-epinephrine metabolism, and Gamperl *et al.*

(Chapter 3, submitted b) have shown that cannulation/confinement may affect heart β-adrenoreceptor density in seawater (SW) acclimated trout. In addition, and Reid and Perry (1991) have shown that cortisol exposure can affect the magnitude of adrenoreceptor-mediated responses in trout erythrocytes. These results suggest that "stress" hormone levels during confinement may influence experimental results. In my study, seawater trout acclimated to low temperatures (3.3 - 5.8°C) were used to ensure that the results from this experiment would have direct relevance to the findings of Gamperl *et al.* (Chapter 1; in press a).

While intra-vascular injections of catecholamines enable researchers to simulate the pulsatile release of endogenous hormone that is concomitant with many types of "stress" (Tang and Boutilier, 1988; Fievet *et al.*, 1990), careful consideration is required to ensure that resultant plasma titers are appropriate for the type or degree of "stress" that one is attempting to mimic. Epple and Nibbio (1985) have found that injected catecholamines result in maximal ("peak") plasma titers within 3 minutes post-injection, and Nekvasil and Olson (1986) have shown that extracellular dilution, of an injected volume, requires approximately 10-15 minutes to be completed. However, despite this information, researchers often select epinephrine doses and/or estimate post-injection plasma titers based on the assumption that the dilution of an injected hormone into the extracellular fluid (300 ml kg^{-1}) requires only 5 minutes (e.g. Perry and Kinkead, 1989); a practice which likely results in erroneous conclusions about the relationship between epinephrine dose (or circulating level) and the measured physiological response. To clearly define the temporal changes in catecholamine titers that are concomitant with

epinephrine injection, I have measured plasma epinephrine and norepinephrine in trout for 60 minutes following the injection of 4.0 μg kg^{-1} epinephrine. Post-injection cortisol titers were also measured to establish whether epinephrine injection induces an endogenous release of cortisol in trout. White and Fletcher (1985) have reported that epinephrine administered *in vivo* causes an increase in circulating cortisol concentrations in the plaice (*Pleuronectes platessa*).

MATERIALS AND METHODS

Animals

Rainbow trout (*Oncorhynchus mykiss*) were obtained from Merlin Fish Farms (Wentworth, Nova Scotia) and held in 1.5 x 1.5 x 1.0 m fibreglass tanks, supplied with dechlorinated freshwater, for several months. Thereafter, fish were gradually transferred to seawater over a one month period, and were maintained in seawater (approx. 3.0°C) for a further six weeks prior to experimentation. Fish were fed daily, to satiation, on a diet of commercially prepared feed pellets (Shurgain Feed Mills, Truro, Nova Scotia), but were fasted for 48 hrs prior to surgery or experimentation. The fish were maintained on a 12h L : 12h D photoperiod throughout the study.

Trout (avg. body wt. 840 ± 35 g) were anaesthetized in MS 222 (0.15 g l^{-1}, 2–4 minutes) and quickly cannulated via the dorsal aorta (Smith and Bell, 1964). Once surgery was completed, fish were placed in individual black Perspex® boxes (45 x 15 x 8 cm) supplied with aerated seawater at a flow rate of 1 l min^{-1}. Uncannulated experimental fish were held, with conspecifics, in a fibreglass tank (approximate density 6.4 kg/m^3) supplied

with seawater at a flow of 5.0 l min^{-1}. Seawater temperature for both groups ranged from 3.3 - 5.8° over the study.

Experimental Protocols

Experiment I: Effect of Cannulation/Confinement

Blood samples (0.8 ml) were obtained from cannulated trout (N = 9) at approximately 11:00, every day, for 7 days post-confinement. "Resting" (pre-confinement) blood samples were collected from rapidly anaesthetized (0.15 g l^{-1} MS 222, 2 min) uncannulated experimental fish (N = 9) by caudal puncture. To ensure accurate resting cortisol levels only three fish were sampled on a particular day. This procedure precluded increases in plasma cortisol concentration due to the serial removal of cohorts (Laidley and Leatherland, 1988). Resting blood samples were not analyzed for catecholamines as it is unrealistic to expect accurate measurements from anaesthetized fish, sampled by caudal puncture.

Experiment II: Effect of Epinephrine Injection

Following 48 hours of recovery from cannulation, trout were slowly (over approx. 15 sec) injected with 4.0 µg kg^{-1} of epinephrine (N = 10) or saline (Hoar and Hickman, 1983)(N = 9) through the dorsal aortic cannula (total injection volume 0.5 - 0.8 ml). The large injection volume represented an initial epinephrine/saline bolus (0.2 - 0.5 ml), immediately followed by a further 0.3 ml of saline. The latter volume of saline ensured that all of the epinephrine was delivered to the fish. Blood samples were collected from

the dorsal aortic cannula prior to injection, and at 2, 4, 6, 10, 30 and 60 minutes post-injection for analysis of plasma cortisol, epinephrine, and norepinephrine. Approximately 0.8 ml of saline was reinjected into the cannula after each blood collection to partially restore blood volume.

In both experiments, blood was centrifuged (10,000 xg for 30 sec) immediately upon collection and separated into two aliquots (approx. 250 µl) for the subsequent determination of cortisol and catecholamine levels. An anti-oxidant solution (8% by vol.; 0.1 mM sodium metabisulfite, 0.3% EDTA; 0.3% ascorbic acid) was added to plasma samples used for catecholamine measurements. All plasma samples were frozen in liquid nitrogen and stored at -85°C until the determination of hormone levels.

Analytical Procedures

Plasma epinephrine and norepinephrine were measured using high performance liquid chromatography (HPLC, Bioanalytical Systems) with electrochemical detection (BAS LC4B detector). For amine separation, a reverse phase (Biophase ODS, 5 µm) C_{18} column was used in conjunction with a monochloroacetic acid mobile phase (0.3 M, pH 3.0) to which EDTA (0.75 g l^{-1}) and octyl sulphate (25 mg l^{-1}) had been added. Samples (250 ul) were thawed and immediately processed using an alumina extraction procedure (BAS LCEC Application Note 14). DHBA (3,4 dihydroxy benzylamine) was used as an internal standard for all plasma samples and catecholamine standards. Recoveries from the alumina, determined on standards, were in the order of 50 - 60%. Values of percent recovery were used in the determination of individual plasma catecholamine

concentrations.

Cortisol levels were measured using a commercial radioimmunoassay kit (ICN Biomedicals, Carson CA) according to Andersen *et al.* (1991).

Statistical Analysis

Statistical differences ($p < 0.05$) between treatments (saline, epinephrine injection) and the number of days post-confinement, for norepinephrine and epinephrine were identified using a one-way analysis of variance with repeated measures (PROC GLM, SAS Institute, Inc.) followed by multiple contrasts. Post-injection (saline, epinephrine) cortisol levels were compared using a similar analysis. However, because cannulated/confined trout and uncannulated trout (values at "rest") were different groups of fish, post-confinement cortisol levels were compared to those in uncannulated trout using T-tests (SAS). Log-transformed data were used to satisfy assumptions of homogeneous variance, although non-transformed data are shown in the text.
Values are expressed as means ± S.E.

RESULTS

Cannulation/Confinement

Plasma cortisol levels in uncannulated, free-swimming, trout were 7.8 ± 1.6 ng ml^{-1}. Upon cannulation/confinement cortisol titers increased greatly, values at 1 day post-confinement exceeding 100 ng ml^{-1} (mean 104 ± 14.2 ng ml^{-1})(Fig A.1). Plasma cortisol concentrations in the cannulated/confined trout declined over the confinement period.

Cortisol titers were approximately 55 ng ml^{-1} from 2–4 days post–confinement, and averaged 25 ng ml^{-1}, 16 ng ml^{-1} and 8 ng ml^{-1} at days 5, 6 and 7 post–confinement, respectively. Cortisol levels were significantly elevated, over those observed for uncannulated trout, for the first 5 days post–confinement (Fig. A.1).

Epinephrine and norepinephrine titers were approximately 12 nM at 1 day post–confinement. Plasma epinephrine concentration decreased to approximately 5 nM at day 2, and remained at this level for the duration of confinement. Plasma norepinephrine levels, however, were more variable. Norepinephrine titers were 21 nM at 2 days post–confinement, and varied from 4 to 10 nM between 3 and 7 days post–confinement. Although plasma norepinephrine concentrations were generally higher than epinephrine concentrations from 2–7 days post–confinement, plasma norepinephrine was significantly lower than epinephrine on the 6^{th} day of confinement (Fig. A.2).

Epinephrine Injection

Epinephrine injection (4.0 μg kg^{-1}) caused an elevation in circulating epinephrine levels, but not in norepinephrine levels; the latter indicating that a catecholaminotrophic release of norepinephrine is not concomitant with epinephrine injection in trout (Fig. A.3). Although the mean plasma epinephrine concentration was 107 ± 26 nM (range 65 – 238 nM) at 2 minutes post–injection, plasma titers decreased rapidly during the post–injection period. Epinephrine titers were 56.5 ± 9.4, 30.3 ± 4.7 and 11.8 ± 1.7 nM at 4, 10 and 30 minutes post–injection, respectively. The value at 30 minutes was not

Figure A.1. Plasma cortisol concentrations in rainbow trout (N = 9) at rest and during 1 – 7 days of confinement in a "black-box". Resting samples were taken from fish prior to cannulation / confinement. Vertical bars represent ± 1 S.E. (*) Indicates a significant difference ($p < 0.05$) as compared to free-swimming fish.

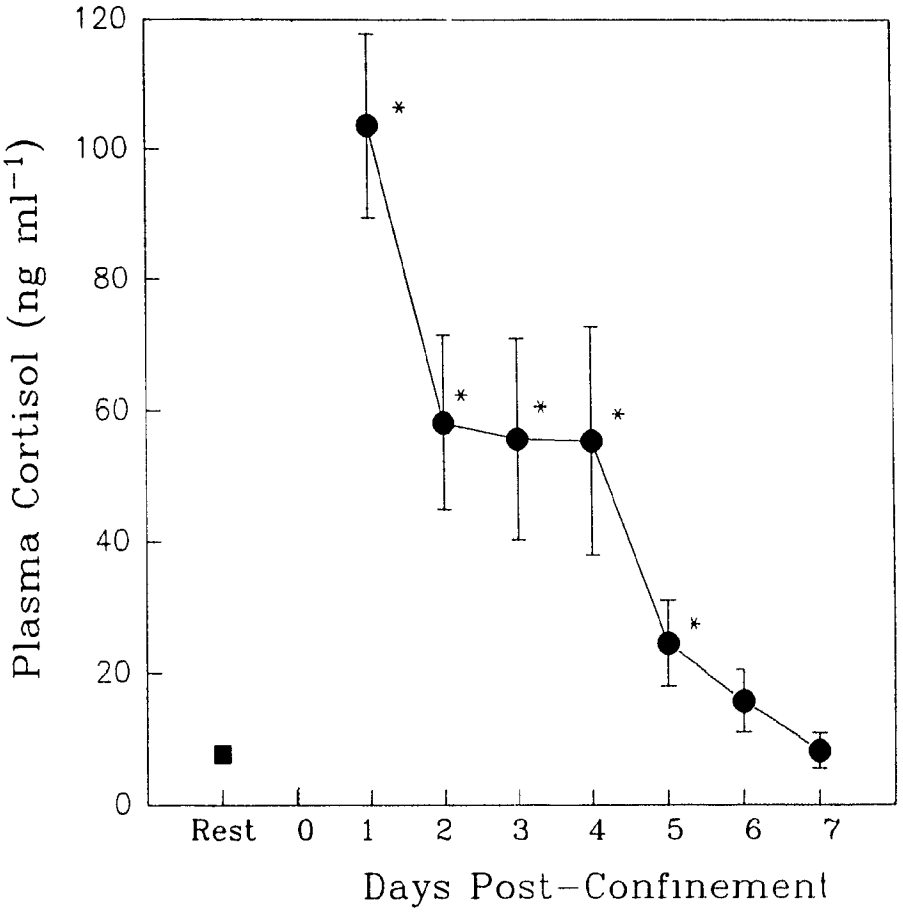
120
100
80
60
40
20
0
Plasma Cortisol (ng ml^{-1})
Rest
0
1
2
3
4
5
6
7
Days Post-Confinement
*
*
*
*
*

Figure A.1

Figure A.2. Plasma epinephrine and norepinephrine concentrations in cannulated rainbow trout (N = 9) from 1 to 7 days post-confinement / cannulation. (*) Indicates a significant difference ($p < 0.05$) between epinephrine and norepinephrine concentrations. Vertical bars represent $\pm$ 1 S.E.

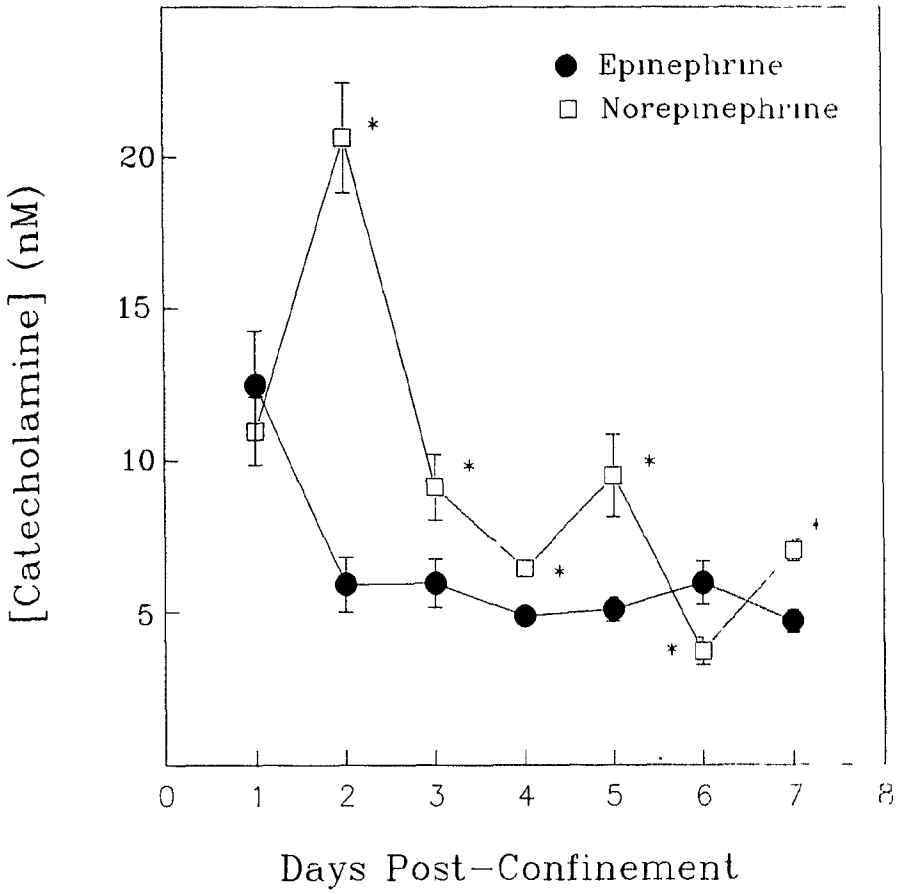
Epinephrine
Norepinephrine
[Catecholamine] (nM)
20
15
10
5
0
1
2
3
4
5
6
7
8
Days Post-Confinement

Figure A.2

Figure A.3. Plasma epinephrine (A) and norepinephrine (B) concentrations in cannulated rainbow trout after injection of saline (N = 9) or 4.0 ug kg^{-1} epinephrine (N = 10). (*) Indicates a significant difference ($p < 0.05$) in hormone level from resting values. Vertical bars represent $\pm$ 1 S.E.

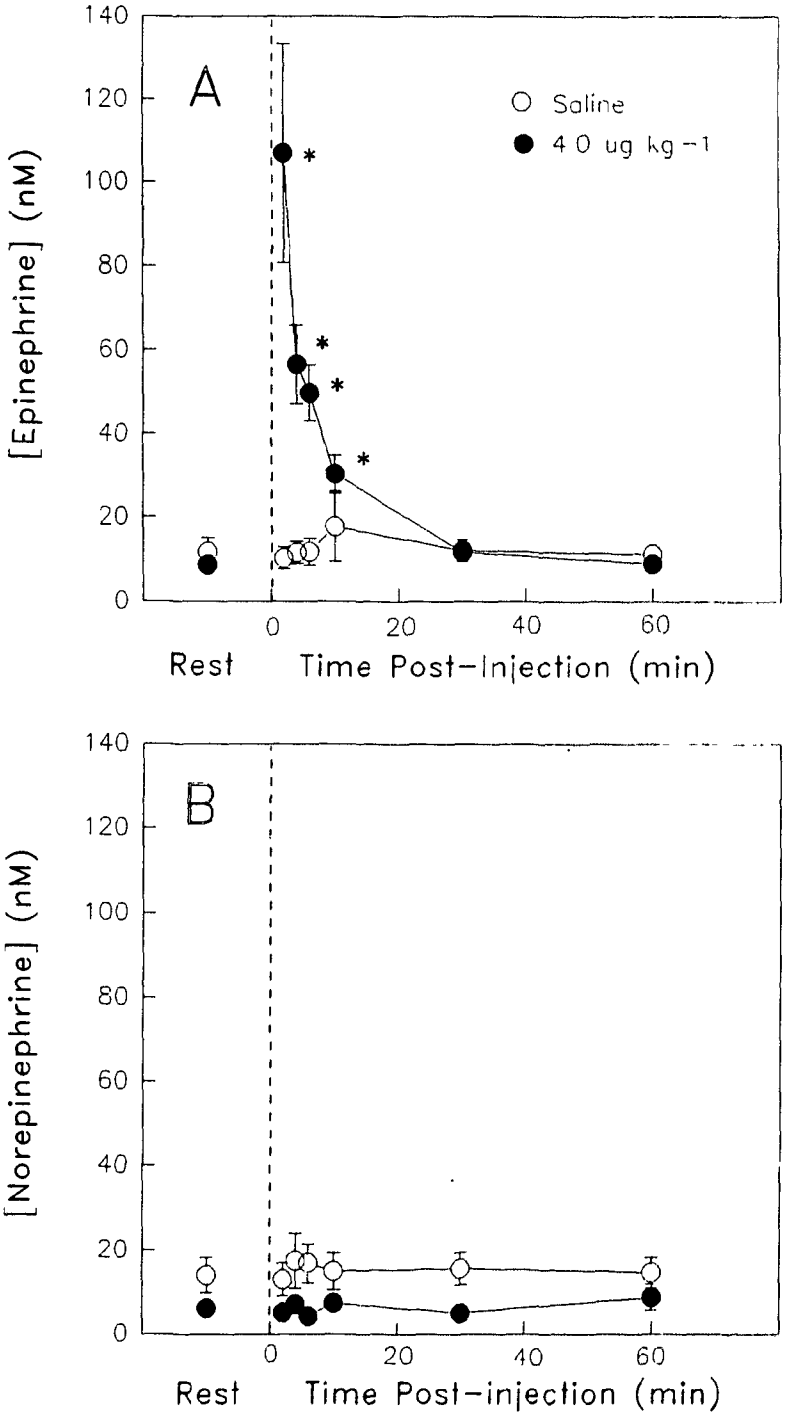
A
Saline
4 0 ug kg -1
[Epinephrine] (nM)
140
120
100
80
60
40
20
0
0
20
40
60
Rest
Time Post-Injection (min)
B
[Norepinephrine] (nM)
140
120
100
80
60
40
20
0
0
20
40
60
Rest
Time Post-injection (min)

Figure A.3

significantly different from pre-injection levels. Saline injection had no effect on circulating levels of epinephrine or norepinephrine.

Injection of epinephrine caused observable (20 ng ml^{-1}) increases in plasma cortisol concentration at 10, 30 and 60 minutes post-injection (Fig. A.4): these increases were significant ($p < 0.05$) at 10 and 20 minutes when compared to saline injected fish, and at 60 minutes when compared to pre-injection levels. Saline injection had no effect on plasma cortisol concentrations from 2 to 30 minutes post-injection. However, plasma cortisol concentrations in saline injected trout were significantly elevated (approx. 25 ng ml^{-1}) over pre-injection levels at 60 minutes post-injection.

DISCUSSION

Plasma cortisol concentrations at 1 day post-confinement in my seawater-acclimated trout (104 ± 14 ng ml^{-1}) are comparable to those in freshwater (FW) salmonids following 12 - 24 hrs of confinement within a net (110 ± 9 ng ml^{-1}; Woodward and Strange, 1987), or after cannulation and confinement within black boxes (~110 ng ml^{-1}; Goss and Wood, 1988: ~ 100 ng ml^{-1}; Andersen *et al.*, 1991). Cortisol titers in my cannulated/confined trout required 6 days to return to levels found in unconfined, free-swimming fish (Fig. A.1). This prolonged elevation in post-confinement cortisol concentrations agrees with previous studies on confined and/or cannulated FW fish. These studies have shown that cortisol titers in confined FW fish fail to reach pre-confinement levels (Brown *et al.*, 1986), stabilize at levels well above those observed in free-swimming fish (~50 ng ml^{-1}; Andersen *et al.*, 1991: ~60 ng ml^{-1}; Redding *et al.*, 1986),

Figure A.4. Plasma cortisol concentrations in cannulated rainbow trout after injection of saline (N = 9) or 4.0 ug kg^{-1} epinephrine (N = 10). (*) Indicates a significant difference ($p < 0.05$) in hormone level from resting values. (+) Indicates a difference as compared to saline injected fish. Vertical bars represent $\pm$ 1 S.E.

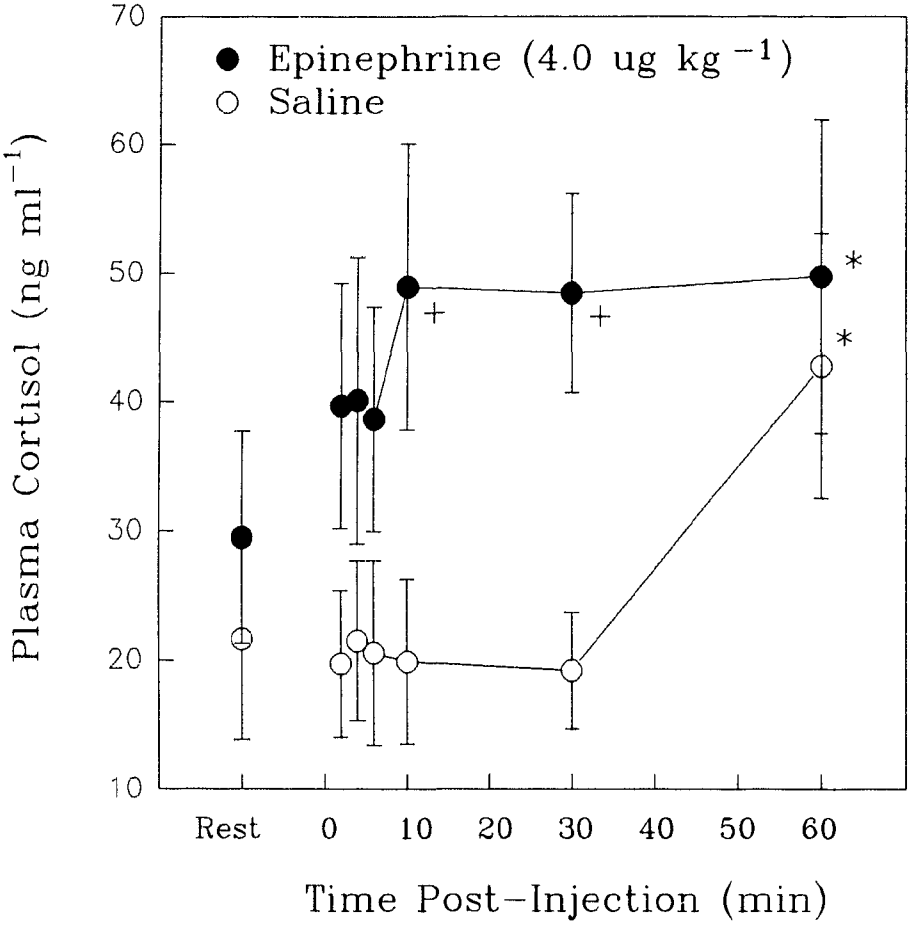
Epinephrine (4.0 ug kg $^{-1}$)
Saline
Plasma Cortisol (ng ml^{-1})
70
60
50
40
30
20
10
Rest
0
10
20
30
40
50
60
Time Post-Injection (min)
+
+
*
*

Figure A.4

or require between one and two weeks to return to pre-confinement levels (Pottinger and Pickering, 1992).

Gamperl and Boutilier (Chapter 4, submitted) observed that cannulated rainbow trout confined in black-boxes for seven days metabolised [^{3}H]-epinephrine faster than trout confined for only two days. Because catecholamines probably remained low (< 10 nM) throughout confinement (present study), temporal changes in plasma catecholamine concentrations could not have caused the reported alterations in [^{3}H]-epinephrine metabolism. In mammals, cortisol is known to impede the uptake of catecholamines into extraneural tissues, where metabolism occurs (Trendelenburg, 1988). Therefore, it is likely that elevated cortisol titers at 2 days post-confinement (55 ng ml^{-1}) inhibited [^{3}H]-epinephrine metabolism. In addition to the proposed effect of cortisol on the rate of epinephrine metabolism, Reid and Perry (1991) have shown that the chronic *in vivo* or acute *in vitro* exposure of erythrocytes to elevated cortisol levels (~100 ng ml^{-1}) may pre-adapt these cells to receive additional, adrenoreceptor-mediated, physiological input. These results demonstrate that: 1) cortisol titers in cannulated/confined fish can modify adrenergically-mediated physiological responses; and 2) that one must consider the potential effects of elevated cortisol levels when designing or interpreting experiments which elucidate the physiological role of circulating catecholamines in fish.

Post-confinement plasma catecholamine concentrations in my trout (generally 5 - 15 nM) are higher than the 0.5 - 2.5 nM levels reported in most studies on confined FW salmonids (Perry *et al.*, 1987; Boutilier *et al.*, 1988; Perry *et al.*, 1989; Gingerich and Drottar, 1989; Perry and Reid, 1991). However, the reasons for this discrepancy are likely

unrelated to seawater acclimation. Tang and Boutilier (1988) report no difference in resting catecholamine levels between confined/cannulated SW and FW trout. Although Milligan *et al.* (1989) have provided some evidence to suggest that resting catecholamine levels are unaffected by season and/or acclimation temperature, the higher resting catecholamine levels in my study may be related to the low acclimation temperature (3–6°C).

Plasma epinephrine levels 2 minutes following the injection of 4.0 μg kg^{-1} averaged 107 nM. This value is comparable to those seen in eels (*Anguilla rostrata* Lesueur; Epple and Nibbio, 1985) and lamprey (*Petromyzon marinus* Linnaeus; Dashow and Epple, 1983) 3 minutes following the injection of 4.0 μg kg^{-1} (109 nM) and 5.0 μg kg^{-1} (118 nM), respectively. However, these peak epinephrine levels are much lower than estimates based on dilution of the injected epinephrine within the plasma. Assuming that injected epinephrine is completely mixed within the plasma at 2 minutes post-injection, and that plasma represents approximately 2.3 vol % per kg (Steffensen and Lomholt, 1992), plasma catecholamine levels should have been 940 nM. Discrepancies between the observed and estimated peak epinephrine concentrations are probably the result of the partial dilution of epinephrine within the extracellular fluid and the high rate of epinephrine metabolism. By two minutes post-injection 60 –70% of an injected dose of epinephrine (4.0 μg kg^{-1}) has been metabolised (Gamperl and Boutilier; Chapter 4, submitted). Values for plasma epinephrine at two minutes post-injection ranged from 65–238 nM. Differences in cardiovascular response patterns, and therefore circulation time, between fish (Wood and Shelton, 1980a: Chapter 1; Gamperl *et al.*, in press a) probably

accounted for the large variation in measured plasma titers.

Epinephrine titers were approximately 50% of peak epinephrine levels at 5 minutes post-injection, and had returned to levels comparable with saline injected fish by 30 minutes post-injection. Tang and Boutilier (1988) found that plasma epinephrine titers in "chased" SW rainbow trout reached maximum values (85 nM) at 2 minutes post-exhaustion and required approximately 50 minutes to return to levels of 10 nM. These results suggest that bolus injections of epinephrine are suitable for imitating the maximum epinephrine titers, and the temporal pattern of epinephrine elevation, that are concordant with acute stress (ie. exhaustive exercise, handling, air exposure etc.). In recent years, researchers have related alterations in physiological variables to plasma catecholamine concentrations taken from fish at 5 minutes (e.g. Perry and Kinkead, 1989) or 15 minutes (e.g. Wright *et al.*, 1989) post-injection. However, while the results from such experiments can yield information about the importance of adrenergic stimulation in regulating physiological processes, they should not be used to define dose-response relationships. The reasons for this conclusion are threefold: 1) the magnitude of an adrenergically-mediated physiological response is probably dependent on maximal (peak) catecholamine titers; 2) plasma catecholamines at 5 and 15 minutes post-injection underestimate peak catecholamine levels by approximately 50 and 75%, respectively (Fig. A1.3); and 3) adjusting administered doses of epinephrine (catecholamines) to achieve plasma levels that are based on measurements taken at 5 to 15 minutes post-injection may lead to supra-physiological or pharmacological *in vivo* levels (ie. at two minutes post-injection).

Injection of epinephrine into SW trout had a small, but insignificant, effect on circulating levels of norepinephrine. The absence of a marked catecholaminotrophic effect is consistent with previous studies on rainbow trout (Perry and Kinkead, 1989; Wright *et al.*, 1989; Aota and Randall, in press), eels (Epple and Nibbio, 1985), and lamprey (Dashow and Epple, 1983). Although epinephrine injection in these species caused a slight catecholaminotrophic release of norepinephrine from the chromaffin tissue, and possibly nerve endings, resultant norepinephrine levels rarely exceeded 10 nM. Exposure of a fish to a naturally or experimentally-induced "stress" usually results in the elevation of both epinephrine and norepinephrine titers (see Gamperl *et al.*, in press c for a review). Because epinephrine injection only results in minimal elevations in plasma norepinephrine (and visa-versa), the results of the present study indicate that researchers interested in evaluating the *in vivo* effects of "stress-released" catecholamines on fish physiology must inject both epinephrine and norepinephrine (the relative proportion of the two amines designed to be specific for the "stress" situation one is trying to duplicate).

In my study, increases in circulating cortisol levels of approximately 20 ng ml^{-1} were induced by epinephrine injection. These data confirm the observations of White and Fletcher (1985) who found that epinephrine administered *in vivo* caused an increase in circulating cortisol concentrations in the plaice. In mammals, *in vivo* and *in vitro* evidence suggests that systemic catecholamines can stimulate increases in circulating cortisol levels through α_1-adrenoreceptor mediated increases in hypothalmic CRF secretion and/or β-adrenoreceptor mediated increases in ACTH release from the pituitary (Axelrod and Reisine, 1984; Plotsky *et al.*, 1989). Although fish possess a blood-brain barrier for

epinephrine (Busacker and Chavin, 1977; Nekvasil and Olson, 1986a), and Derry (1975) has shown that epinephrine and norepinephrine increase adenyl cyclase activity in the pars distalis of teleosts, little information is available on mechanisms mediating catecholamine effects on cortisol levels in fish. The presence of an epinephrine (catecholamine) induced elevation in plasma cortisol suggests that some post-injection physiological responses (e.g. hyperglycaemia) may involve a complex interaction between these two hormones (M.M. Vijayan and T.W. Moon, submitted). Therefore, investigators who wish to study the effect of catecholamines (epinephrine injection) on physiological variables should attempt to distinguish between the direct effects of catecholamines and the indirect effects of cortisol. To clearly understand the *in vivo* relationship between acute elevations in plasma catecholamines and alterations in specific physiological variables, inhibition of cortisol/ACTH release may be necessary.

Cortisol levels in saline injected fish increased between 30 and 60 minutes post-injection. This result, however, is likely related to the repeated removal of blood (0.8 ml) and not the injection procedure. Redding et al. (1986) found no increase in eel (*Anguilla anguilla*) plasma cortisol concentration 2 hours following the intra-arterial injection of saline. Brown et al. (1986) demonstrated that serial blood sampling (7 times within three hours) can elevate plasma cortisol concentrations by 30 ng ml^{-1}.

In summary, this study demonstrates that cortisol concentrations in cannulated trout, acclimated to 3-6°C, remain elevated until 6 days post-confinement, and indicates that up to a week may be required before cannulated fish are completely acclimated to black-box confinement. Because epinephrine injection results in maximal (peak)

epinephrine concentrations shortly after injection (i.e. within 2 minutes), measurement of plasma titers during this period is essential for interpreting the results from experiments which utilise epinephrine injection. Elevations in plasma cortisol, which are inherent with cannulation/confinement and epinephrine injection, may influence adrenergically-mediated physiological responses.

REFERENCES

Andersen, D.E., Reid, S.D., Moon, T.W. and Perry, S.F. (1991). Metabolic effects associated with chronically elevated cortisol in rainbow trout (*Oncorhynchus mykiss*). *Can. J. Fish. Aquat. Sci.* **48**, 1811–1817.

Aota, S. and Randall, D.J. (1993). The effects of exogenous catecholamines on the ventilatory and cardiac responses of normoxic and hyperoxic rainbow trout, *Oncorhynchus mykiss J. Comp. Physiol. B.*

Ascoli, M. and Segaloff, D.L. (1986). Effects of collagenase on the structure of the lutropin/ choriogonadotropin receptor. *J. Biol. Chem.* **261**, 3807–3815.

Ask., J.A., Stene–Larsen, G., and Helle, K.B. (1980). Atrial β_2–adrenoreceptors in the trout *J. Comp. Physiol. B.* **139**, 109-115.

Ask., J.A., Stene–Larsen, G. and Helle, K.B. (1981). Temperature effects on the β_2–adrenoreceptors of the trout atrium. *J. Comp. Physiol. B.* **143**, 161–168.

Ask., J.A. (1983). Comparative aspects of adrenergic receptors in the hearts of lower vertebrates. *Comp. Biochem. Physiol.* **76A**, 543–552.

Axelrod, J. and Reisine, T.D. (1984). Stress hormones: their interaction and Regulation. *Science*. **224**, 452–459.

Axelrod, J., Weil–Malherbe, H and Tomchick, R. (1959). The physiological diposition of [^{3}H]–epinephrine and its metabolite metanephrine. *J. Pharmac. Exp. Ther.* **127**, 251–256.

Axelsson, M. and Farrell, A.P. (1993). Coronary blood flow *in vivo* in the coho salmon (*Oncorhynchus mykiss*). *Am. J. Physiol.* **264**, R963–R971.

Barron, M G., Tarr, B.D. and Hayton, W.L. (1987). Temperature–dependence of cardiac output and regional blood flow in rainbow trout, *Salmo gairdneri* Richardson. *J. Fish. Biol.* **31**, 735–744.

Booth, J.H. (1979). The effects of oxygen supply, epinephrine, and acetylcholine on the distribution of blood flow in trout gills. *J. Exp. Biol.* 83, 31–39.

Boutilier, R.G., Iwama, G K. and Randall, D.J. (1986). The promotion of catecholamine release in rainbow trout, *Salmo gairdneri*, by acute acidosis: Interactions between red cell pH and haemoglobin oxygen–carrying capacity. *J. Exp. Biol.* **123**, 145–157.

Boutilier, R.G., Dobson, G., Hoeger, U. and Randall, D.J. (1988). Acute exposure to graded levels of hypoxia in rainbow trout (*Salmo gairdneri*): Metabolic and respiratory adaptations. *Resp. Physiol.* **71**, 69–82.

Brown, S.B., Eales, J.G. and Hara, T.J. (1986). A protocol for estimation of cortisol plasma clearance in acid-exposed rainbow trout (*Salmo gairdneri*). *Gen. Comp. Endocrinol.* **62**, 493–502.

Busacker, G.P. and Chavin, W. (1977). Uptake, distribution, and turnover of catecholamine radiolabel in the goldfish, *Carassius auratus* L. *Can. J. Zool.* **55**, 1656–1670.

Burt, D.R. (1985). Criteria for receptor identification. *In* "Neuroreceptor receptor binding." (H.I. Yamamura, S.J. Enna, and M.J. Kuhar, Eds.). Raven Press, New York.

Burton, A.C. (1972). *Physiology and Biophysics of the Circulation*, 2nd ed. Chicago: Yearbook Medical Publishers.

Cameron, J.N. and Davis J.C. (1970). Gas exchange in rainbow trout (*Salmo gairdneri*) with varying blood oxygen capacity. *J. Fish. Res. Bd. Can.* **27**, 1069–1085.

Cameron, J.S. and Brown, S.E. (1981). Adrenergic and cholinergic responses of the isolated heart of the goldfish, *Carassius auratus*. *Comp. Biochem. Physiol.* **70C**, 109–115.

Cech Jr., J.J., Rowell, D.M. and Glasgow, J.S. (1977). Cardiovascular responses of the winter flounder *Pseudopleuronectes americanus* to hypoxia. *Comp. Biochem. Physiol.* **57A**, 123–125.

Colletti, A.E. and Olson, K.R. (1988). Catecholamine metabolism by the perfused rainbow trout gill. *J. Exp. Zool. 248*, 177–184.

Dashow, L. and Epple, A. (1983). Effects of exogenous catecholamines on plasma catecholamines and glucose in the sea lamprey, *Petromyzon marinus*. *J. Comp. Physiol.* **152**, 35–41.

Davie, P.S. and Farrell, A.P. (1991a). The coronary and luminal circulations of the myocardium of fishes. *Can. J. Zool.* **69**, 1993–2001.

Davie, P.S. and Farrell, A.P. (1991b). Cardiac performance of an isolated heart preparation from the dogfish (*Squalus acanthias*): effects of hypoxia and coronary artery perfusion. *Can. J. Zool.* **68**, 1822–1828.

Davie, P.S. and Franklin, C.E. (1993). Preliminary observations on blood flow in the coronary arteries of two school sharks (*Galeorhinus australis*). *Can. J. Zool.* **71**, 1238–1241.

Davie, P.S., Farrell, A.P. and Franklin, C.E. (1992). Cardiac performance of an isolated eel heart: effects of hypoxia and responses to coronary artery perfusion. *J. Exp. Zool.* **262**, 113–121.

Daxboeck, C. (1982). Effect of coronary artery ablation on exercise performance in *Salmo gairdneri*. *Can. J. Zool.* **60**, 375–381.

Deery, D.J. (1975). Effect of catecholamines and synthetic mammalian hypothalmic hormones on the adenyl cyclase activity of the pituitary of the teleost, *Carassius auratus*. *Gen. Comp. Endocrinol.* **25**, 395–399.

Deighton, N.M., Hamilton, C.A., Howie, C.A. and Reid, J.L. (1989). Effects of short-term exposure to noradrenaline on adrenoreceptor responses. *Eur. J. Pharmacol.* **169**, 95–101.

Dunn, G., Koshikawa, N., Duncan, M.J. and Campbell, I.C. (1988). An examination of experimental design in relation to receptor binding assays. *Br. J. Pharmacol.* **94**, 693–698.

Elfellah, M.S., Deighton, N. and Reid, J.L. (1988). Regulation of β–adrenoreceptors by catecholamines in the rabbit skeletal muscle. *Eur. J. Pharmacol.* **157**, 215–220.

Epple, A. and Nibbio, B. (1985). Catecholaminotropic effects of catecholamines in a teleost fish, *Anguilla rostrata*. *J. Comp. Physiol. B.* **155**, 285–290.

Farrell, A.P. (1981). Cardiovascular changes in the lingcod (*Ophiodon elongatus*) following adrenergic and cholinergic drug infusions. *J. exp. Biol.* **91**, 293–305.

Farrell, A.P. (1984). A review of cardiac performance in the teleost heart: intrinsic and humoral regulation. *Can. J. Zool.* **62**, 523–536.

Farrell, A.P. (1987). Coronary flow in a perfused rainbow trout heart. *J. Exp. Biol.* **129**, 107–123.

Farrell, A.P. and Graham, M.S. (1986). Effects of adrenergic drugs on the coronary circulation of Atlantic salmon (*Salmo salar*). *Can. J. Zool.* **64**: 481–484.

Farrell, A.P. and Steffensen, J.F. (1987) Coronary ligation reduces maximum sustained swimming speed in chinook salmon, *Oncorhynchus tshawytscha*. *Comp. Biochem. Physiol.* **87A**, 35–37.

Farrell, A.P. and Jones, D.R. (1992). The heart. In *Fish Physiology, Vol. XIIA* (ed. W.S. Hoar, D.J. Randall and A.P. Farrell), pp. 1–88. New York, London: Academic Press.

Farrell, A.P., MacLeod, K. and Driedzic, W.R. (1982). The effects of preload, afterload, and epinephrine on cardiac performance in the sea raven, *Hemipterus americanus*. *Can. J. Zool.* **60**, 3165–3171.

Farrell, A.P., MacLeod, K. 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., Johansen, J.A. and Graham, M.S. (1988a). The role of the pericardium in cardiac performance of the trout (*Salmo gairdneri*). *Physiol. Zool.* **61**, 213–221.

Farrell, A.P., Hammons, A.M., Graham, M.S., and Tibbits, G.F. (1988b). Cardiac growth in rainbow trout, *Salmo gairdneri*. *Can. J. Zool.* **66**, 2368–2373.

Farrell, A.P., Small, S. and Graham, M.S. (1989). Effect of hypoxia and heart rate on the performance of a perfused trout heart. *Can. J. Zool.* **67**, 274–280.

Farrell, A.P., Johansen, J.A., Steffensen, J.F., Moyes, C.D., West, T.G. and, Suarez, R.K. (1990). Effects of exercise training and coronary ablation on swimming performance, heart size, and cardiac enzymes in rainbow trout, *Oncorhynchus mykiss*. *Can. J. Zool.* **68**, 1174–1179.

Feigl, E.O. (1983). *Coronary Physiology*. *Physiol. Rev.* **63**, 1–205.

Fievet, B., Caroff, J. and Motais, R. (1990). Catecholamine release controlled by blood oxygen tension during deep hypoxia in trout: effect on red cell Na/H exchanger activity. *Resp. Physiol.* **79**, 81–90.

Franklin, C.E. and Davie, P.S. (1992). Myocardial power output of an isolated eel (*Anguilla dieffenbachii*) heart preparation in response to adrenaline. *Comp. Biochem. Physiol.* **101C**, 293–298.

Fritsche, R. and Nilsson, S. (1989). Cardiovascular responses to hypoxia in the Atlantic Cod, *Gadus morhua*. *Exp. Biol.* **48**, 153–160.

Gamperl, A.K., Pinder, A.W. and Boutilier, R.G. (in press a). Effect of coronary ablation and adrenergic stimulation on *in vivo* cardiac performance in trout (*Oncorhynchus mykiss*). *J. Exp. Biol.*

Gamperl, A.K., Vijayan, M.M., and Boutilier, R.G. (in press b). Epinephrine, norepinephrine, and cortisol concentrations in cannulated seawater-acclimated trout *(Oncorhynchus mykiss Walbaum).* following black-box confinement and epinephrine injection *J. Fish. Biol.*

Gamperl, A.K., Vijayan, M.M. and Boutilier, R.G. (in press c). Experimental control of stress hormone levels in fishes: Techniques and applications. *Rev. Fish. Biol. Fish.*

Gamperl, A.K. and Boutilier, R.G. (submitted). Effect of acute and chronic epinephrine administration on the clearance and metabolism of [^{3}H] epinephrine in trout (*Oncorhynchus mykiss*). *J. Comp. Physiol. B.*

Gamperl, A.K., Pinder, A.W. and Boutilier, R.G. (submitted a). Influence of hypoxia and adrenaline administration on coronary blood flow and cardiac performance in seawater rainbow trout (*Oncorhynchus mykiss*). *J. exp. Biol.*

Gamperl, A.K., Wilkinson, M. and Boutilier, R.G. (submitted b). β-adrenoreseptors in the trout (*Oncorhynchus mykiss*) ventricle: characterization, quantification, and the effects of repeated catecholamine exposure. *Gen. Comp. Endocrinol.*

Gesser, H. (1985). Effects of hypoxia and acidosis on fish heart performance. In *Respiration and Metabolism* (ed. R. Gilles), pp. 402-410. Berlin: Springer-Verlag.

Gesser, H., Andresen, P., Brams, P. and Sund-Larsen, J. (1982). Inotropic effects of adrenaline on the anoxic or hypercapnic myocardium of rainbow trout and eel. *J. Comp. Physiol.* **147**, 123-128.

Gingerich, W.H. and Drottar, K.R. (1989). Plasma catecholamine concentrations in rainbow trout (*Salmo gairdneri*) at rest and after anaesthesia and surgery. *Gen. Comp. Endocrinol.* **73**, 390-397.

Goodman Gilmann, A., Rall, T.W., Nies, A.S. and Taylor, P. (1990). *The pharmacological basis of Therapeutics* (8th ed). New York: Pergamon Press Inc.

Goss, G.G. and Wood, C.M. (1988). The effects of acid and acid/aluminum exposure on circulating cortisol levels and other blood parameters in the rainbow trout, *Salmo gairdneri. J. Fish. Biol.* **32**, 63-76.

Graham, M.S. and Farrell, A. P. (1989). The effects of temperature acclimation and adrenaline on the performance of a perfused trout heart. *Physiol. Zool.* **62**, 38-61.

Graham, M.S. and Farrell, A. P. (1990). Myocardial oxygen consumption in trout acclimated to 5°C and 15°C. *Physiol. Zool.* **63**, 536-554.

Grohmann, M. and Trendelenburg, U. (1984) The substrate specificity of uptake$_2$ in the rat heart. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **328**, 164-173.

Haddad, C., Wilkinson, M., Roeder, L.M., Tildon., J.T. and J.A. Armour. (1987). Binding of the hydrophillic β-adrenergic antagonist [^{3}H] CGP-12177 to cardiac tissue slices: characterization and ontogenetic studies in dogs. *Can. J. Physiol. Pharmacol.* **65**, 1928-1933.

Hedberg, A., Minneman, K.P. and Molinoff, P.B. (1980). Differential distribution of beta-1 and beta-2 adrenergic receptors in cat and guinea pig heart. *J. Pharmacol. Exp. Ther.* **212**, 503 - 508.

Helgason, S. St. and Nilsson, S. (1973). Drug effects on pre- and post-branchial blood pressure and heart rate in a free-swimming marine teleost, *Gadus morhua*. *Acta. Physiol. Scand.* **88**, 533-540.

Hendensen, I.W., Sa'di, M.N. and Hargreaves, G. (1974). Studies on the production and metabolic clearance rates of cortisol in the European eel, *Angulla anguilla* (L.). *J. Steroid Biochem.* **5**, 701-707.

Hipkins, S.F. (1985). Adrenergic responses of the cardiovascular system of the eel, *Anguilla australis, in vivo. J. Exp. Zool.* **235**, 7-20.

Hoar, W.S. and Hickman Jr., C.P. (1983). *A Laboratory Companion for General and Comparative Physiology.*, 3rd ed. New Jersey: Prentice-Hall. p. 307.

Holeton, G.F. and Randall, D.J. (1967). The effect of hypoxia upon the partial pressure of gases in the blood and water afferent and efferent to the gills of rainbow trout. *J. Exp. Biol.* **46**, 317-327.

Holmgren, S. (1977). Regulation of the heart of a teleost, *Gadus morhua*, by autonomic nerves and circulating catecholamines. *Acta. Physiol. Scand.* **99**, 62-74.

Horackova, M. and Wilkinson, M. (1992). Characterization of cell-surface β-adrenergic ([^{3}H] CGP-12177) binding in adult rat ventricular myocytes: lack of regulation by β-agonists at physiological concentrations. *Pflügers. Arch.* **421**, 440-446.

Hughes, G.M., Le Bras-Pennac, Y. and Pennac, J-P. (1988). Relationships between swimming speed, oxygen consumption, plasma catecholamines and heart performance in rainbow trout (*S. gairdneri* R.). *Exp. Biol.* **48**, 45-49.

Jones, D.R. and Randall, D.J. (1978). The respiratory and circulatory systems during exercise. In *Fish Physiology, Vol. VII* (ed. W.S. Hoar and D.J. Randall), pp. 425-501. New York, London: Academic Press.

Jones, D.R., Langille, B.L., Randall, D.J. and Shelton, G. (1974). Blood flow in the dorsal and ventral aortas of the cod, *Gadus morhua*. *Am. J. Physiol.* **226**, 90–95.

Kiceniuk, J.W. and Jones, D.R. (1977). Oxygen transport system in trout (*Salmo gairdneri*) during sustained exercise. *J. exp. Zool.* **69**, 247–260.

Kita, J. and Itazawa, Y. (1989). Release of erythrocytes from the spleen during exercise and splenic constriction by adrenaline infusion in the rainbow trout. *Jap J. Icthyology*. 36, 48–52.

Keen, J.E., Vianzon, D–M., Farrell, A.P. and Tibbits, G.F. (1993). Thermal acclimation alters both adrenergic sensitivity and adrenoreceptor density in cardiac tissue of rainbow trout. *J. exp. Biol.* **181**, 27–47.

Laidley, C.W. and Leatherland, J.F. (1988). Cohort sampling, anaesthesia and stocking–density effects on plasma cortisol, thyroid hormone, metabolite and ion levels in rainbow trout, *Salmo gairdneri* Richardson. *J. Fish. Biol.* **33**, 73–88.

Larsson, A.L. (1973). Metabolic effects of epinephrine and norepinephrine in the eel *Anguilla anguilla* L. *Gen. Comp. Endocrinol.* **20**, 155–167.

Lassen, N.A. and Perl, W. (1979). *Tracer kinetic methods in medical physiology*. New York: Raven Press.

Lefkowitz, R.J. (1978). Identification and regulation of alpha– and beta–adrenergic receptors. *Fed. Proc.* **37**, 123–129.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275.

Mader, S.L., Downing, C.L. and Van Lunteren, E. (1991). Effect of age and hypoxia on β–adrenergic receptors in the rat heart. *J. Appl. Physiol.* **71**, 2094–2098.

Mahon, L.C., McKernan, R.M. and Insef, P.A. (1987). Metabolism of α– and β–adrenergic receptors *in vitro* and *in vivo*. *Ann. Rev. Pharmacol. Toxicol.* **27**, 215–235.

Manalan, A.S., Besch, S.R. and Watanabe, A.M. (1981). Characterization of [^{3}H] (±) carazolol binding to β–adrenergic receptors: application to study of β–adrenergic receptor subtypes in canine ventricular myocardium and lungs. *Circ. Res.* **49**, 326–336.

Mauz, A.B.M. and Pelzer, H. (1990). β-Adrenoreceptor-binding studies of the cardioselective β blockers bisoprolol, H-1 42 BS, and HX-CH 44 BS to heart membranes and intact ventricular myocytes of adult rats: two β_1-binding sites for bisoprolol. *J. Cardio. Pharmacol.* **15**, 421-428.

Mazeaud, M.M., Mazeaud, F. and Donaldson, E.M. (1977). Primary and secondary effects of stress in fish: Some new data with a general review. *Trans. Amer. Fish. Soc.* **106**, 201-212.

Mazeaud, M.M. and Mazeaud, F. (1981). Adrenergic responses to stress in fish. In *Stress and Fish* (Pickering, A.D., ed.), pp. 49-75. London: Academic Press.

Mazeaud, M.M., Mazeaud, F. and Donaldson, E.M. (1977). Primary and secondary effects of stress in fish: Some new data with a general review. *Trans. Amer. Fish. Soc.* **106**, 201-212.

Meurs, H., Van Den Bogaard, W., Kauffman, H.K. and Bruynzeel, P.L.B. (1982). Characterization of (-)-[^{3}H]-dihydroalprenolol binding to intact and broken cell preparations of human peripheral blood lymphocytes. *Eur. J. Pharmacol.* **85**, 185-194.

Middeke, M., Remien, J., Block, L.H., Kirzinger, S., Landrock, A. and Holzgreve, H. (1983). $Beta_2$-adrenoreceptor density on membranes and on intact mononuclear cells in essential hypertension. *Res. Exp. Med.* **183**, 227-232.

Milligan, C.L., M.S. Graham, M.S. and Farrell, A.P. (1989). The response of trout red cells to adrenaline during seasonal acclimation and changes in temperature. *J. Fish. Biol.* **35**, 229-236.

Minard, F.N. and Grant, D.S. (1972). A convenient method for the chromatographic anlaysis of norepinephrine, dopamine and serotonin. *Biochem. Med.* **6**, 46-52.

Molenaar, P., McPherson, G.A., Malta., E. and Raper, C. (1985). Comparison of β-adrenoreceptor populations in cat and guinea pig left atria. *J. Pharm. Pharmacol.* **37**, 257-260.

Mommsen, T.P., Walsh, P.J., Perry, S.F. and Moon, T.W. (1988). Interactive effects of catecholamines and hypercapnia on glucose production in isolated trout hepatocytes. *Gen. Comp. Endocrinol.* **70**, 63-73.

Moon, T.W., Walsh, P.J. and Mommsen, T.P. (1985). Fish hepatocytes: a model metabolic system. *Can. J. Fish. Aquatic. Sci.* **42**, 1772-1782.

Nekvasil, N.P. and Olson, K.R. (1986a). Plasma clearance, metabolism, and tissue accumulation of ^{3}H-labeled catecholamines in trout. *Am. J. Physiol.* **250**, R519-R525.

Nekvasil, N.P. and Olson, K.R. (1986b). Extraction and metabolism of circulating catecholamines by the trout gill. *Am. J. Physiol.* **250**, R526-R531.

Nilsson, S. (1983). *Autonomic Nerve Function In the Vertebrates, Zoophysiology, Vol XIII.* New York, Berlin: Springer-Verlag.

Neumann, P., Holeton, G.F. and Heisler, N. (1983). Cardiac output and regional blood flow in gills and muscles after exhaustive exercise in rainbow trout (*Salmo gairdneri*). *J. exp. Biol.* **105**, 1-14.

Nikinmaa, M., Cech Jr., J.J. and McEnroe, M. (1984). Blood oxygen transport in stressed striped bass (*Morone saxatilis*): role of beta-adrenergic responses. *J. Comp. Physiol. B.* **154**, 365-369.

Nilsson, S. and Grove, G.D. (1974). Adrenergic and cholinergic innervation of the spleen of the cod: *Gadus morhua. Eur. J. Pharmacol.* **28**, 135-143.

Nonnotte, G., Maxine, V., Truchot, J.P., Williot, P. and Peyraud, C. (1993). Respiratory responses to ambient hypoxia in the sturgeon, *Acipenser baeri. Resp. Physiol.* **91**, 71-82.

Ottolenghi, C., Puviani, A.C., Baruffaldi, A. and Brighenti, L. (1984). Epinephrine effects on carbohydrate metabolism in catfish, *Ictalurus melas. Gen. Comp. Endocrinol.* **55**, 378-386.

Pearson, M.P. and Stevens, E.D. (1991) Size and hematological impact of the splenic resevoir in rainbow trout, *Oncorhynchus mykiss. Fish Physiol. Biochem.* **9**, 39-50.

Perry, S.F. and Vermette, M.G. (1987). The effects of prolonged epinephrine infusion on the physiology of the rainbow trout, *Salmo gairdneri*. I. Blood respiratory, acid-base and ionic states. *J. Exp. Biol.* **128**, 235-253.

Perry, S.F. and Kinkead, R. (1989). The role of catecholamines in regulating arterial oxygen content during acute hypercapnic acidosis in rainbow trout (*Salmo gairdneri*). *Resp. Physiol.* **77**, 365-378.

Perry, S.F. and Reid, S.D. (1992). Relationship between blood O_2 content and catecholamine levels during hypoxia in rainbow trout and American eel. *Am. J. Physiol.* **262**, R240-R249.

Perry, S.F., Daxboeck, C. and Dobson, G.P. (1985). The effect of perfusion flow rate and adrenergic stimulation on oxygen transfer in the isolated, saline-perfused head of rainbow trout (*Salmo gairdneri*). *J. Exp. Biol.* **116**, 251-269.

Perry, S.F., Malone, S. and Ewing, D. (1987). Hypercapnic acidosis in the rainbow trout (*Salmo gairdneri*). I. Branchial ionic fluxes and blood acid-base status. Can. J. Zool. **65**, 888-895.

Perry, S.F., Kinkead, R., Gallaugher, P. and Randall, D.J. (1989). Evidence that hypoxemia promotes catecholamine release during hypercapnic acidosis in rainbow trout, *Resp. Physiol.* **77**, 351-364.

Pettersson, K. and Nilsson, S. (1980). Drug induced changes in cardio-vascular parameters in the Atlantic cod, *Gadus morhua*. *J. Comp. Physiol.* **137**, 131-138.

Peyraud-Waitzenegger, M. (1979). Simultaneous modifications of ventilation and arterial PO_2 by catecholamines in the eel, *Anguilla anguilla* L.: Participation of α and β effects. *J. Comp. Physiol.* **129**, 343-354.

Plotsky, P.M., Cunningham, E.T. and Widmaier, E.P. (1989). Catecholaminergic modulation of corticotropin-releasing factor and adrenocorticotropin secretion. *Endocrine Rev.* **10**, 437-458.

Porzig, H. (1982). Are there differences in the β-receptor-adenylate cyclase systems of fragmented membranes and living cells? *Trends Pharmacol. Sci.* **3**, 75-78.

Pottinger, T.G. and Pickering, A.D. (1992). The influence of social interaction on the acclimation of rainbow trout, *Oncorhynchus mykiss* (Walbaum) to chronic stress. *J. Fish. Biol.* **41**, 435-447.

Primmett, D.R.N., Randall, D.J., Mazeaud, M. and Boutilier, R.G. (1986). The role of catecholamines in erythrocyte pH regulation and oxygen transport in rainbow trout (*Salmo gairdneri*) during exercise. *J. Exp. Biol.* **122**, 139-148.

Randall, D.J. and Smith, J.C. (1967). The regulation of cardiac activity in fish in a hypoxic environment. *Physiol. Zool.* **39**, 104-113.

Randall, D.J. and Stevens, E.D. (1967). The role of adrenergic receptors in cardiovascular changes associated with exercise in salmon. *Comp. Biochem. Physiol.* **21**, 415-424.

Randall, D.J. and Perry S.F. (1992). Catecholamines. In *Fish Physiology, Vol. XIIB.* (eds. W.S. Hoar, D.J. Randall, and A.P. Farrell), pp. 255-300. New York: Academic Press.

Redding, M.J., Patino, R. and Schreck, C.B. (1984). Clearance of corticosteroids in yearling coho salmon, *Oncorhynchus kisutch*, in fresh water and seawater and after stress. *Gen. Comp. Endocrinol.* **54**, 433–443.

Redding, M.J., DeLuze, A., Leloup–Hatey, J. and J. Leloup (1986). Suppression of plasma thyroid hormone concentrations by cortisol in the European eel *Anguilla anguilla*. *Comp. Biochem. Physiol. A.* **83**, 409–413.

Reid, S.D., Moon, T.W. and Perry, S.F. (1991). Characterization of β–adrenoreceptors of rainbow trout (*Oncorhynchus mykiss*) erythrocytes. *J. exp. Physiol.* **158**, 199–216.

Reid, S.D., Moon, T.W. and Perry, S.F. (1992). Rainbow trout hepatocyte β–adrenoreceptors, catecholamine responsiveness, and effects of cortisol. *Am. J. Physiol.* **262**, R794–R799.

Reid, S.D., Le Bras, Y. and Perry, S.F. (1993). The *in vitro* effect of hypoxia on the trout erythrocyte β–adrenergic signal transduction system. *J. exp. Biol.* **176**, 103–116.

Ristori, M.T. and Laurent, P. (1985). Plasma catecholamines and glucose during moderate exercise in the trout: Comparison with bursts of violent activity. *Exp. Biol.* **44**, 247–253.

Roth, R.A. (1982). Flow dependence of norepinephrine extraction by isolated perfused lungs. *Am. J. Physiol.* **242**, H844–H848.

Sladeczek, F., Bockaert, J. and Mauger, J–P. (1983). Differences between agonist and antagonist binding to $Alpha_1$–adrenergic receptors of intact and broken–cell preparations. *Mol. Pharmacol.* **24**, 392–397.

Smith, D.G., Berger, P. and Evans, B.K. (1981). Baroreceptor control of heart rate in the conscious toad, *Bufo marinus*. *Am. J. Physiol.* **241**, R307–R311.

Smith, F.M. and Davie, P.S. (1984). Effects of sectioning cranial nerves IX and X on the cardiac response to hypoxia in the coho salmon, *Oncorhynchus kisutch*. *Can. J. Zool.* **62**, 766–768.

Smith, L.S. and Bell, G.R. (1964). A technique for prolonged blood sampling in free–swimming salmon. *J. Fish. Res. Bd. Can.* **21**, 1775–1790.

Staehelin, M. and Hertel, C. (1983). [^{3}H] CGP–12177, a β–adrenergic ligand suitable for measuring cell surface receptors. *J. Recep. Res.* **3**, 35–43.

Steffensen, J.F. and Lomholt, J.P. (1992). The secondary vascular system. In *Fish Physiology* Vol. XIIA (eds. Hoar, D.J. Randall, and A.P. Farrell. eds.), pp. 185–217. London: Academic Press.

Steffensen, J.F. and Farrell, A.P. (1993). Cardiac and swimming performance of coronary-ligated rainbow trout, *Oncorhynchus mykiss*, exposed to hypoxia. *J. Comp. Physiol.*

Stevens, E.D. and Randall, D.J. (1967). Changes in blood pressure, heart rate, and breathing rate during moderate swimming activity in the rainbow trout. *J. Exp. Biol.* **46**, 307-315.

Stuart, R.E., Hedtke, J.L. and Weber, L.J. (1983). Physiological and pharmacological investigation of the nonvascularized marine teleost heart with adrenergic and cholinergic agents. *Can. J. Zool.* **61**, 1944-1948.

Su, Ying-Fu, Harden, T.K. and Perkins, J.P. (1980). Catecholamine-specific desensitization of adenylate cyclase. *J. Biol. Chem.* **255**, 7410-7419.

Tang, Y. and Boutilier, R.G. (1988). Correlation between catecholamine release and degree of acidotic stress in trout. *Am. J. Physiol.* **255**, R395-R399.

Tang, Y., Nolan, S. and Boutilier, R.G. (1988). Acid-base regulation following acute acidosis in sea-water adapted rainbow trout, *Salmo gairdneri*: A possible role for catecholamines. *J. Exp. Biol.* **134**, 297-312.

Tetens, V., Lykkeboe, G. and Christensen, N.J. (1988). Potency of adrenaline and noradrenaline for β-adrenergic proton extrusion from red cells of rainbow trout, *Salmo gairdneri*. *J. exp. Biol.* **134**, 267-280.

Tibbits, G.F., Kashihara, H., Thomas, M.J., Keen, J. and Farrell, A.P. (1990). Ca^{2+} transport in myocardial sarcolemma from rainbow trout. *Am. J. Physiol.* **259**, R453-R460.

Trendelenburg, U. (1988). The extraneural uptake and metabolism of catecholamines. In *Handbook of Experimental Pharmacology 90, Catecholamines I.* (ed. U. Trendelenburg and N. Weiner), pp. 279-319. New York, Berlin: Springer-Verlag.

Ungell, A-L., and Nilsson, S. (1979). Metabolic degradation of [^{3}H]-adrenaline in the Atlantic Cod, *Gadus Morhua. Comp. Biochem. Physiol.* **64C**, 137-141.

Ungell A-L., and Nilsson, S. (1983). Catabolism and excretion of [^{3}H]-adrenaline in the spiny dogfish, *Squalis acanthias. Comp. Biochem. Physiol.* **74C**, 319-322.

Vatner, D.E., Vatner, S.F., Nejima, J., Uemura, N., Sussanni, E.E., Hintze, T.H. and Homcy, C.J. (1989). Chronic epinephrine elicits desensitization by uncoupling the β-receptor. *J. Clin. Invest.* **84**, 1741-1748.

Vermette, M.G. and Perry, S.F. (1988). Effects of prolonged epinephrine infusion on blood respiratory and acid-base states in the rainbow trout: Alpha and beta effects. *Fish. Physiol. Biochem.* **4**, 189-202.

Voelkel, N.F., Hogstrand, L., Reeves, J.T., McCurty, I.F. and Molinoff, P.B. (1981). Effects of hypoxia on density of β-adrenergic receptors. *J. Appl. Physiol.* **50**, 363-366.

Voisin, P.J., Girault, J.M., Labouesse, J. and Viratelle, O.M. (1987). β-adrenergic receptors of cerebellar astrocytes in culture: intact cells versus membrane preparation. *Brain. Res.* **404**, 65-79.

Watson-Wright, W.M., Armour, J.A., Johnstone, D.E. and Wilkinson, M.W. (1989). Myocardial slice: a physiological approach to β-adrenergic ([3H] CGP-12177) receptor binding in hamster and guinea pig heart. *J. Pharmacol. Meth.* **22**, 37-47.

Whitby, L.G., Axelrod, J., and Weil-Malherbe, H. (1961). The fate of ^{3}H-norepinephrine in animals. *J. Pharmacol. Exp. Ther.* **132**, 193-201.

White, A. and Fletcher, T.C. (1985). The influence of hormone and inflammatory agents on C-reactive protein, cortisol and alanine aminotransferase in the plaice (*Pleuronectes platessa* L.). *Comp. Biochem. Physiol.* **80C**, 99-104.

Wilkinson, M., Macdonald, M.C., Landymore, K. and Wilkinson, D. (1991). Opioid receptors in micropunches of fresh hypothalmic tissue: a binding assay for everyman. *Neuroendo. Res. Methods* **2**, 723-748.

Wood, C.M. (1968). The effect of temperature on the responses to hypoxia of the rainbow trout (*Salmo gairdneri*). B.Sc. Thesis, Department of Zoology, University of British Columbia, B.C.

Wood, C.M. and Shelton, G. (1980a). Cardiovascular dynamics and adrenergic responses of the rainbow trout *in vivo*. *J. exp. Biol.* **87**, 247-270.

Wood, C.M. and Shelton, G. (1980b). The reflex control of heart rate and cardiac output in the rainbow trout: interactive influences of hypoxia, haemorrhage, and systemic vascular tone. *J. exp. Biol.* **87**, 271-284.

Wood, C.M., Pieprzak, P. and Trott, J.N. (1979). The influence of temperature and anaemia on the adrenergic and cholinergic mechanisms controlling heart rate in the rainbow trout. *Can. J. Zool.* **57**, 2440-2447.

Woodward, C.C. and Strange, J.J. (1987). Physiological stress responses in wild and hatchery-reared rainbow trout. *Trans. Am. Fish. Soc.* **116**, 574-579.

Wright, P.A., Perry, S.F. and Moon, T.W. (1989). Regulation of hepatic gluconeogenesis and glycogenolysis by catecholamines in rainbow trout during environmental hypoxia. *J. Exp. Biol.* **147**, 169–188.

Zivin, J.A. and Waud, D.R. (1982). How to analyze binding, enzyme and uptake data: the simplest case, a single phase. *Life Sci.* **30**, 1407–1422.