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THE RAINBOW TROUT MUSCLE β_2-ADRENOCEPTOR SYSTEM: IMPACT OF β_2-AGONIST FEEDING

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ABSTRACT

The presence and functionality of β-adrenoceptors (β-ARs) were examined in red and white muscles of the rainbow trout, *Oncorhynchus mykiss*. Specific binding assays performed on red and white muscle membrane preparations revealed the presence of a single class of binding sites with similar affinities (K_d) in both muscles with significantly higher number of binding sites (B_{max}) in red compared with white muscle. Competition assays with various selective and non-selective β-adrenergic agonists and antagonists revealed an "atypical" β-AR pharmacology which could simply represent a non-mammalian β-AR classification or the presence of more than one β-AR subtype with similar affinities that could not be resolved with specific binding assays. Furthermore, the functionality of these receptors was assessed using adenylyl cyclase (ACase) activity. ACase/cAMP assays showed a dose-dependent increase in cAMP production in the presence of increasing concentrations of β_2-adrenergic agonists (β_2-AAs) in both red and white muscles with significantly higher basal production in the red compared with the white muscle. Moreover, this agonist-induced increase in cAMP production was blocked by the β-adrenergic antagonist propranolol (PROP). Forskolin (FSK), a direct ACase activator increased cAMP production by 7 to 14-fold above basal and approximately 3-fold above all β-AAs tested.

The impact of feeding β_2-AAs on rainbow trout red and white muscles, β_2-AR binding characteristics and mRNA expression was assessed in parallel with fractional protein synthetic rates. Feeding 40 ppm of β_2-AAs for 30-37 days had no significant effect or apparent anabolic effect on any body or physiological parameters measured. There were no significant differences in the numbers of binding sites (B_{max}), but a significant change in the affinity (K_d) in β-ARs of red and white muscle membrane isolated from β_2-AA-fed trout. These changes in affinity may reflect a change or modulation in red and white muscle β-AR subtype; however, this could not be confirmed. Similarly, no change in β_2-AR mRNA levels was observed with β_2-AA feeding. However, β_2-AA treatments did significantly increase red and white muscle fractional protein synthesis rates in whole protein, myofibrillar protein and sarcoplasmic soluble protein fractions.

This study was successful in showing the presence of functional β-ARs coupled to ACase in red and white muscles of the rainbow trout. However, no changes in β-AR numbers or mRNA levels were observed with β_2-AA treatment, but significant changes in affinity, and more importantly increases in fractional protein synthesis rates, provide evidence for the importance of β-ARs in red and white muscles of the rainbow trout.

RÉSUMÉ

La présence et la fonctionnalité des récepteurs β-adrénergiques (β-AR) ont été examinées dans le muscle rouge et blanc de la truite arc-en-ciel, *Oncorhynchus mykiss*. Les analyses d'interactions spécifiques ligand-récepteur exécutées sur des préparations de membranes plasmiques provenant de muscles rouges et blancs ont indiqué la présence d'une seule classe de récepteurs avec une affinité (K_d) semblable dans les deux muscles contenant un nombre sensiblement plus élevé de récepteurs (B_{max}) dans le muscle rouge comparativement au muscle blanc. Des analyses de compétitions ligand-radioligand effectuées à l'aide de divers agonistes et antagonistes sélectifs et non-sélectifs pour certains sous-type de β-AR indiquent une pharmacologie "atypique" comparativement à celle obtenue chez les mammifères. Ceci pourrait représenter soit un sous-type de β-AR "atypique" ou possiblement la présence de plusieurs sous-types de β-ARs avec des affinités semblables qui ne pourraient pas être résolues

avec des analyses d'interactions spécifiques ligand-récepteur. De plus, la fonctionnalité de ces récepteurs a été évaluée en mesurant l'activité de l'adénylyl-cyclase (ACase) par la production d'AMPc. À l'aide de concentration augmentant d'agonistes spécifiques aux β_2-AR (β_2-AA), l'ACase/AMPc a démontré une activité croissante en fonction de la dose de β_2-AA dans les muscles rouges et blancs avec une production basale sensiblement plus élevée dans le muscle rouge comparativement au muscle blanc. D'ailleurs, cette augmentation de la production induite par l'agoniste a été bloquée à l'aide d'un antagoniste aux β-ARs, le propranolol (PROP). Une drogue activant directement l'Acase, le forskolin (FSK) a lui aussi augmenté la production d'AMPc, de 7 à 14 fois comparativement à la production basale et approximativement 3 fois plus que la production obtenue à l'aide des β_2-AAs utilisés.

De plus, une évaluation de l'impact d'une administration orale de β_2-AA sur les caractéristiques de liage aux β-ARs, l'expression d'ARNm des β-ARs en parallèle avec une mesure du taux fractionnel de la synthèse protéique dans les muscles rouges et blancs de la truite arc-en-ciel a été entreprise. L'alimentation de 40 ppm de β_2-AAs par jours pendant une période de 30 à 37 jours n'a eu aucun effet anabolisant significatif au niveau des paramètres corporels et physiologiques mesurés ou calculés. Il n'y a eu aucun impact sur le nombre de β-AR (B_{max}), mais un changement subtil a été observé au niveau de l'affinité (K_d) des β-ARs dans les préparations de membranes plasmiques provenant de muscles rouges et blancs isolés de truites nourrient aux β_2-AAs. Ces changements d'affinité peuvent refléter un changement ou une modulation de sous-type de β-ARs dans le muscle rouge et blanc; cependant, ceci n'a pas pu être confirmé. De même, aucun changement des niveaux d'ARNm de β_2-ARs suivant l'administration orale de β_2-AAs n'a été observé. Cependant, les traitements de β_3-AAs ont augmenté de façon significative le taux fractionnel de la synthèse protéique du muscle rouge et

blanc dans les fractions protéiques contenant la totalité des protéines, les protéine myofibrillères et les protéines solubles retrouvées dans le réticulum sarcoplasmique.

Cette étude a réussi à démonter la présence de β-AR fonctionnels couplés à l'ACase dans le muscle rouge et blanc de la truite arc-en-ciel. Cependant, aucun changement au niveau du nombre de β-ARs ou au niveau de l'expression de l'ARNm de β_2-ARs n'a été observé suite à l'administration d'une dose orale de β_2-AA, par contre, des changements subtils au niveau de l'affinité des β-ARs ainsi qu'une augmentation des taux fractionnels de synthèse protéique fournissent une indication de l'importance de la présence et du rôle joué par les β-ARs dans le muscle rouge et le muscle blancs de la truite arc-en-ciel.

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Abbreviations	Full Name
$[AA]_{Plasma}$	Plasma amino acid concentration
$[CLEN]_{Plasma}$	Plasma clenbuterol concentration
$[Gluc]_{Plasma}$	Plasma glucose concentration
$[Prot]_{Plasma}$	Plasma protein concentration
^{125}I-CYP	(-)-^{125}I-cyanopindolol
^{3}H-CGP	(±)-^{3}H-CGP 12177
^{3}H-DHA	(-)-^{3}H-dihydroalprenolol
^{3}H-Phe	L-[2,6-^{3}H]-Phenylalanine
α-AR	α-Adrenoceptor
α_1-AR	α_1-Adrenoceptor
α_2-AR	α_2-Adrenoceptor
ACase	Adenylate cyclase
ADR	(-)-Adrenaline
AR	Adrenoceptor
ATL	Atenolol
BAAM	Bromoacetyl alprenolol methane
β-AR	β-Adrenoceptor
βARK	β Adrenergic receptor kinase
β_1-AR	β_1-Adrenoceptor
β_2-AR	β_2-Adrenoceptor
β_2-AA	β_2-Adrenergic agonist
β_3-AR	β_3-Adrenoceptor
BCA	Bicinchoninic acid
B_{max}	Maximum number of binding sites
β-PEA	β-Phenyethylamine
BRL	BRL 37,334
BSA	Bovine serum albumin

BW	Body Weight
BW_f	Final body weight
BW_i	Initial body weight
CA	Catecholamine
cAMP	Cyclic adenosine monphosphate
CF	Condition Factor
CF_f	Final condition factor
CF_i	Initial condition factor
CGP	(±)-CGP 12177A
CL	CL 316,243
CLEN	Clenbuterol
COMT	Catechol-*o*-methyl transferase
CSI	Cardio-somatic index
DAG	diacylglycerol
DNA	Deoxyribonucleic acid
DOB	(±)-Dobutamine
DTT	DL-Dithiothreitol
EIA	Enzyme Imuno-Assay
FP	Free pool
FSK	Forskolin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
G6PDH	Glucose 6'-phosphate dehydrogenase
GH	Growth hormone
GIT	Gastrointestinal tract
G-proein	Guanine nucleotide binding protein
G_i-protein	G-protein involved in the α_2-AR signal transduction pathway
G_q-protein	G-protein involved in the α_1-AR signal transduction pathway
G_s-protein	G-protein involved in the β-AR signal transduction pathway
GPCR	G-Protein coupled receptor
Hk	Hexokinase
HSI	Hepato-somatic index
ICI	(±)-ICI 118,551

IGF-I	Insulin-like growth factor-I
IP_3	inositol-1,4,5-trisphosphate
ISO	Isoproterenol
K_d	Affinity (Concentration at 50% of maximum number of binding sites)
K_i	Displacement affinity (Concentration displacing 50% of specific binding)
k_s	Rate of fractional protein synthesis
L	Length
L_f	Final length
L_i	Initial length
MAO	Monoamine oxidase
MitoP	Mitochondrial protein
mRNA	Messenger ribonucleic acid
MyofP	Myofibrillar protein
NOR	(-)-Noradrenaline
NSB	Non-specific binding
PCA	Perchloric acid
PHL	Phenylephrine
PHT	Phentolamine
PKA	Protein kinase A
PKC	Protein kinase C
PLC β	Phospholipase C β
PMSF	Phenylmethyl-sulfonyl fluoride
post-MitoP	Post-mitochondrial protein
PROC	Procaterol
PROP	Propranolol
RACT	Ractopamine
RBC	Red blood cell
RM	Red muscle
RPA	RNase protection assay
S1	Supernatant 1
S2	Supernatant 2
S3	Supernatant 3

S4	Supernatant 4
SB	Specific binding
SGR	Specific growth rate
SGR_{exp}	Specific growth rate exponential equation
SGR_{lin}	Specific growth rate linear equation
SSP	Sarcoplasmic soluble protein
T_4	Thyroxine
TB	Total binding
TIM	Timolol
W_{Heart}	Heart weight
W_{Liver}	Liver weight
WM	White muscle
WP	Whole protein

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Michel, May 2002

CHAPTER 1. Introduction

1.1 Rationale

The aquaculture industry has undergone rapid worldwide expansion during the past 15 years and with it a need to optimize production and efficiency has arisen as in any other traditional livestock industry (Vandenberg and Moccia, 1998). Recently a variety of approaches involving manipulation of the endocrine system to influence growth and body composition have been studied. Amongst these manipulations, selective β_2-adrenergic agonists (β_2-AAs) such as clenbuterol (CLEN) and ractopamine (RACT) have been shown to enhance muscle growth (protein accretion) and reduce lipid deposition in animals of agricultural and economical importance (for reviews see Yang and McElligott, 1989; Kim and Sainz, 1991; Reeds and Mersmann, 1991; Beerman, 1993, 2002; Mersmann, 1998; Moody *et al.*, 2000; Mommsen and Moon, 2001; Mills, 2001; 2002), including modest responses in teleost fish (Mustin and Lovell, 1993; Webster *et al.*, 1995; Vandenberg and Moccia, 1998; Vandenberg *et al.*, 1998). In mammalian muscle, there is now convincing evidence that the effects of β_2-AAs are mediated directly on the target tissues through β_2-adrenoceptors (β_2-ARs) and the intricate details of the cellular signaling pathway involved are being investigated, including cAMP, protein phosphorylation (via protein kinase A), activation of cAMP responsive elements (CRE) by cAMP response element binding protein (CREB) (Moody *et al.*, 2000; Beermann, 2002; Mills, 2002). However, the current lack of data on the teleost muscle β-adrenergic system, presence of a muscle cell β-AR, functional subsequent transduction pathway from the receptor, and specific mechanisms of action altering protein accretion, makes the use of such agonists in aquaculture problematic. To date, ractopamine (RACT) has been approved for use in pigs by the Food and Drug Administration (USA) and is being marketed as Paylean® by Elanco

Animal Health products; and Zilpaterol, manufactured by Hoechst Roussel Vet is being sold in South Africa and Mexico (Moody *et al.*, 2000). However, before any of these agonists are even considered for use in fish, further efforts are required to remedy the lack of mechanistic and basic fish muscle β-adrenergic system knowledge.

1.2. Hypothesis

This study has two main hypotheses, which are tested in Chapter 2 and 3, respectively.

H1: Rainbow trout red and white muscles have β_2-ARs and upon β_2-AA binding, a functional transduction cascade will result in the increased production of the second messenger cAMP (Chapter 2).

H2: Feeding rainbow trout β_2-AAs, CLEN and RACT, will enhance muscle growth by increasing muscle protein synthesis. However, such sustained stimulation of β_2-ARs by β_2-AAs will cause down-regulation of the receptors and decrease β_2-AR mRNA expression (Chapter 3).

1.3 Objectives

To test the two main hypotheses above, this study will attempt to attain the following objectives.

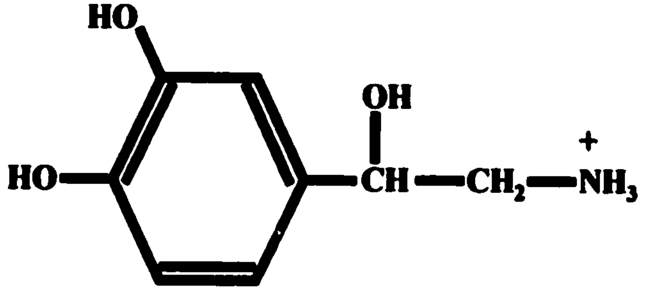
O1: The main objectives of Chapter 2 are to 1) show the presence of β-ARs in red and white muscles of the rainbow trout; 2) pharmacologically determine the subtype(s) of β-AR; and, 3) demonstrate the causative association between β-AA binding and activation of the subsequent transduction pathway.

O2: The main objectives of Chapter 3 are to determine the impact of feeding rainbow trout β_2-AAs on 1) muscle β_2-ARs binding characteristics and 2) muscle β_2-AR mRNA expression, and 3) muscle fractional protein synthesis rates.

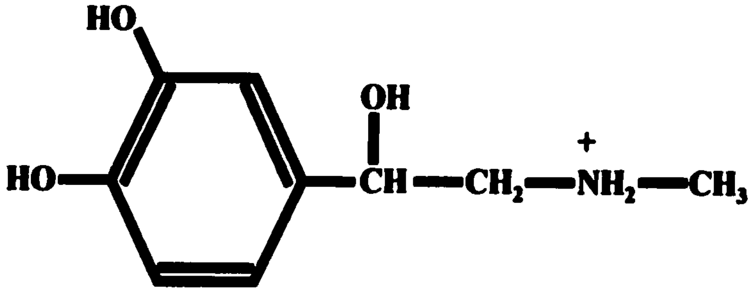
1.4 Overview of the Adrenergic System

1.4.1 Catecholamine Hormones

In vertebrates, including teleost fish, the circulating catecholamine (CA) hormones, noradrenaline (NOR; Fig. 1.1A) and adrenaline (ADR; Fig. 1.1A), are synthesized via the Blaschko pathway and stored in membrane-bound granules. NOR is mainly a neurotransmitter released by adrenergic neurons and can circulate in the blood as neuronal overflow, whereas ADR is released from chromaffin cells upon preganglionic, autonomic neural stimuli (Randall and Perry, 1992; Epple, 1993; Reid *et al.*, 1998). A wide variety of acute and chronic, external and internal factors, including anticipation of stressful activity, can elicit the release of CAs in fish (Wendelaar Bonga, 1997; Reid *et al.*, 1998; Fabbri *et al.*, 1998). However, response to CAs in specific tissues requires the presence of a receptor, binding of the CA to the receptor, as well as the presence of a functional transduction pathway. Almost all fish tissues are potentially able to remove CAs from the blood as convincing evidence shows a highly conserved adrenergic system, as most vertebrates possess these receptors (Fabbri and Moon, 1994). Therefore it is not surprising that by altering levels of CAs, fish modulate numerous physiological processes including the metabolism of carbohydrates, lipids and protein (for reviews see Sheridan, 1994; Fabbri and Moon, 1994; Fabbri *et al.*, 1998). The two hormones can be metabolized in most tissue by the degrading enzymes monamine oxidase (MAO) and catechol-*o*-methyl transferase (COMT); these will be discussed below (see section 1.5.1.2).



A) NORADRENALINE



B) ADRENALINE

Figure 1.1: Diagrammatic representation of the chemical structures of the endogenous circulating catecholamine hormones, noradrenaline (A) and adrenaline (B).

1.4.2 Catecholamine Hormone Receptors and Transduction Pathway

1.4.2.1 Adrenoceptors

CAs exert their numerous effects on target cells by binding to membrane receptor proteins called adrenoceptors (ARs; Alquist, 1948). Much knowledge has been gathered over the years of intensive research in this field. This work has been extensively reviewed including the fact that ARs belong to the superfamily of seven membrane spanning domain proteins, which are a subset of the large family of guanine nucleotide binding protein (G-protein) coupled receptors (GPCR) (Strosberg, 1996). These receptors consist of > 400 amino acids in a continuous chain. Models indicate seven hydrophobic transmembrane domains that anchor the receptor in the plasma membrane. The amino terminus and three loops connecting adjacent transmembrane domains are extracellular while the carboxy terminus and three loops connecting adjacent transmembrane domains are intracellular. Mammalian ARs are classified into α_1- (A, B and C), α_2- (A, B, C and D) and β-AR, which are further subdivided into β_1, β_2 and β_3 according to their pharmacological and molecular properties (Strosberg, 1993; Hieble *et al.*, 1995; Heible and Ruffolo, 1995; Kaumann and Molenaar, 1997). A phylogenetic tree has even been established for the mammalian ARs and their signaling transduction pathway (Fryxell, 1995).

The signaling pathway is grouped in three different and distinct categories, where all the β-AR subtypes share a common pathway and the α_1-AR and α_2-AR are distinct. A general representation of the signaling pathways is given for a liver cell (Fig. 1.2); upon agonist binding, the β-AR stimulates adenylyl cyclase (ACase) activity by coupling to G_s, the α_2-AR inhibits ACase by coupling to G_i and the α_1-AR stimulates phospholipase C (PLC β) by coupling to G_q. ACase converts ATP (not shown) into cyclic adenosine monophosphate

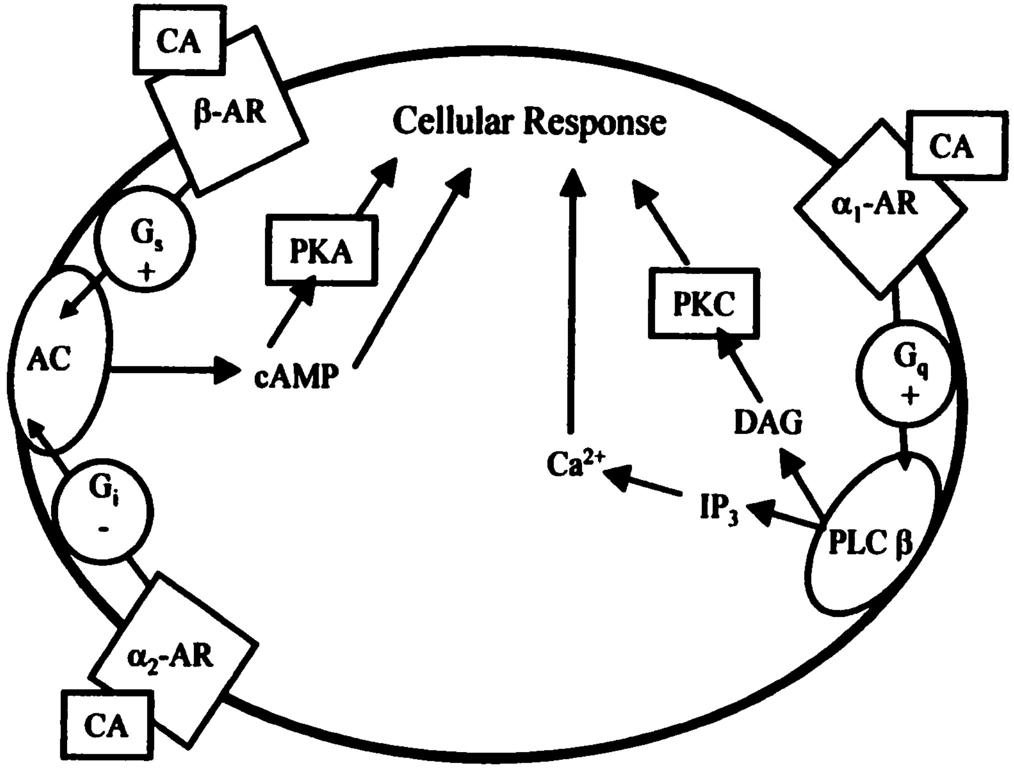


Figure 1.2: Diagrammatic representation of the general view of the signal transduction pathway of catecholamine (CA) binding to α- and β-adrenoceptors (α- and β-ARs) on a liver cell (modified from Fabbri *et al.*, 1998). Arrows indicate the direction of the signaling pathway and within the different G-proteins is indicated a positive (+) or a negative (-) effect on adenylyl cyclase (AC) or phospholipase β (PLC β). Additional abbreviations: cAMP, cyclic adenosine monophosphate; IP_3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PKA, protein kinase A; PKC, protein kinase C.

(cAMP) which in turn binds to the regulatory protein kinase A (PKA) subunit freeing the catalytic subunits to modulate various cellular responses by phosphorylation events. PLC β converts phosphatidylinositol-4phosphate (not shown) into diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3), DAG directly activates protein kinase C (PKC), while IP_3 increases intracellular Ca^{2+} ion levels to further activate PKC. PKC, as with PKA, modulates various cellular responses by phosphorylation (Fabbri *et al.*, 1998).

1.4.2.2 β-Adrenoceptors in Mammals

The family of β-AR includes three subtypes, β_1, β_2 and β_3. These subtypes have been cloned from a number of mammalian species (Table 1.1) and share approximately 30-50% sequence homology. Sequence homology is higher for the same subtype across species and is approximately 80-90%, than homology across subtypes. Sequence differences provide for variation in affinity for various ligands, for receptor activation and possibly signal (Moody *et al.*, 2000). The pharmacology of β-AR subtypes exhibit ligand selectivity such that the rank order of affinity for NOR is β_1-AR > β_2-AR > β_3-AR, while that for ADR is β_1-AR ≈ β_2-AR > β_3-AR, but ADR has a greater affinity for the β_2-AR than does NOR (Mills, 2001). These receptors have also been pharmacologically characterized using selective and non-selective agonists and antagonists in numerous species and various tissues. Tissue subtype distribution and proportion greatly varies across mammalian species, sex of the animals, age and season. Within mammals in general, β_1-ARs are located mainly in the heart but are also found in platelets, the salivary glands, non-sphincter part of the gastrointestinal tract (GIT) and skeletal muscle; and the β_2-ARs are located on a number of tissues including blood vessels, bronchi, GIT, liver, and skeletal muscle; the β_3-ARs are found predominantly in white adipose and brown adipose tissues, the GIT and skeletal muscle (Roberts and Summers, 1998; Moody *et al.*, 2000; Mills, 2001; Beermann, 2002; Mills, 2002). The β_2-AR is by far the most thoroughly

Table 1.1: Accession numbers of various cloned and sequenced mammalian β-adrenoceptors (β-ARs).

	Accession Number		
Species	β_1-AR	β_2-AR	β_3-AR
Human (*Homo sapiens*)	J03019	J02960	X72861
Monkey (*Macaca mulatta*)	X75540	L38905	U63592
Mouse (*Mus musculus*)	L10084	X15643	X72862
Rat (*Rattus novergicus*)	J05561	X17607	S73473
Cattle (*Bos taurus*)	AF188187	Z86037	X85961
Pig (*Sus scrofa*)	AF042454	AF000134	U55858^{P}
Sheep (*Ovis aries*)	AF072433	-	-
Dog (*Canis familiaris*)	-	-	U92468

Table adapted from Moody et al. (2000). Accession numbers refer to GenBank nucleotide database (http://www.ncbi.nlm.nih.gov). Partial sequence indicated by ^{P} represents a 303bp fragment; all other sequences are full-length.

characterized β-AR subtype. Using computer modeling, site-directed mutagenesis and photoaffinity labeling (Strosberg, 1997; Summers *et al.*, 1997), different membrane-spanning domains of the β_2-AR have been implicated in receptor subtype ligand binding specificity to agonists and antagonists (Fig. 1.3). Some of the key features in Figure 1.3 will be discussed further as they pertain to receptor agonist-induced desensitization processes.

Interestingly and pertinent to the present study is the confirmed presence of all three β-AR subtypes in skeletal muscle of most mammals studied, but the precise reason for the presence of three different β-ARs is still controversial (Kim and Sainz, 1992). A study in rats by Roberts and Summers (1998) has shown increased cAMP production to be predominantly β_2-AR-mediated with small contributions by β_1-AR but no effect by β_3-AR.

1.4.2.3 β-Adrenoceptors in Fish

In fish, few radioligand binding studies, in very few species have been performed to date, so little is known about the tissue distribution of ARs (Fabbri *et al.*, 1998). The binding technique is considered the most appropriate for studying the number and affinity of receptors, but in non-mammalian organisms these studies are hindered by the lack of suitable pharmacological agents as all pharmacological agents to date have been developed specifically with application in mammals. Even in mammalian species other than rodents or humans, some discrepancies exist in ligand selectivity for different receptor subtypes. For example, ICI 188,551, a selective β_2-AR antagonist in rodents and humans, has similar affinity for pig β_1- and β_2-AR; similarly, BRL 37344, a selective β_3-AR agonist in rat, is selective for pig β_2-AR (Liang and Mills, 2001). In fish, similar discrepancies have also been reported where agonists behave as antagonists and even specific α-AR agonists and antagonists may interact with β-AR (Fabbri *et al.*, 1998). Whether a given selective ligand shows high or low affinity for a receptor or receptor subtype, and is able to signal through G-proteins, is highly dependent upon the AR

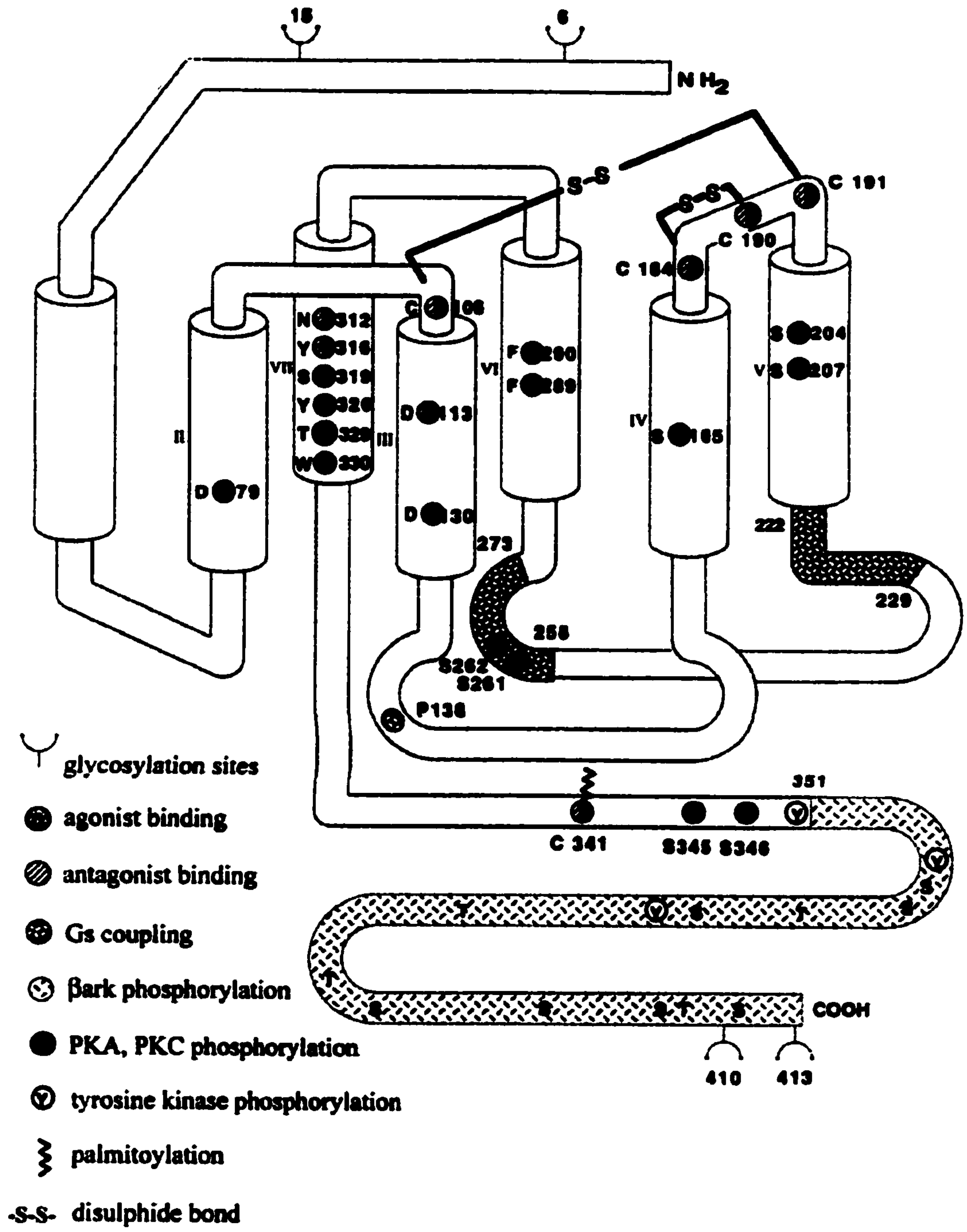


Figure 1.3: Diagramatic representation of the current model of the β_2-adrenoceptor showing major functional domains. Large diameter sections represent membrane-spanning domains (I-VII) with the outside of the cell, NH_2 head, and inside, the COOH tail. Amino acids are identified using the single letter convention and key amino acids are numbered. Figure from Summers *et al.* (1997).

amino acid sequence, which differs across mammalian species and even further in non-mammalian species (Fabbri *et al.*, 1998). The presence, binding characteristics and in some fish the subtype of β-ARs have been confirmed pharmacologically and reported mostly in liver, heart, red blood cell and a few additional tissues (Table 1.2). Additionally, molecular biology techniques have only recently begun to be utilized for the study of fish AR, so there are very few details regarding sequence homology (Fabbri *et al.*, 1998). To date, six sequences are accessible through GenBank (Table 1.3) and only one, the rainbow trout β_2-AR, is a complete coding sequence while the others are only partial sequences. Two other complete β-AR sequences from rainbow trout, the β_{3a}- and the β_{3b}-AR and partial sequences for the Atlantic salmon β_{3b}-AR, and the black bullhead catfish β_3-AR have also been obtained (J.G. Nickerson, pers. commun.). In addition, the recent publicly available puffer fish genome (Fugu genome database), permitted the confirmation of a β_2- and β_3-AR in this fish species. Similar findings are expected in the zebra fish, once the genome is more readily accessible. The subtype of each fish β-AR is based upon an amino acid blast search (GenBank), however some of these receptors, especially in the jawless fish do not group specifically with any particular β-AR subtype and are, therefore reported as general β-ARs.

1.5 Overview of the β_2-Adrenergic Agonists

1.5.1 β_2-Adrenergic Agonists

1.5.1.1 Structure and General Chemical Properties of β_2-Adrenergic Agonists

If a β_2-AA is to have β_2-AR biological activity, structurally (Fig. 1.4A) it must have a substituted six-member aromatic ring, a hydroxyl group bound to the *β*-carbon, positively charged nitrogen in the ethylamine side-chain and a bulky substituent (*R*) on the aliphatic nitrogen (Hieble *et al.*, 1995; Smith, 1998). The aromatic ring attached through the *β*-carbon is

Table 1.2: β-Adrenoceptors (β-ARs) characterized to date in various fish tissues.

Tissue	Species	Radioligand	K_d (nM)	B_{max} (fmol • mg protein^{-1})^a (sites • cell^{-1})^b	Subtype	Reference
Liver	Australian lungfish (*Neoceratodus forsteri*)	^{125}I-CYP	0.02 0.5	100^a (at 5nM)	β_2-AR^c	Janssens and Grigg, 1988
	Black bullhead catfish (*Ictalurus melas*)	^{3}H-DHA	2.04	46.7^a	-	Fabbri *et* al., 1992
		^{3}H-CGP	0.64	1.22^a	β_2-AR^d	Dugan, 2002 (unpublished data)
	Carp (*Cyprinus carpio*)	^{125}I-CYP	0.083	45^a (at 0.6 nM)	-	Janssens and Lowrey, 1987
	European eel (*Anguilla anguilla*)	^{3}H-CGP	1.31	7,000^b	-	Fabbri *et al.*, 2001
	Rainbow trout (*Oncorhynchus mykiss*)	^{3}H-CGP	55	1,696^b	β_2-AR^e	Reid *et al.*, 1992
		^{3}H-CGP	0.36	8.61^a	-	Fabbri *et al.*, 1995a
		^{3}H-CGP	0.26	14.0^a	-	Dugan and Moon, 1998

		^{3}H-CGP	0.29	-	β_2-AR^{f}	Nickerson *et al.*, 2001
Heart	Rainbow trout (*Oncorhynchus mykiss*)	^{125}I-CYP	0.13	1000^{b}	-	Keen *et al.*, 1993
		^{3}H-CGP	0.25	12,000^{b}	β_2-AR^{g}	Gamprell *et al.*, 1994
		^{3}H-CGP	0.16	24^{a}	-	Gamprell *et al.*, 1998
(compact myocardium)	Chinook salmon (*Oncorhynchus tshawytscha*)	^{3}H-CGP	0.25	50^{a}	-	Gamprell *et al.*, 1998
(spongy myocardium)		^{3}H-CGP	0.25	60^{a}	-	Gamprell *et al.*, 1998
Gill	Rainbow trout (*Oncorhynchus mykiss*)	^{3}H-CGP	0.11	38.26^{a}	-	Dugan, 2002 (unpublished data)
Red Blood Cell	European eel (*Anguilla anguilla*)	^{3}H-DHA	1.36	315^{a}	-	Bennett and Rankin, 1985
	Rainbow trout (*Oncorhynchus mykiss*)	^{3}H-CGP	4	1065^{b}	-	Gilmour *et al.*, 1994
		^{3}H-CGP	2.5	884^{b}	-	Reid and Perry, 1995

	Tilapia (*Oreochromis mossambicus*)	^{3}H-CGP	1.2	1,900[b]	-	Chang and Liao, 1994
White Muscle	Black bullhead catfish (*Ictalurus melas*)	^{3}H-CGP	17	18[a]	atypical β-AR[h]	Lortie and Moon, 1999 (unpublished data)
Enterocytes	Black bullhead catfish (*Ictalurus melas*)	^{3}H-CGP	9	12[a]	atypical β-AR[h]	Lortie and Moon, 1999 (unpublished data)

Tissue preparations are plasma membrane preparation for tissue B_{max} values indicated by [a] and whole cell or isolated cells for tissue B_{max} values indicated by [b]. Radioligand abbreviations, ^{3}H-CGP, ^{3}H-DHA and ^{125}I-CYP represent, ^{3}H-CGP 12177, ^{3}H-dihydroalprenolol and ^{125}I-cyanopindolol, respectively. Reported binding values are from control experimental conditions from the different studies. The subtypes are determined using: [c] [propranolol (PROP), isoproterenol (ISO), adrenaline (ADR), noradrenaline (NOR), phenylephrine (PHL), phentolamine (PHT)]; [d] [ICI 118,551 (ICI), bromoacetyl alprenolol methane (BAAM), alprenolol (ATL), CGP 12177 (CGP), PHT, procaterol (PROC), ISO, dobutamine (DOB), ADR, NOR]; [e] (ISO, ADR, NOR, PHL); [f] (ICI, BAAM, ATL, CGP, PROC, DOB, ADR, NOR); [g] (ICI, ATL, PHT, ALP, IOS, timolol (TIM), ADR, NOR, ISO); and [h] (ICI, BAAM, ATL, PHT, ISO, DOB, ADR, NOR).

Table 1.3: β-Adrenoceptors (β-ARs) partial or complete coding sequences reported to date in various fish species.

Species	Sequence name or subtype	Nucleotide Length (bp)	Accession Number	Reference
Atlantic hagfish (*Myxine glutinosa*)	hagfish β-AR	672^P	AJ005436	Vernier, 1998
Sea lamprey (*Petromyzon marinus*)	lamprey β-AR A	650^P	AJ005437	Vernier, 1998
	lamprey β-AR B	794^P	AJ005438	Vernier, 1998
Rainbow trout (*Oncorhynchus mykiss*)	β_2-AR	1230^c	AY044093	Nickerson *et al.*, 2001
	β_{3a}-AR	1287^c	-	Nickerson, upublished data
	β_{3b}-AR	1287^c	-	Nickerson, unpublished data
Atlantic salmon (*Salmo salar*)	β_{3b}-AR	750^P	-	Nickerson, unpublished data
Black bullhead catfish (*Ictalurus melas*)	β_3-AR	400^P	-	Nickerson, unpublished data

Channel catfish (*Ictalurus punctatus*)	β_2-AR	528^p	AF127775	Blackshaw and Snyder, 1999
	β_1-AR	172^p	AI431389	Blackshaw, 1999
Japanese puffer fish (*Fugu rubripes*)	β_2-AR	approx 1230^c	-	Fugu database
	β_3-AR	approx 1287^c	-	Fugu database

Complete coding sequence length indicated by ^{c} and partial coding sequence length is indicated by ^{p}. Accession numbers refer to the GenBank nucleotide database (http://www.ncbi.nlm.nih.gov). The puffer fish sequence were obtained by searching the Fugu database (http://fugu.hgmp.mrc.ac.uk) with the rainbow trout β_2-AR and β_{3a}-AR/β_{3b}-AR sequences, followed by a blast search on the GenBank data base to confirm receptor subtype similarity.

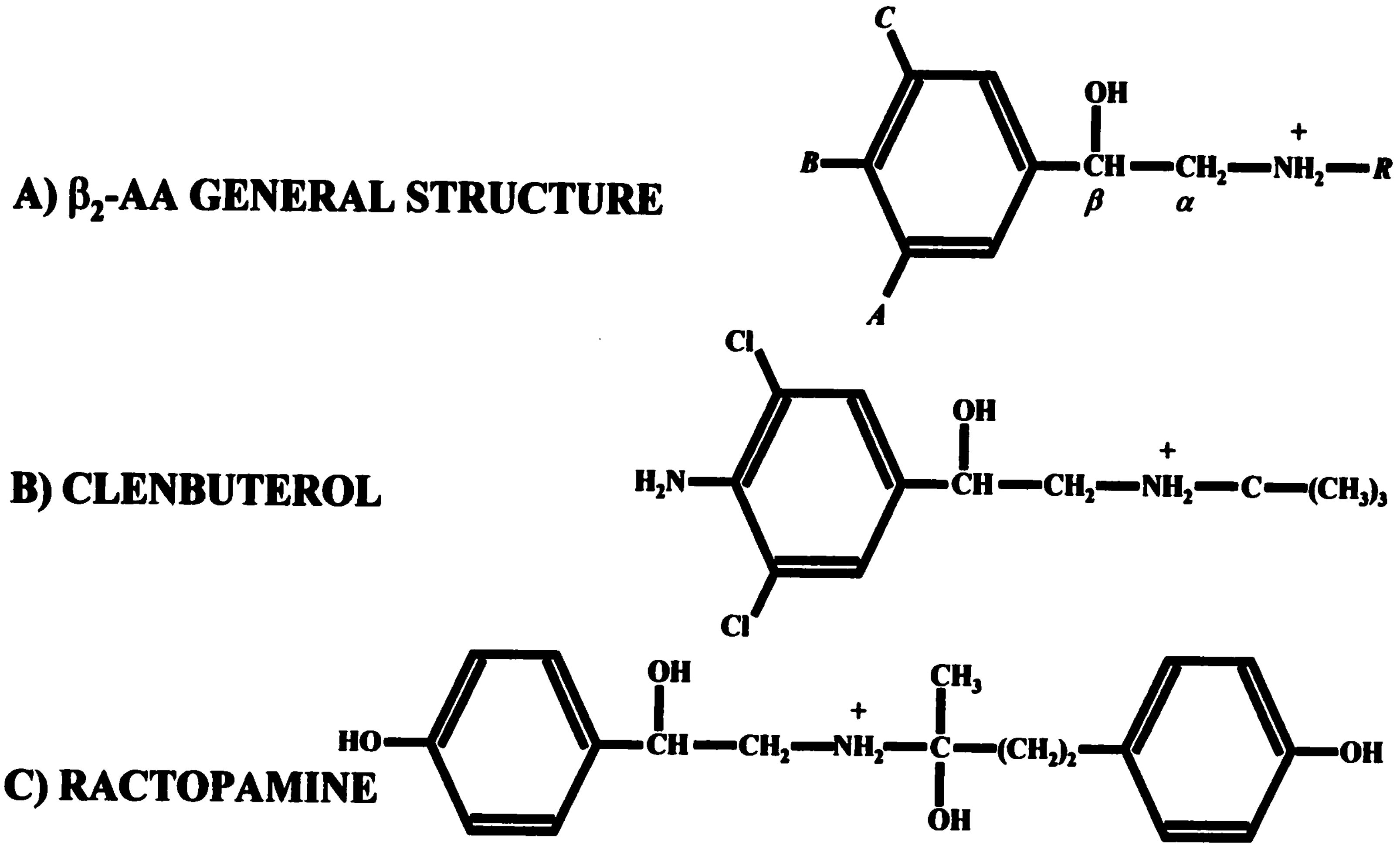


Figure 1.4: Diagrammatic representation of the general chemical structure of β_2-adrenergic agonists (β_2-AAs; A) and the chemical structures of the β_2-adrenergic agonists clenbuterol (B) and ractopamine (C) commonly used to enhance muscle growth.

essential for biological activity and is substituted with hydroxyl groups (as in RACT; Fig. 1.4C), halogens (as in CLEN; Fig. 1.4B), amines (as in CLEN; Fig. 1.4B), hydroxymethyl groups, cyano groups or various combinations of the above (Smith, 1998). The substitutions are important in dictating the longevity of the β_2-AA in tissues and the efficacy at the receptor level. For example, ADR (Fig. 1.1B), is rapidly deactivated by the enzyme COMT which is specific for the hydroxyl group in position *C*, neither RACT nor CLEN have a hydroxyl group at position *C* meaning neither are substrates for COMT thus preventing their deactivation. Finally, the *β*-carbon is chiral and therefore two stereoisomers exist, which at the receptor level is important since only one steroisomer is biologically active (Ruffolo, 1991; Smith, 1998).

1.5.1.2 Absorption, Elimination and Residues of β_2-Adrenergic Agonists

Peak plasma levels of β_2-AA generally occur within 1-3 h after oral administration in humans and livestock (Smith, 1998). For example plasma CLEN levels reached peak plasma concentration around 0.2 to 2.5 nM, 2 to 7 h following a single oral dose in cattle, while prolonged oral administration at the same dose resulted in doubling the plasma levels (CLEN, 5-10 ppm, Meyer and Rinke, 1991; Stoffel and Meyer, 1993). A similar study monitored CLEN levels throughout a 15 day treatment period; maximal plasma concentrations occurred after 10 days with further accumulation over time (Sauer *et al.*, 1995). While plasma levels of β_2-AAs are good evidence of absorption, it does not measure the extent of absorption as a portion of these β_2-AAs are excreted in the urine and accumulate in tissues (Beermann, 1993; Smith, 1998).

Studies in cattle using radioisotopically labeled CLEN (3 ppm; Smith and Paulson, 1997) showed rapid increases in blood reaching peak values within 1 h after the oral dose. By 48 h after dosing, less than 50% of the total dose administered had been excreted in the urine and only 2% was found in the feces. The remainder of the radioactivity accumulated in the carcass and intestinal tract. The absorption of clenbuterol was extensive as recovery of the radioactivity in urine and feces accounted for 76% of the total radioactivity administered. Similar results with other β_2-AAs in other mammalian species follow similar trends (see review by Smith, 1998).

Residues in tissues have also been studied in mammals, for obvious health hazards to human consuming meat from treated livestock and to assess proper withdrawal periods following treatment before the meat could be marketed. Pertinent to this study is one such assessment in rainbow trout tissues (Brambilla *et al.*, 1994); following a treatment of 5 ppm CLEN for 21 days, peak residues levels reached approximately 400 ppb in the liver, 70 ppb in the white muscle and 90 ppb in the skin. Thirty days following withdrawal, levels dropped to 25 ppb in the liver and 5 ppb in the white muscle and skin.

1.5.1.3 Repartitioning Effects of β_2-Adrenergic Agonists

β_2-AAs are often referred to as "repartitioning agents" because of their ability to re-direct nutrients away from adipose tissue and towards muscle (Moody *et al.*, 2000). In general reviews in the livestock literature on the subject (Beermann, 1993; Moody *et al.*, 2000; Mills, 2001; 2002; Beermann, 2002) summarize the effects of β_2-AAs as 1) increasing rate of weight gain, 2) improving feed utilization, 3) increasing leanness, and 4) increasing the "dressed" percentage (sometimes referred to as carcass weight). It is demonstrated at least in mammals, that β_2-AAs increase protein accretion in muscle by a transient increase in muscle protein synthesis and a more prolonged decrease in muscle protein degradation; while in the adipose tissue there is an increase in lipolysis and decrease lipogenesis (Yang and McElligott, 1989; Beermann, 2002).

1.5.2 The Effects of β_2-Adrenergic Agonists on Muscle

1.5.2.1 Effects on Muscle β_2-Adrenoceptors: Agonist-Induced Desensitization

Agonist-induced receptor desensitization is defined as "the attenuation of response despite continued presence of the stimulus" (Mills, 2002) and has been demonstrated in many tissues including mammalian skeletal muscle. Many mechanisms contribute to desensitization but they are in general divided into acute uncoupling responses and chronic down-regulation. In the case of the β_2-ARs, uncoupling of the receptor occurs when 1) the receptor is phosphorylated by PKA (see Fig. 1.3; dark circles) at the level of the 3rd intracellular loop and the COOH tail; or 2) when another group of kinases, the G-protein coupled receptor (GPCR) kinases such as β-adrenergic receptor kinase (βARK) phosphorylate agonist-occupied receptors at the level of the COOH tail (Fig. 1.3; cross dashes) (Liggett and Lefkowitz, 1994). The phosphorylation of the receptor ultimately results in impeding further G-protein interaction and, therefore subsequent activation of the receptor signal transduction pathway. This process is initiated very quickly, within seconds to minutes of agonist exposure and is recovered quickly once the stimulus is removed. However, the more pertinent mechanism to the present study is the chronic down-regulation of the receptor. The mechanism of receptor down-regulation occurs in conditions of chronic exposure to agonist and may involve a decline in synthesis rates of the receptor and/or an increase in rate of degradation. The degradation of the receptor involves endocytosis and hydrolysis in lysosomes. The process of endocytosis requires phosphorylation by βARK and subsequent attachment of β-arrestin, a protein that binds to clatherin in "coated pit regions" of the plasma membrane (Lefkowitz, 1998). However, the author points to a very complex system where endocytosis can lead to possible recycling of the receptors to the plasma membrane in addition to degradation in lysosomes. Down-regulation, in contrast to uncoupling, is considered to occur after hours to days of agonist exposure and recovery from down-regulation generally extends over a longer period of time (Mills, 2002).

1.5.2.2 Effects on Muscle Protein Metabolism

As previously stated, β_2-AAs impact protein accretion in muscle mainly through a transient increase in muscle protein synthesis and a more prolonged decrease in muscle protein degradation or a combination of both (Kim and Sainz, 1992; Beermann, 2002). Using radioisotopically labeled amino acid tracers, many early mammalian studies failed to provide evidence of increases in protein synthesis, despite a clear increase in muscle mass, and therefore, concluded that the increase in synthesis might be transient and that decreased rates of degradation could amount to the observed gain in weight (in rats, Reeds *et al.*, 1986; in sheep, Bohorov *et al.*, 1987; MacRae *et al.*, 1988). However, later studies did confirm an effect of β_2-AAs on fractional protein synthesis rates (in mouse, Bates and Pell, 1991; in pigs, Bergen *et al.*, 1989). More recently, the idea of a transient increase proposed by earlier studies was supported by a closed hind-limb perfusion experiment in cattle (Byrem *et al.*, 1998). This study showed a transient increase in amino acid removal from the blood by hind-limb skeletal muscle following perfusion with a β_2-AA increasing to a maximum in the first 14 days but declining thereafter. Protein degradation is usually determined indirectly by means of the difference between measured muscle protein accretion and protein synthesis rates, rates of urinary excretion, or by measurement of protease activity in muscle. Generally, results point towards no change in degradation to slight decreases following treatment with β_2-AAs (Beermann, 2002).

Other alternatives for measurements of muscle protein synthesis and degradation include molecular techniques for measuring mRNA expression of myofibrillar protein genes or genes coding for specific enzymes important in the regulation of muscle protein synthesis or degradation. Increases in mRNA levels of α-actin were reported in muscle of pigs fed RACT

(Bergen *et al.*, 1989; Helferich *et al.*, 1990; Grant *et al.*, 1993). Increased mRNA levels of myosin were also observed in cattle fed CLEN and RACT (Smith *et al.*, 1989; 1995). Moreover, in the cattle closed hind-limb perfusion experiments, skeletal muscle had increased mRNA levels for calpastatin, an endogenous inhibitor of the calpains, which are important proteases involved in muscle protein degradation (Sun *et al.*, 1994).

1.5.2.3 β_2-Adrenergic Agonists Mechanism of Action in Mammalian Muscle: Direct vs. Indirect

The question of whether the β_2-AA-induced muscle hypertrophy is mediated through a direct and/or an indirect mechanism of action has been the focus of numerous studies (for reviews see Yang and McElligott, 1989; Beermann, 1993; Mersmann, 1998; Moody *et al.*, 2000; Beermann, 2002). By definition, a direct mechanism of action implies direct β_2-AA binding to a muscle β_2-AR subsequently activating the downstream signaling transduction pathway resulting. in muscle hypertrophy (increased protein accretion). Studies aimed at providing evidence for a direct mechanism of action include experiments indicating the dependence of the activation of β_2-ARs by β_2-AAs using selective and non-selective β-adrenergic antagonists. Reeds *et al.* (1988) used the non-selective β-adrenergic antagonist propranolol (PROP) and the selective β_2-adrenergic antagonist ICI 118,551 (ICI), in combination with CLEN feeding in rats and observed a blocking of the β_2-AA induced muscle hypertrophy. MacLennen and Edwards (1989) obtained a similar result using the same agonists and antagonists. It must be noted that the concentration of antagonists used were 10 to 100 times the concentration of clenbuterol. Choo *et al.* (1992) used equivalent concentrations of the selective β_2-adrenergic antagonist ICI and successfully blocked the clenbuterol-induced muscle hypertrophy in rats. These experiments, however, do not completely resolve direct or indirect mechanism of action, but only show dependency of the activation of β_2-ARs somewhere in the animal. Despite this, there is evidence that muscle responds directly to β_2-AAs as many studies have shown quantitative measurements of cAMP responses to β_2-AAs in muscle slices (Sillence and Matthews, 1994; Roberts and Summers, 1998), cultured myoblasts and myotubes (Shappell *et al.*, 2000). Moreover, there is extensive documentation of muscle β_2-AR desensitization or down-regulation following chronic exposure to β_2-AAs, which implies direct stimulation at the level of the muscle (Smith, 1989; Spurlock *et al.*, 1994). Taken together, these findings point towards a direct mechanism of action, but fail to rule out an indirect mechanism of action.

A novel technique, the "closed arterial hind-limb perfusion technique", is to date the best evidence by far for a direct mechanism of action of the β_2-AAs on muscle (Byrem *et al.*, 1998). The authors define this technique as "the direct continuous infusion of a compound into the arterial circulation to achieve a local elevated concentration of the compound relative to its concentration in the systemic circulation" (Byrem *et al.*, 1998, p. 993). Continuous infusion of β_2-AAs directly enhanced chronic protein accretion in muscle of cattle (cimaterol; Byrem *et al.*, 1998). The response in rates of amino acid uptake by hind-limb muscle was a transient increase for the first 14 days of treatment then gradually decreasing after 21 days. Even after 21 days, the rates in the treatment group were still significantly higher than the control group. Direct assessments of muscle protein accretion rates were also determined and were significantly higher (approx. 61 to 130% higher on day 7 and 14 respectively) in the β_2-AA treated animals (Byrem *et al.*, 1998). Calculated estimates of cumulative protein accretion predicted a 10 to 15% difference in protein mass between treated and control animals and was confirmed by direct analysis of the muscles taken after 21 days (Byrem *et al.*, 1998). This study provides very good evidence and arguments for β_2-AAs mediating their effects on muscle by a direct interaction with muscle.

Despite the arguments mentioned above, the indirect mechanisms of action are being considered by many since the complexity of hormonal influence on skeletal muscle growth is well established, even in fish (Mommsen and Moon, 2001). By definition, an indirect mechanism of action implies β_2-AAs binding to tissue or cell β_2-ARs other than muscle tissue and subsequently activating the downstream signaling transduction pathway resulting in the release of a hormone or factor which in turn increases the rate of muscle protein accretion. Many studies in mammals have monitored growth hormone (GH), thyroxine (T_4), insulin-like growth factor-I (IGF-I) and insulin levels following chronic treatments with β_2-AAs. The results are somewhat inconclusive as an increase (sustained increase, Beermann *et al.*, 1987; latent increase, Chikhou *et al.*, 1991), decrease (transient decrease, Chikhou *et al.*, 1991) and no change (Dawson *et al.*, 1993) in GH, increase (Beermann *et al.*, 1987) and decrease (O'Connor *et al.*, 1991) in T_4, slight decrease (Beermann *et al.*, 1987; Chikhou *et al.*, 1991) and no change (O'Connor *et al.*, 1991; Dawson *et al.*, 1993; Young *et al.*, 1995) in IGF-I, and decrease (O'Connor *et al.*, 1991) in insulin levels were observed. There is even one study in rainbow trout that reported no change from 1 to 8 weeks and a slight decrease after 12 weeks in GH following treatments with RACT (Vandenberg *et al.*, 1998). Other studies suggested increased tissue sensitivity to the various hormones (Beermann *et al.*, 1987); similar non-conclusive observations were made with insulin (Anderson *et al.*, 1991). However, studies performed on endocrine-altered animal models add support for a direct mechanism of action independent of any hormone modulation (Mills, 2001, 2002; Beermann, 2002). β_2-AA-induced muscle growth is significantly increased in genetically GH-deficient (Bates and Pell, 1991), in hypophysectomized (Thiel *et al.*, 1987), in diabetic (McElligott *et al.*, 1987), in adrenolectomized (Buttery and Dawson, 1987) and in hypothyroid (Beermann, 2002) animals. Moreover studies combining treatments with β_2-AAs and GH (Jones *et al.*, 1989; Maltin *et al.*, 1990; Hansen *et al.*, 1997) found additive effects, which suggest different responses and possibly separate mechanism of action of β_2-AAs and GH (Beermann, 2002). In summary, the combination of 1) the apparently negligible indirect effects of β_2-AAs in the endocrine-altered animal models, and 2) the direct effects of β_2-AAs monitored using the hind-limb perfusion, strongly supports a direct mechanism of action independent of other hormones or factors. The general hypothesized and confirmed aspects of the β_2-AR signal transduction pathway involved in a direct mechanism of action on mammalian skeletal muscle are summarized in Figure 1.5.

This thesis will provide some insight into the rainbow trout muscle β_2-AR system. The focus will be to 1) show the presence of β-ARs in red and white muscles of the trout; 2) pharmacologically determine the subtype(s) of β-ARs; 3) demonstrate the causative association between β_2-AA binding and activation of the subsequent signaling transduction pathways; and to determine the impact of β_2-AA feeding on 4) muscle β_2-AR binding characteristics, 5) β_2-AR mRNA expression and 6) muscle fractional protein synthesis rates.

Figure 1.5: Cartoon illustrating hypothesized direct mechanisms of action of β_2-adrenergic agonists (β_2-AAs) in mammalian skeletal muscle. Included are skeletal muscle cell, blood vessel and myosatellite cell. Arrows with a solid line indicate confirmed interaction while a dashed line represents indications but not conclusive interactions. A triangle arrow end indicates a positive effect on the indicated parameter while a dotted arrow end represents an inhibition. Note the letters "DR" on the β_2-AR indicating down-regulation of the receptor upon chronic exposure to agonist. Abbreviations are from top to bottom, left to right: BF, blood flow; AA, amino acids; β-Ant, β-adrenergic antagonist; β_2-AA, β_2-adrenergic agonist; β_1-, β_2- and β_3-AR, β_1-, β_2- and β_3-adrenoceptors; DR, down-regulation; PKA, protein kinase A. Diagram is based on reviewed literature reported by Yang and McElligott (1989), Mersmann (1998), Mills (2001; 2002) and Beermann (2002).

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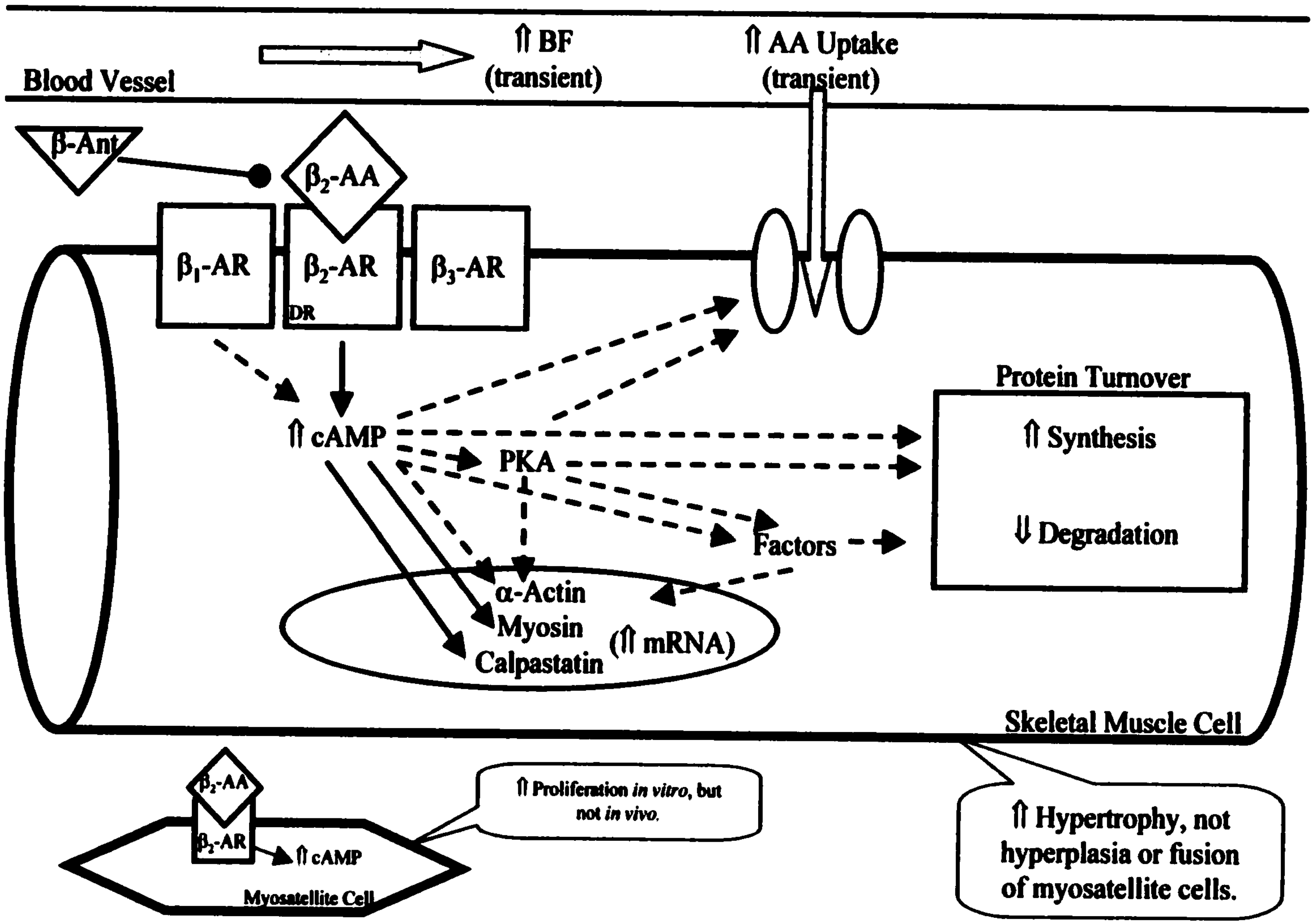
Blood Vessel
⇑ BF (transient)
⇑ AA Uptake (transient)
β-Ant
β_2-AA
β_1-AR
β_2-AR
DR
β_3-AR
⇑ cAMP
PKA
Factors
α-Actin
Myosin
Calpastatin
(⇑ mRNA)
Protein Turnover
⇑ Synthesis
⇓ Degradation
Skeletal Muscle Cell
β_2-AA
β_2-AR
⇑ cAMP
Myosatellite Cell
⇑ Proliferation *in vitro*, but not *in vivo*.
⇑ Hypertrophy, not hyperplasia or fusion of myosatellite cells.

CHAPTER 2. The Rainbow Trout Muscle β-Adrenergic System: Characterization and Signaling

2.1 Introduction

The adrenergic system is key to integrating and modulating many aspects of vertebrate, including fish, metabolism (Fabbri *et al.*, 1998). The endogenous circulating catecholamine (CA) hormones, adrenaline (ADR) and noradrenaline (NOR) exert effects on target cells or tissues by binding to specific hormone receptors called adrenoceptors (ARs), which in turn activate intracellular transduction pathways. Catecholamines (CAs), AR pharmacological/molecular classification and underlying transduction pathways in fish and mammals were covered extensively in Chapter 1. Briefly, both subtypes of ARs, α-AR and β-AR, are found in fish and mammals. The characterization and steps involved in the signal transduction system of fish ARs from the binding of CA to α- and β-ARs to the ultimate effects of specific enzyme phosphorylation, is derived from the study of isolated tissues and especially hepatocytes (Fabbri and Moon, 1998). Studies on the effects of CA and the AR subtype(s) distribution in other metabolically important fish tissues, however, have received little or no attention. Of all the tissues in fish, skeletal muscle comprises the largest tissue compartment representing more than 50% of total body weight, a proportionally larger component when compared with other vertebrates (Johnston, 1982). There are more total insulin and IGF-I receptors in skeletal muscle than liver of fish (Navarro *et al.*, 1999); a similar situation may exist for ARs.

Pharmacological and molecular studies confirmed the presence of β_1-, β_2- and β_3-AR subtypes in rat white (glycolytic type II) and red (oxidative type I) skeletal muscles (Elfellah and Reid, 1987; Liggett *et al.*, 1988; Kim *et al.*, 1991; Kaumann and Molenaar, 1997; Roberts and Summers, 1998). β_2-Adrenergic agonists (β_2-AAs) binding leads to increased intracellular cAMP concentrations, primarily through the β_2-AR subtype with slight and no cAMP increase observed using β_1- and β_3-AR agonists, respectively (Roberts and Summers, 1998). Subsequent to changes in cAMP, protein phosphorylation (by protein kinase A) and activation of cAMP responsive elements (CRE) by cAMP response element binding protein (CREB), are believed to impact protein turnover by a transient stimulation of protein synthesis and a longer-lasting reduction in protein degradation (Beerman, 1993; Yang and McElligott, 1989; Kim and Sainz, 1991; Reeds and Mersmann, 1991; Mersmann, 1998; Navegantes *et al.*, 2001; Mills, 2001; 2002; Beermann, 2002). However, a mechanistic gap persists between phosphorylation of proteins or activation of CRE by cAMP and subsequent changes in protein turnover (Beerman, 1993; Moody *et al.*, 2000; Mills, 2001; 2002; Beermann, 2002).

Recent molecular studies have demonstrated the presence of a putative β_2-AR from rainbow trout that shares a high degree of amino acid sequence conservation with other vertebrate β_2-ARs and is highly expressed in liver, red and white muscles, but less so in gill, spleen and kidney with no detectable expression in red blood cells (Nickerson *et al.*, 2001). No pharmacological characterization of β-ARs in teleost skeletal muscle has been reported, nor has its subsequent coupling to the second messengers.

2.2 Hypothesis

Rainbow trout red and white muscles have β_2-ARs and upon β_2-AA binding, a functional transduction cascade results in the increased production of the second messenger cAMP.

2.3 Objectives

The main objectives of this study are to 1) show the presence of β-ARs in red and white muscles of the rainbow trout, 2) pharmacologically characterize the subtype(s) of β-AR and 3) demonstrate the causative association between β_2-AA binding and activation of the subsequent transduction pathway.

2.4 Materials and Methods

2.4.1 Animals

Female rainbow trout (*Oncorhynchus mykiss*), weighing approximately 125-200 g, were obtained from Linwood Acres Trout Farm (Campellcroft, ON). Fish were transported to the University of Ottawa Aquatic Care Facility and were maintained in fibreglass holding tanks (1275 L) of well aerated, dechloraminated City of Ottawa tap water at 13.0 ± 1.0 °C. Fish were subjected to a constant 12L:12D photoperiod and fed five times a week with commercial trout pellets [Martin Mills 5 PT, 5 mm in size and composed of: 41.0% crude protein (min); 11.0% crude fat (min); 3.5% crude fibre (max); 1.0% calcium (actual); 0.85% phosphorus (actual); 0.45% sodium (actual); 6,800 IU • kg^{-1} vitamin A (min); 2,100 IU • kg^{-1} vitamin D (min); 80 IU • kg^{-1} vitamin E (min); 200 IU • kg^{-1} vitamin C (min)]. Receptor characterization experiments were conducted between October and December and adenylyl cyclase (ACase) experiments were performed in January and February using fish acquired in September.

2.4.2 Muscle Membrane Preparation

Rainbow trout were netted from the tank and killed by a sharp blow to the head. The skin was removed, the superficial red muscle (RM) and underlying epaxial white muscle (WM) were quickly and carefully excised, freeze clamped between aluminium blocks cooled in liquid N_2 and stored at -80 °C until membranes were prepared within one week. The muscle tissue samples were crushed to a fine powder using a porcelain mortar with pestle kept at liquid N_2 temperatures. The powder was weighed and suspended in 5 vol of ice cold Basic Hanks' medium [in mM: 136.9 NaCl, 5.4 KCl, 0.8 $MgSO_4 \times 7H_2O$, 0.33 $Na_2HPO_4 \times 7H_2O$, 0.44 KH_2PO_4, 5.0 Hepes, 5.0 Hepes-Na, 1.0 $NaHCO_3$, and 0.43 phenylmethyl-sulfonyl fluoride (PMSF)] adjusted to pH 7.63. All subsequent procedures were carried out on ice (4 °C), unless specified otherwise. The muscle was homogenized with 6 strokes (approximately 10 sec • stroke^{-1}) of a Potter-Elvehjem Teflon-glass homogenizer attached to a commercial drill (Black & Decker) running at low speed. The resulting homogenate was centrifuged at 400 x g for 10 min in a Sorvall RC 5B Plus (SS 34 rotor) at 4 °C. The supernatant was filtered through nitex nylon mesh (250 μm; Sefar America Inc., Kansas City, MO) and the pellet was discarded. The filtrate was centrifuged at 38,000 x g for 30 min in the Sorvall RC 5B Plus (SS 34 rotor) at 4 °C. The resulting supernatant was discarded and the final pellet resuspended in approximately 2 vol of ice cold Basic Hanks' medium (pH 7.63) and aliquotted into 1.5 mL conical plastic centrifuge tubes. Membranes were frozen in liquid N_2 and stored at -80 °C until assayed within 2 weeks of preparation.

2.4.3 β-Adrenoceptor Characterization

2.4.3.1 Specific Binding Assays

Specific binding assays were performed by following established and validated methods used in previous studies of trout ARs (Dugan and Moon, 1998). Frozen membranes were thawed on ice. Aliquots of the membrane samples were assayed for protein using the Bicinchoninic Acid (BCA) assay (Sigma, St. Louis, MO) with bovine serum albumin (BSA) as a standard and a SPECTRAmax PLUS 384 (Molecular Device, Sunnyvale, CA) microplate spectrophotometer. Protein concentrations were adjusted to 250-350 μg • 50 μL^{-1}. The radiolabelled, hydrophilic, non-specific β-adrenoceptor antagonist ^{3}H-CGP [(-)-4-(3-tbutylamino-2-hydroxypropoxy)-[5,7-^{3}H]benzimidazol-2-one); ^{3}H-CGP; Amersham Canada Ltd., Oakville, ON; specific activity 46.0 Ci • mmol^{-1}] was used to characterize β-AR binding sites. Fifty μL of trout white and red muscle membranes, containing 250-350 μg protein, were incubated in 5 mL polystyrene round-bottom clear tubes (Falcon) for 60 min at room temperature (approx. 19.0 °C) in a final volume of 150 μL and in the presence of varying concentrations of ^{3}H-CGP (approx. 0.1 to 5 nM) to estimate total binding (TB) while non-specific binding (NSB) was determined in the presence of 10 μM CGP 12177A (Sigma). All incubations were performed in Basic Hanks' (pH 7.63). Binding assays were terminated by aspirating the incubations through a cell harvester (Brandel 24R) onto pre-rinsed (ice-cold 0.9% w/v NaCl) borosilicate filters (#32 Mandel Scientific) and repeated washing (3x) with ice cold 0.9% w/v NaCl. The membranes collected on the borosilicate filters were then placed in polyethylene scintillation vials containing 4 mL scintillation cocktail (Safety-Solve; Research Products International, Mount Prospect, IL). The vials were left in the dark for at least 24 h and the radioactivity was determined using a Beckman Coulter LS6500 multi-scintillation counter.

2.4.3.2 Competition Assays

Competition assays using 50 μL of muscle membranes (containing 200-300 μg protein) were incubated as above, in the presence of a constant concentration of 1nM ^{3}H-CGP (approx. 14,500 dpm). Displacement of ^{3}H-CGP was determined in the presence of five concentrations (10, 1, 0.1, 0.01 and 0.001 μM) of β-adrenergic antagonists [(±)-ICI 118,551, atenolol, (±)-CGP 12177A, propranolol] or β-adrenergic agonists [(±)-dobutamine, procaterol, clenbuterol, ractopamine, BRL 37,334, CL 316,243, (-)-adrenaline and (-)-noradrenaline; all from Sigma except for ractopamine, provided by Eli-Lilly, Greenfield, IN, and CL 316,243, provided by Dr Jean Himms-Hagen, Dept of Biochemistry, Microbiology and Immunology, Faculty of

Medicine, University of Ottawa], and terminated after a period of 60 min at room temperature. All subsequent manipulations were carried out exactly as stated in the binding assay methodology (2.4.3.1) with the exception that all agonists were kept in tubes wrapped with aluminium foil and assays were carried out in the dark.

2.4.4 β-Adrenoceptor Coupling to Adenylyl Cyclase

Adenylyl cyclase (ACase) activity was assayed by adapting methods described for eel liver membranes (Fabbri *et al.*, 1998; 2001), rockfish enterocytes and brain membranes (Mommsen and Mojsov 1998) and rat liver membranes (Unson *et al.*, 1996). Membrane protein (45-55 μg) was incubated in medium (in mM: 12.5 $MgCl_2$, 5.0 ATP, 20.0 creatine phosphate, 0.025 GTP, 6.0 theophylline, 50.0 Tris-HCl and 6 units • mL^{-1} creatine phosphokinase and 0.1 mg • mL^{-1} bovine serum albumin) in the presence of various β-adrenergic agonists [(-)-isoproterenol, clenbuterol, ractopamine] and antagonists [propranolol, (±)-CGP 12177A] and the ACase activator, forskolin. The antagonist propranolol was pre-incubated for 10 min to assure proper blocking of the ARs. After 10 min at room temperature (approx. 19.0 °C), the reaction was stopped by boiling for 3 min and the samples were subsequently frozen in liquid N_2 and stored at –80 °C until assayed for cAMP content within one week. Again the agonists were kept in tubes wrapped with aluminium foil and assays were carried out in the dark.

Proteins were precipitated by centrifugation (14,000 x g, 5 min, Beckman-Coulter Microfuge R, F241.5 rotor at 4 °C). Cyclic adenosine monophosphate (cAMP) was determined in supernatants following a modified method that extended the EIA kits from Amersham (Mississauga, ON) (T.P. Mommsen, pers. commun.). This included diluting the antiserum (2x) with additional 3,3',5,5'-tetramethylbenzidine substrate solution (TMB substrate; Sigma) and purchasing an additional anti-rabbit IgG coated plate (Cayman Chemical, Ann Arbor, MI).

This permitted twice the number of analyses per cAMP kit, and preliminary studies validated this procedure. The plates were read at 450 nm with a SPECTRAmax PLUS 384 and the cAMP production was expressed as pmol cAMP • mg protein^{-1} • 10 min^{-1}. Membrane protein was assayed as previously described using the BCA protein assay.

2.4.5 Statistics

Receptor saturation and competition data were analyzed by the EBDA and LIGAND computer programs (Munson and Rodhard, 1980). All further data conversions used Microsoft Excel 2000, graphs were plotted using SigmaPlot 2000 (Jandel Scientific) and statistical differences were evaluated using appropriate tests with SigmaStat 2.0 (Jandel Scientific). A value of $P < 0.05$ was accepted to indicate significant differences.

2.5 Results

2.5.1 β-Adrenoceptor Characterization

2.5.1.1 Specific Binding Assays

Specific binding assays were performed to assess the affinity (K_d) and number (B_{max}) of β-adrenergic binding sites on rainbow trout red and white muscle membranes by incubating isolated membranes with increasing concentrations of [^{3}H]-CGP (Fig. 2.1A and 2.1B, respectively). In both tissues, specific binding increased as the concentration of radiolabelled ligand increased to eventually saturate at approximately 1.5 nM. Specific binding was also higher than non-specific binding up to concentrations near 4 nM. Scatchard analysis (Fig. 2.1C) was linear (EBDA; Munson and Rodhard, 1980) and indicated the presence of a homogeneous class of binding sites in both tissues (LIGAND, at $P < 0.05$; Munson and Rodhard, 1980). The K_d (mean ± SEM in nM) was 0.14 ± 0.03 (n = 6) in red and 0.18 ± 0.03 (n = 6) in white muscle membranes. The B_{max} (mean ± SEM in fmol • mg protein^{-1}) was 3.22 ±

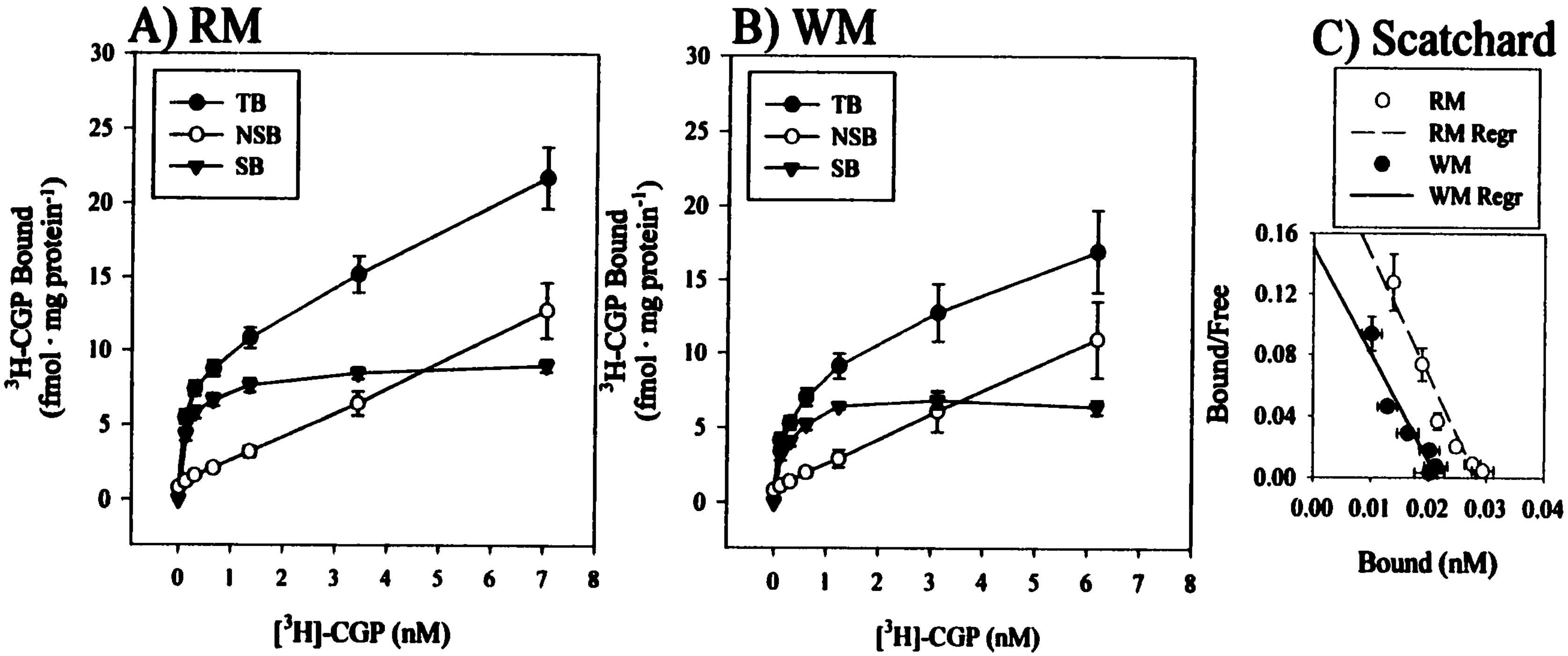

Figure 2.1: Radiolabelled saturation curves representing specific binding assays performed on rainbow trout red (A) and white (B) muscle membranes. Assays contained between 250-350 μg protein • 50 μL^{-1} and incubations were for 60 min with increasing concentrations of ^{3}H-CGP in the absence (total binding, TB; solid circles) and in the presence of 10 μM (±)-CGP 12177A (non-specific binding, NSB; open circles). Specific binding (SB; solid triangles, down) was assessed by subtracting the NSB from the TB. Scatchard plot (EBDA; Munson and Rodhard, 1980) of the same data (C) for red (RM, open circles and dashed line) and white (WM, solid circles and solid line) muscle membranes; Regr = calculated regression line. Values represent means ± SEM of 6 experiments (each experiment from an individual animal) done in duplicates.

0.11 (n = 6) in red and 2.60 ± 0.13 (n = 6) in white muscle membranes. A paired t-test revealed no significant difference between K_d values ($P > 0.05$) but a significant difference between B_{max} values of red and white muscle membrane ARs (Table 2.1).

2.5.1.2 Competition Assays

Competition assays were performed on rainbow trout white and red muscle membranes using classic mammalian AR agonists (Fig. 2.2A RM; Fig. 2.3A WM) and antagonists (Fig. 2.2B RM; Fig. 2.3B WM). The mammalian antagonists classified as mixed β-antagonists, CGP and PROP, displaced ^{3}H-CGP most effectively in both tissues, to approximately 30% of total binding at the highest concentration used (10 μM). The β_2-antagonist ICI and the β_2-agonists CLEN and RACT were less effective, displacing to less than 50% of total binding. The endogenous adrenergic agonists, ADR and NOR both displaced to approximately 60% of total binding with a slightly higher displacement by ADR. The remaining agonists and antagonists displaced less than 60% of total binding, although there were some quantitative differences between DOB (a β_1-agonist) and PROC (a β_2-agonist) in red and white muscles (Fig. 2.2A and 2.3A).

The concentration of ligands causing 50% displacement of specific binding (K_i) were determined using EBDA (Table 2.2). The K_i values for the agonists were in the following order: CLEN ≈ RACT > PROC ≈ NOR ≈ DOB ≈ ADR with no significant displacement by BRL and CL in the RM, and CLEN ≈ RACT > ADR ≈ NOR > PROC > BRL with no significant displacement by DOB and CL in the WM. The K_i values for the antagonists were in the following order: CGP > PROP > ICI and no significant displacement by ATL in red or white muscle membranes.

Table 2.1: Binding affinities (K_d) and maximum number of binding sites (B_{max}) for ^{3}H-CGP (β-adrenoceptors) in rainbow trout red and white muscle membranes.

	K_d (nM)	B_{max} (fmol • mg protein^{-1})
Red Muscle	0.14 ± 0.03	3.22 ± 0.11[a]
White Muscle	0.18 ± 0.03	2.60 ± 0.13[a]

All values are mean ± SEM, n = 6. There is a significant difference between red and white muscle B_{max} values, represented by [a] (paired t-test, $P < 0.05$).

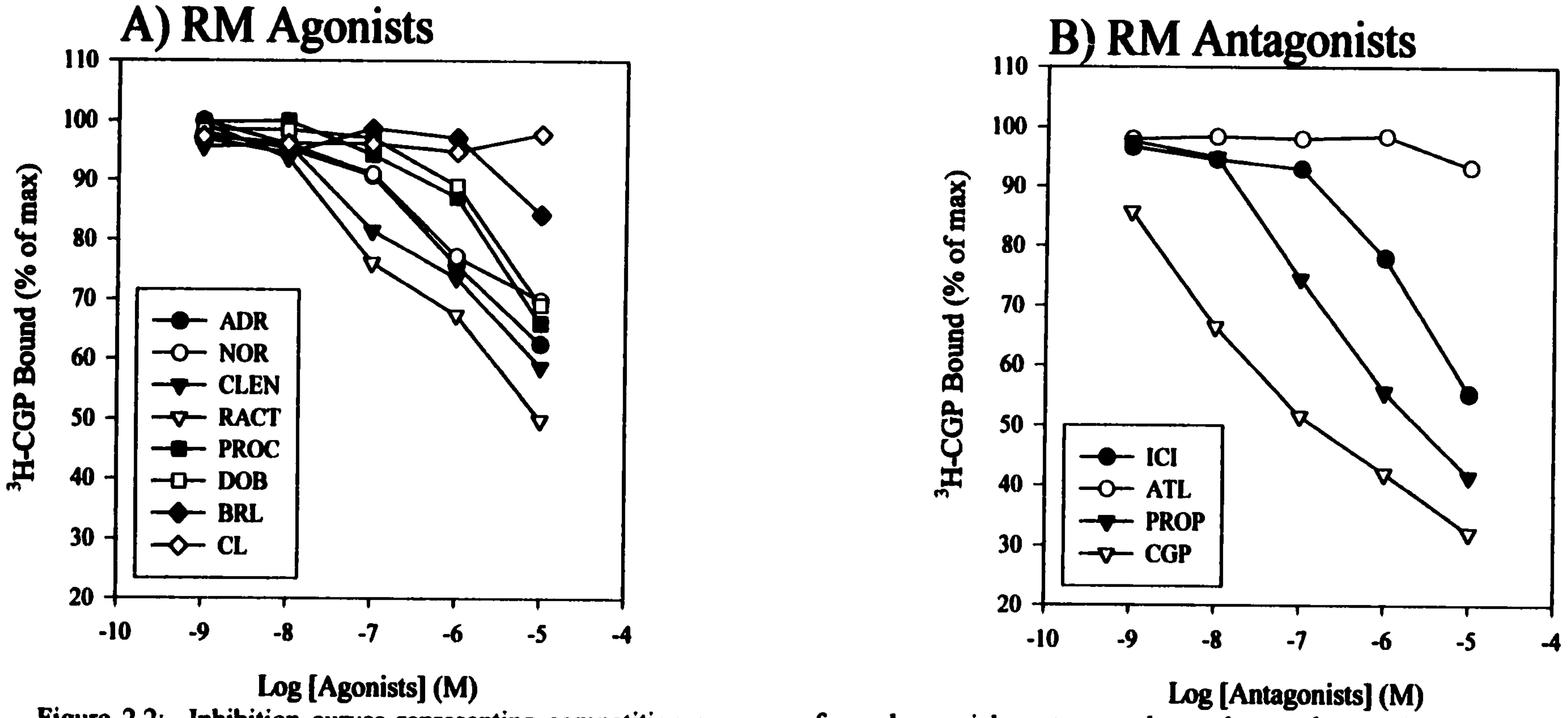

Figure 2.2: Inhibition curves representing competition assays performed on rainbow trout red muscle membranes (A, agonists and B, antagonists). Assays contained between 250-350 μg protein • 50 μL^{-1} and incubations were for 60 min at a constant concentration of 1 nM ^{3}H-CGP in the absence (total binding) and in the presence of five concentrations (10 μM, 1 μM, 0.1 μM, 0.01 μM and 0.001 μM) of agonists [(-)-adrenaline (ADR solid circles), (-)-noradrenaline (NOR open circles), clenbuterol (CLEN solid triangles, down), ractopamine (RACT open triangles, down), procaterol (PROC solid squares), dobutamine (DOB open squares), BRL 37,334 (BRL solid diamonds) and CL 316,243 (CL open diamonds)] and antagonists [(±)-ICI 118,551 (ICI solid circles), atenolol (ATL open circles), propranolol (PROP solid triangles, down) and (±)-CGP 12177A (CGP open triangles, down)]. Values represent means of 4 experiments (each experiment from an individual animal) done in duplicates. The variations (approx. ± 5 %) for individual data points are omitted for clarity.

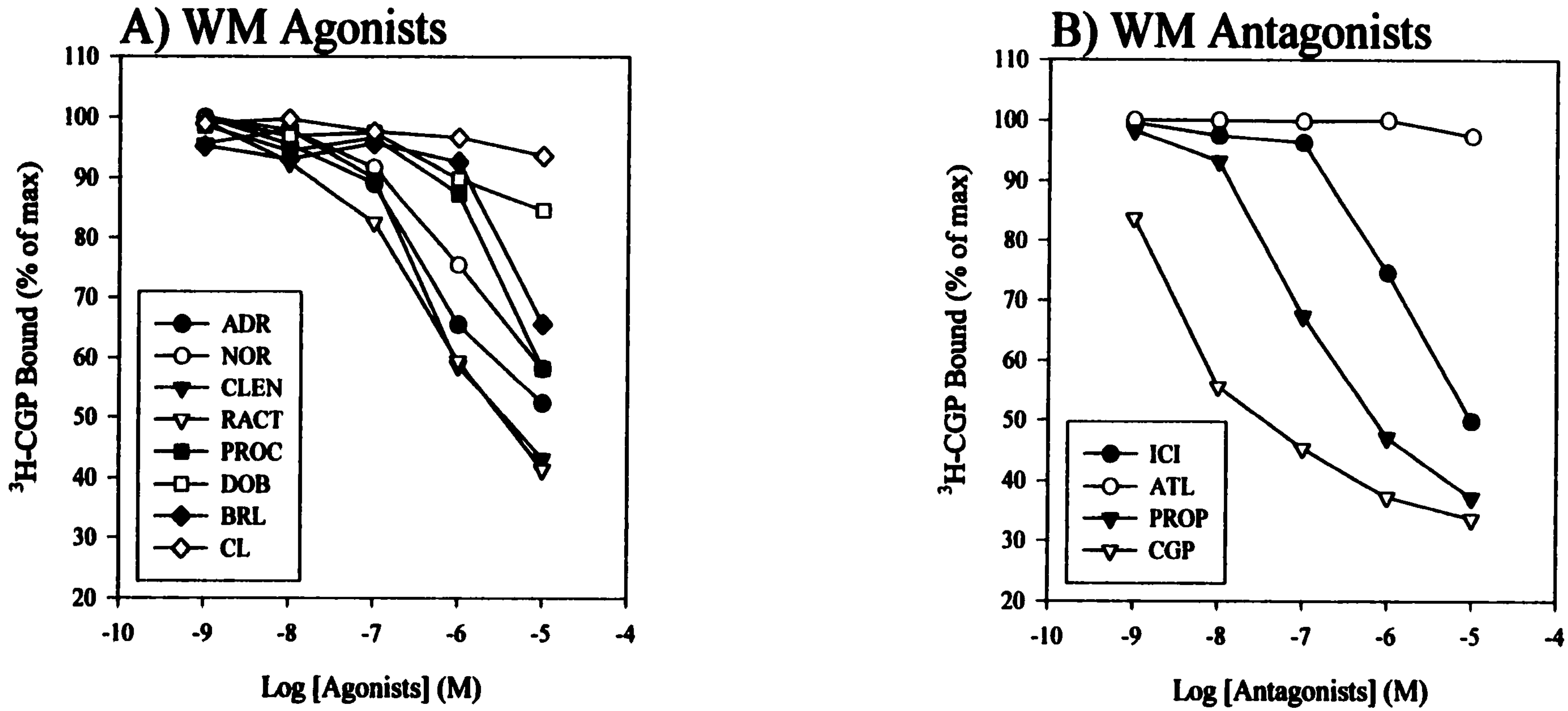

Figure 2.3: Inhibition curves representing competition assays performed on rainbow trout white muscle membranes (A, agonists and B, antagonists). Conditions and abbreviations are as noted on legend of Fig. 2.2.

Table 2.2: Competitive displacement parameters (K_i) for ^{3}H-CGP in rainbow trout red and white muscle membranes using various agonists and antagonists.

	K_i (nM)	
Agonists	**Red Muscle**	**White Muscle**
ADR (β_2-AR > β_1-AR)	3297.84 ± 2294.95	201.43 ± 61.19
NOR (β_1-AR > β_2-AR)	1578.74 ± 790.11	332.38 ± 144.29
CLEN (β_2-AR)	331.57 ± 100.48	83.38 ± 9.98
RACT (β_2-AR ≈ β_1-AR)	109.99 ± 50.33	136.14 ± 103.17
PROC (β_2-AR)	1245.59 ± 362.63	710.21 ± 69.20
DOB (β_1-AR)	1902.11 ± 580.00	-
BRL (β_3-AR)	-	1187.94 ± 481.90
CL (β_3-AR)	-	-
Antagonists		
CGP (non-selective β-AR)	3.01 ± 0.77	1.27 ± 0.80
PROP (non-selective β-AR)	37.25 ± 6.59	13.90 ± 1.66
ICI (β_2-AR)	472.44 ± 53.74	203.11 ± 24.06
ATL (β_1-AR)	-	-

All values are mean ± SEM, n = 4. Concentration of agonist or antagonist causing 50% displacement of specific binding (K_i) was calculated using the EBDA computer program (Munson and Rodhard, 1980). Agonists or antagonists causing less than 50% displacement of specific binding even at the highest concentration tested (10 µM) are represented by a dash.

2.5.2 β-Adrenoceptor Coupling to Adenylyl Cyclase

Adenylyl cyclase assays were performed on rainbow trout red and white muscle membranes to assess cAMP production. Assays were undertaken with increasing concentrations of β_2-agonists (Fig. 2.4A RM; 2.4B WM), and in the presence of 1 μM β_2-agonists with or without 100 μM of the general β-antagonist PROP (Fig. 2.5A RM; Fig. 2.5B WM). Basal activities of RM cAMP production (mean ± SEM in pmol cAMP • mg protein^{-1} • 10 min^{-1}) were 24.67 ± 3.06 and the β_2-agonists-induced significant increases in cAMP production at a concentration of 1 μM CLEN and RACT (Fig. 2.4A). Similarly in WM basal values of cAMP production were 9.64 ± 3.45 and increased significantly at concentrations of 0.1 and 0.01 μM CLEN and RACT, respectively (Fig. 2.4B). Cyclic AMP concentrations increased in a dose-dependent manner (reaching 61.26 ± 8.19 and 59.52 ± 8.75 in RM and 57.99 ± 5.10 and 66.29 ± 11.85 in WM) up to 10 μM CLEN and RACT, respectively.

Pre-incubating the membranes with 100 μM PROP for 10 min blocked the significant increase in cAMP production elicited by 1 μM of the β_2-agonists (CLEN, RACT and ISO) in both red (Fig. 2.5A) and white (Fig. 2.5B) muscle membranes. Significant differences in cAMP production were observed between basal and β_2-agonists and similarly between PROP alone and β_2-agonists. Furthermore, no significant differences in cAMP production were observed between basal and PROP with β_2-agonists, also between PROP alone and PROP with β_2-agonists (CLEN, RACT and ISO). The β-antagonist CGP was also tested alone, and with PROP and no significant differences in cAMP production were observed in either red and white muscle membranes (data not shown). Additionally, 10 μM of the ACase activator forskolin (FSK) significantly increased cAMP production by approximately 7- and 14-fold above basal in red and white muscle membranes, respectively (Fig. 2.5A, B). This represents

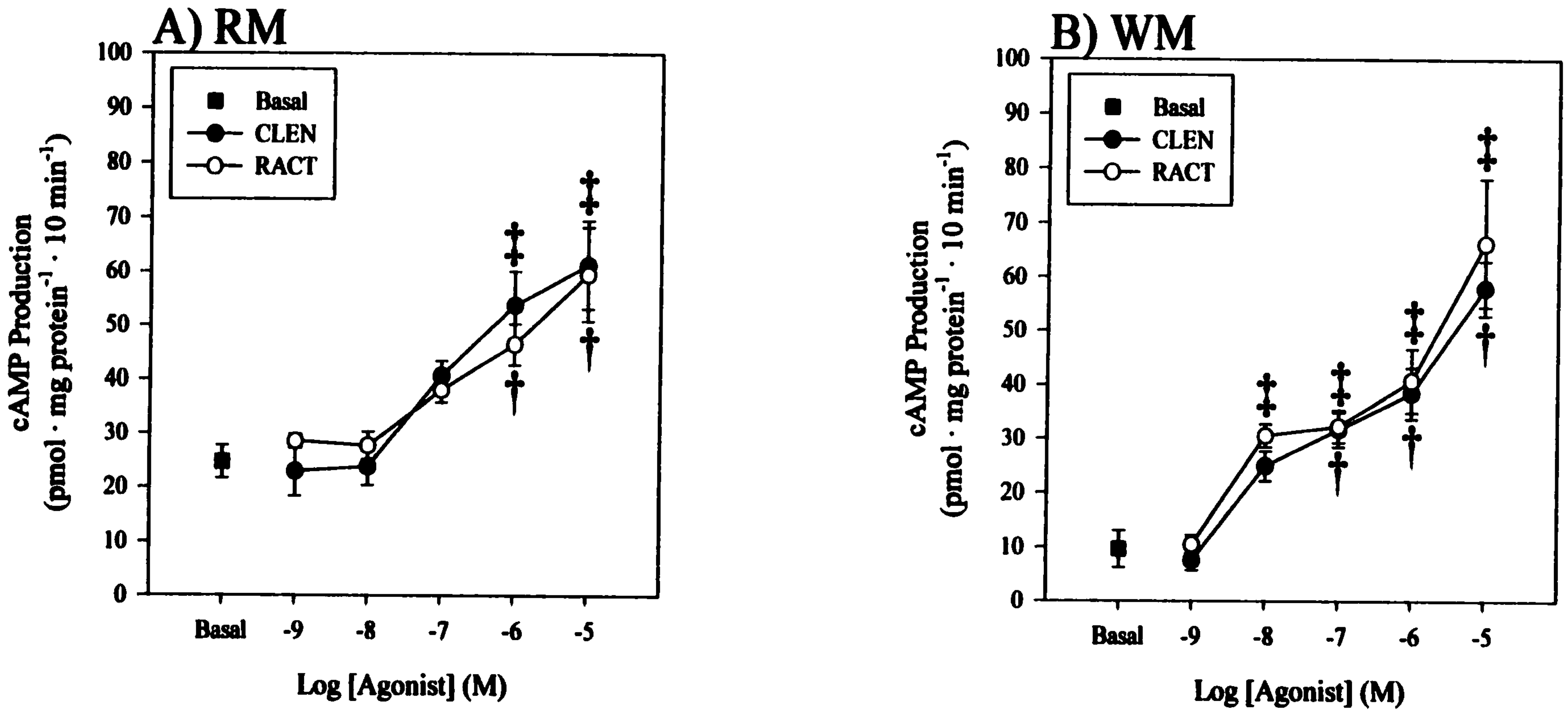

Figure 2.4: Effects of increasing concentrations of adrenergic agonists [clenbuterol (CLEN, solid circles) and ractopamine (RACT, open circles)] on red (A) and white (B) muscle membrane cAMP production. Assays containing 45-55 μg protein were incubated for 10 min in the presence or absence of agonists. Means ± SEM are presented for four independent experiments (each experiment from an individual animal) measured in duplicates. Levels of significance (repeated measures one-way ANOVA, $P < 0.05$) for increasing concentrations of CLEN and RACT compared with basal values are represented by † and ‡, respectively (A and B). There is a significant difference in basal cAMP production between red (A) and white (B) muscle membranes (paired t-test, $P < 0.05$).

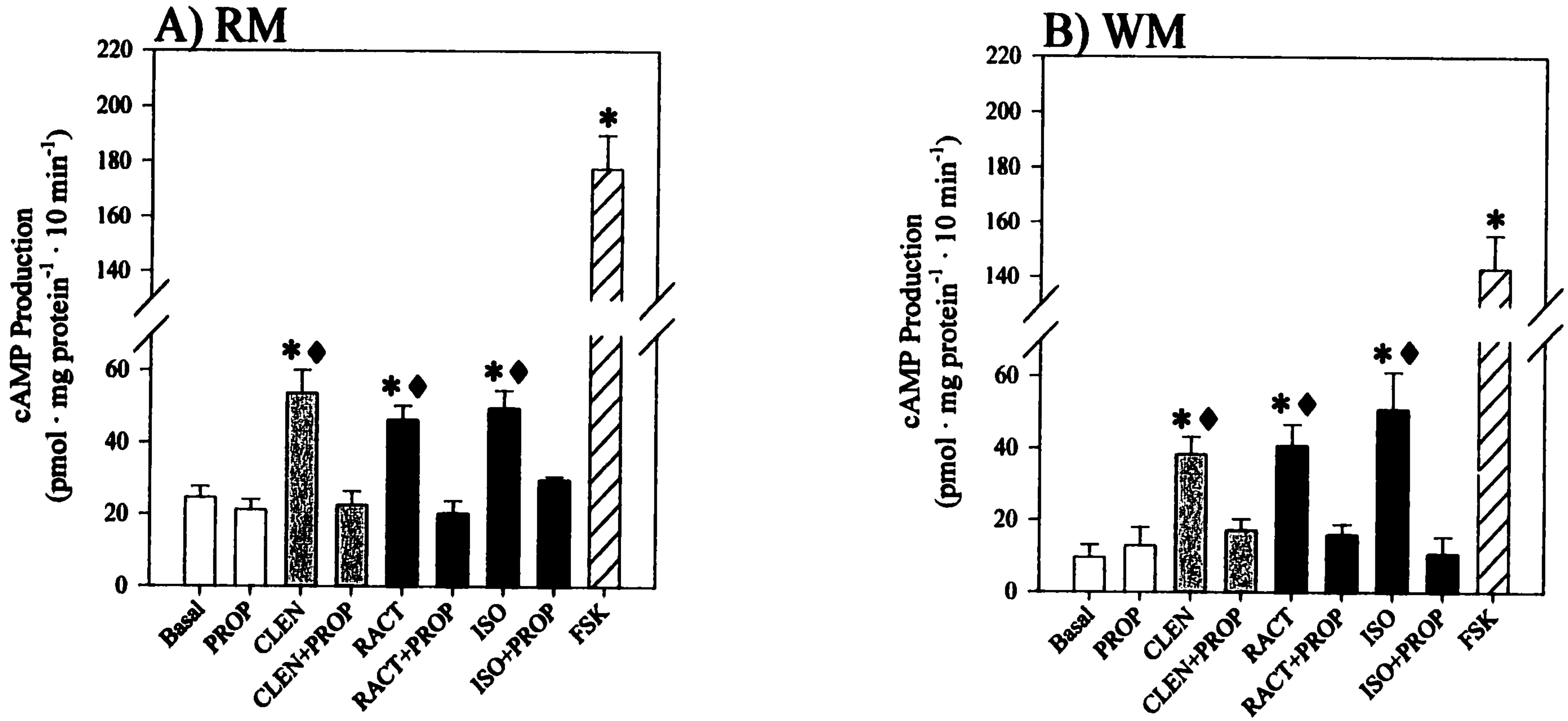

Figure 2.5: Effects of 10 μM forskolin (FSK) and 1 μM concentrations of various adrenergic agonists [clenbuterol (CLEN), ractopamine (RACT) and (-)-isoproterenol (ISO)] with or without 10 μM concentration of the adrenergic antagonist propranolol (PROP) on red (A) and white (B) muscle membrane ACase activities. Assays containing 45-55 μg protein were incubated for 10 min in the presence or absence of agonists, while those incubated with the antagonist PROP were pre-incubated for 5 min prior to 10 min incubation with the agonists. Means ± SEM are presented for four independent experiments (each experiment from an individual animal) measured in duplicates. Levels of significance in comparison to basal or PROP alone and agonist plus PROP are represented by * and ◆, respectively (repeated measures one-way ANOVA, $P < 0.05$). There is a significant difference in basal cAMP production between red (A) and white (B) muscle membranes (paired t-test, $P < 0.05$).

approximately a 3-fold higher activation than any of the agonists used. There is a significantly higher basal cAMP production in red compared to white muscle, however, in presence of PROP this difference was abolished. Cyclic AMP production in presence of FSK was not significantly different between red and white muscle.

2.6 Discussion

The main objectives of this study were to show the presence of β-ARs in RM and WM of the rainbow trout, identify the β-AR subtype(s) present on the basis of pharmacology and show causative association between β-AA binding and activation of the subsequent signaling pathway. β-Adrenergic binding sites and increases in cAMP upon β_2-AA binding have been characterized in mammalian red (oxidative type I) and white (glycolytic type II) skeletal muscles. However, this is the first characterization of β-adrenergic binding sites and coupling to cAMP in skeletal muscles of a teleost fish.

2.6.1 β-Adrenoceptor Characterization

The use of different radioactive ligands (^{3}H-CGP, ^{3}H-DHA and ^{125}I-CYP) and different muscle preparations makes it difficult to compare the present study with previous studies. However, one study using membranes isolated from adult and juvenile rats and the radioligand ^{3}H-CGP, did find similar results (Jenson *et al.*, 1995). In adult rat membranes, ^{3}H-CGP affinity (K_d in nM) was 0.37 and 0.31 and the number of binding sites (B_{max} in fmol • mg protein^{-1}) was 9.38 and 4.74 in red (soleus) and white (extensor digitorum longus) skeletal muscles, respectively. Similar values in juvenile rats were K_d values (in nM) 0.27 and 0.24 and B_{max} values (in fmol • mg protein^{-1}) 11.21 and 5.45 in red (soleus) and white (epitrochlearis) skeletal muscles, respectively. Rat red skeletal muscle had similar affinity values but more binding sites in both adult and juvenile rats. Comparing the values obtained from red and white muscles of the rainbow trout (Table 2.1) indicate slightly higher affinities but approximately 2 to 5-fold lower B_{max} values than that of the rat. The trend of higher binding site numbers in red compared with white muscle does remain, as reported in other mammalian studies that used other radiolabelled ligands and/or preparations (Williams *et al.*, 1984; Fell *et al.*, 1985; Kim *et al.*, 1991; Polla *et al.*, 2001). The smaller number of cell surface hormone receptors in fish compared with mammals is a consistent observation (Navarro *et al.*, 1999).

Furthermore, comparing the values obtained from red and white muscles (Table 2.1) with those of rainbow trout liver reveasls slightly higher affinity (K_d) than that of liver (approx. 0.16 nM vs 0.4 nM) but B_{max} values approximately 3-fold lower than that of liver (approx. 3 vs 9 fmol • mg protein^{-1}; Fabbri *et al.*, 1995a). Despite this higher number of binding sites in liver, skeletal muscle comprises approximately 50% of total body weight (Johnston, 1982) compared to approximately 1% BW for liver (Dugan and Moon, 1998). Therefore, when considering total receptor numbers, the 50-fold difference between tissue masses compensates for the 3-fold lower number of receptors; meaning muscle contains the bulk of the ARs in the body of rainbow trout.

Previous studies report mainly β_2-ARs and possibly β_1-ARs on adult and juvenile rat skeletal muscle (Kim *et al.*, 1991; Jenson *et al.*, 1995) with β_3-ARs also on some mammalian skeletal muscles (Roberts *et al.*, 1993; Sillence *et al.*, 1993; Summers *et al.*, 1995). Competition assays using classical mammalian β-AR agonists and antagonists and rainbow trout red and white muscle membranes revealed the presence of an atypical β_2-AR subtype (Fig. 2.2, Table 2.3). The order of potency for the antagonists (CGP > PROP > ICI with no displacement by ATL) clearly shows typical β_2-AR characteristics in both red and white trout muscles. The mixed β-AR antagonists CGP and PROP displace best, closely followed by the β_2-AR antagonist ICI and no displacement with the β_1-AR antagonist ATL. However the order of potency for the agonists in both red and white muscles is ambiguous and would suggest an "atypical" β_2-AR. In red muscle (CLEN ≈ RACT > PROC ≈ NOR ≈ DOB ≈ ADR with no displacement by BRL and CL) the β_2-AAs CLEN, RACT and PROC displaced best, supporting the antagonist result. NOR has a slightly higher affinity than ADR, which would suggest β_1-AR characteristics (Mills, 2002). Also some displacement by the β_1-AA DOB indicates the presence of β_1-AR characteristics. Similarly in white muscle (CLEN ≈ RACT > ADR ≈ NOR > PROC > BRL with no displacement by DOB and CL) the β_2-AAs CLEN and RACT displaced best, which supports the results of the antagonist experiments. However the β_2-AA PROC would be expected to displace better than the endogenous CAs, but does not. ADR has a slightly higher affinity than NOR, again supporting the presence of the β_2-AR subtype. Also some displacement by the β_3-AA, BRL, would support a β_3-AR component. This study is not the first to report ambiguities when using mammalian pharmacological agents in non-mammalian organisms and this may indicate non-mammalian pharmacological classification (Fabbri *et al.*, 1998).

In liver of the rainbow trout (Nickerson *et al.*, 2001; S.G. Dugan, pers. commun.) the orders of potency for the antagonists (CGP >> ICI with no displacement by ATL) and the agonists (CLEN > ADR > PROC > RACT with no displacement by NOR and DOB) both support a strict β_2-AR pharmacology. Some quantitative differences exist between agonist and antagonist displacement in trout liver and muscle, but in general trends are similar. The most significant differences are much lower K_i values for RACT in muscle (approx. 120 nM) than liver (7090 nM; S.G. Dugan, pers. commun.). Furthermore when comparing liver and muscle, we observe for the antagonists, similar to identical values for CGP, 2-5 fold lower values for ICI, similarly no displacement for ATL, while for the agonists, similar to slightly lower values for CLEN. Similar in red but much lower in white for ADR, approximately 3 fold lower values for PROC and much lower values for RACT, in red and white muscle compared to liver. Also, NOR displaced in both red and white muscle but not in liver, and DOB displaced in red muscle but not in white muscle or liver. This may also be evidence for the presence of more than one β-AR (β_1- β_2- and/or β_3-ARs), with similar affinities since the Scatchard analysis (Fig. 2.1C) revealed only a single class of binding sites over the range of concentrations used in the specific binding assays. In cattle, competitive ligand binding studies suggested the presence of β_1- and β_2-ARs but saturation analysis indicated one binding site without distinguishing between them (Sillence and Matthews, 1994). Furthermore, studies in pigs and rodents reported RACT to have slightly less affinity for β_1- compared with β_2-AR (Colbert *et al.*, 1991; Moody *et al.*, 2000) which could explain the higher affinity in red and white muscles, if we assume more than one subtype in muscle compared with liver which is exclusively β_2-AR. In addition, molecular evidence indicates the expression of a rainbow trout putative β_2-ARs in liver and red and white muscles (Nickerson *et al.*, 2001), while a rainbow trout putative β_3-AR is expressed in red and white muscles but not liver (J.G. Nickerson, pers. commun.). Also possible contamination by red blood cells, which are known to have β-ARs, could change the pharmacology and may explain differences observed between muscle types. Therefore, the results of the agonist displacement studies need to be evaluated with caution, as it is possible that more than one β-AR exists in fish muscle as in mammalian skeletal muscle. Also the coupling of these different subtypes to the signaling transduction pathway could be different as was shown in rat muscle (Roberts and Summers, 1998).

Interestingly the two β_2-AA, CLEN and RACT, used in studies to enhance muscle growth in the meat industry (mammals and fish; Beermann, 1993; Mustin and Lovell, 1993; Vandenberg and Moccia, 1998; Vandenberg *et al.*, 1998), displaced with high affinity the radiolabelled ligand ^{3}H-CGP. Binding of these two β_2-AAs to rainbow trout muscle membrane β-ARs would imply possible direct effects upon skeletal muscle and the need for further investigations of these agents for the aquaculture industry.

2.6.2 β-Adrenoceptor Coupling to Adenylyl Cyclase

To establish coupling between β-AR occupancy and the receptor transduction pathway, the production of cAMP was determined. This study used the ACase/cAMP assay on muscle membrane preparations (similar to studies carried out on hepatic membranes of fish; Fabbri *et al.*, 1992; 1995a; 1995b; 2001) rather than the whole muscle or transverse muscle slices commonly used in mammalian studies due to the anatomical differences between fish and mammalian skeletal muscles (Johnston, 1982). Direct comparisons of cAMP production rates between this study and the mammalian literature are difficult to make, but obvious qualitative comparisons are possible. Roberts and Summers (1998), using soleus muscle slices from young rats, reported dose-dependent cAMP production with the β_2-AA (-)-isoproterenol (ISO) with 50% of the maximum response (i.e. the response at 10 μM) reached at concentrations of 10 to 100 nM. CLEN and RACT in both red and white muscle membranes of the rainbow trout (Fig. 2.4A and 2.4B, respectively) increased cAMP concentration in a dose dependant fashion but saturation at 10 μM was not achieved. As in the mammalian study, cAMP production in red and white muscle of trout was blocked by the non-selective β-adrenergic antagonist PROP (Fig. 2.5A and 2.5B, respectively). FSK, which directly stimulates mammalian ACase independent of the hormone receptor (Zhang *et al.*, 1997), activated mammalian ACase by 7-8 fold at 10 μM compared to values reached using 10 μM ISO. However in both red and white muscle membranes of the rainbow trout, FSK at a concentration of 10 μM produced approximately 3-fold higher cAMP production rate compared with values reached using 1 μM of CLEN, RACT or ISO and 7-14 fold above basal cAMP production. The difference in potency of FSK may reflect specificity of this agent to mammalian ACase rather than fish ACase, but also tissue preparation and the actual amount of ACase.

Studies in fish liver have reported basal ACase/cAMP activities (in pmol cAMP • mg protein^{-1} • 10 min^{-1}) of 20, 40, 7.6 and stimulated (1 to 10 μM ADR) of 40-50, 63, 20.5 in American eel (Fabbri *et al.*, 1998; 2001), bullhead catfish (Fabbri *et al.*, 1992) and rainbow trout (Fabbri *et al.*, 1995a), respectively. In comparison, basal values in trout RM were similar to eel while in WM basal values were more similar to rainbow trout liver. Stimulated values (1 μM ISO) in RM and WM were similar to bullhead liver stimulated values. These levels of agonist stimulated-cAMP production in red and white muscles were obtained with 10 times less agonist compared with liver. As the number of liver β_2-ARs is approximately 3-fold higher than in the muscle, the coupling between the AR and ACase in muscle may be greater than in liver.

It is interesting that RM had significantly higher basal rates of cAMP production than WM. However this trend was abolished in samples incubated with the antagonist PROP and the ACase activator FSK. We know of no similar observation in the mammalian literature, but coupled with the higher number of β-AR in RM may indicate a greater sensitivity or amplification of the message conveyed by ADR, NOR or any β-AA to ACase.

2.7 Conclusions

This study supports the presence of a single class of saturable β-adrenergic ^{3}H-CGP binding sites in both red and white muscle membranes of rainbow trout; there appears to be a greater number of binding sites in red than in white muscle. Mammalian β-adrenergic agonists and antagonists used support these binding sites as "atypical" β_2-ARs or implicate the presence of more than a single β-AR subtype in these muscles. The rainbow trout muscle β-AR transduces its cellular message through a G-protein, ultimately activating the ACase/cAMP pathway. The increase in cAMP upon stimulation with β_2-AAs was dose-dependent and blocked using the antagonist PROP. This study is the first to report the presence of functional β-ARs and the causative association between β-AA binding and subsequent receptor specific transduction in red and white muscles of fish. The precise effects of elevated levels of second messenger following chronic administration of β-AAs on muscle β-AR binding characteristics and β-AR mRNA expression as well as muscle protein synthesis are reported in Chapter 3.

CHAPTER 3. The Impact of Feeding β_2-Adrenergic Agonists on Rainbow Trout Red and White Muscles

3.1 Introduction

The potential for β_2-adrenergic agonists (β_2-AA) to modify growth rate and body composition in mammals and birds has been investigated. The most significant effects of β_2-AA are modifications to growth with increased accretion of skeletal muscle protein and decreased body fat (see reviews: Yang and McElligott, 1989; Kim and Sainz, 1991; Reeds and Mersmann, 1991; Beerman, 1993; Mersmann, 1998; Moody *et al.*, 2000; Mills, 2001; 2002; Beermann, 2002). The question of whether the binding of these β_2-AAs to β_2-adrenoceptors (β_2-ARs) mediates this growth response in animals is not fully established. Research has been directed at understanding how β_2-ARs might mediate increased growth and protein accretion and whether these effects are exerted directly, on adipose and muscle tissue or mediated by secondary factors (Mills, 2002).

The current hypothesized model of how β_2-AAs increase muscle protein accretion in mammalian muscle points towards a direct mechanism. That is, β_2-AAs bind to muscle β_2-ARs with subsequent activation of downstream transduction pathways (Moody *et al.*, 2000; Mills 2001, 2002; Beermann, 2002). The evidence and arguments for this direct mechanism were presented in Section 1.5.2.3, but will be summarized briefly here. Studies aimed at providing evidence for a direct mechanism of action include experiments indicating the dependence of activation of β_2-ARs by β_2-AAs using selective and non-selective β-adrenergic antagonists. Most report blocking of the muscle hypertrophy using 10 to 100 times higher concentrations of antagonists than agonists (Reeds *et al.*, 1988; MacLennan and Edwards, 1989) and equivalent concentrations of antagonists and agonists (Choo *et al.*, (1992). These experiments however do not completely resolve the issue of a direct or indirect mechanism of action, but only show dependency of the activation of the a β_2-AR somewhere in the animal. Despite this, there is evidence that muscle responds directly to β_2-AA. Many studies have shown quantitative measurements of cAMP response in various muscle preparations (Sillence and Matthews, 1994; Roberts and Summers, 1998; Shappell *et al.*, 2000). Moreover, there is extensive documentation of muscle β_2-AR desensitization or down-regulation following chronic exposure to β_2-AAs, which implies direct stimulation at the level of the muscle (Smith, 1989; Spurlock *et al.*, 1994). Together these findings point towards a direct mechanism of action, but still fail to rule out an indirect mechanism of action. However, a novel technique, the "closed arterial hind-limb perfusion technique", is to date the best evidence by far for a direct mechanism of action of the β_2-AAs on muscle as it focuses on muscle alone, excluding possible input by other hormones or factors (Byrem *et al.*, 1998). The continuous infusion of cimaterol directly increased muscle amino acid uptake and enhanced muscle protein accretion in cattle (Byrem *et al.*, 1998).

In addition, studies attempting to show an indirect mechanism also point towards the direct route of action. Many studies in mammals have monitored growth hormone (GH), thyroxine (T_4), insulin-like growth factor-I (IGF-I) and insulin levels following chronic treatments with β_2-AAs. The results are inconclusive as increases, decreases and no change are observed with no clear response (Beermann, 2002). However, studies performed on endocrine-altered animal models (GH-deficient, hypophysectomized, diabetic, adrenolectomized and hypothyroid animals) have greatly clarified the issue as β_2-AAs treatments all show muscle hypertrophy in these animals (Mills, 2002; Beermann, 2002). Moreover studies combining treatments with β_2-AAs and GH found additive effects, which suggest different responses and possibly separate mechanism of action of β_2-AAs and GH (Beermann, 2002). In summary, the combination of 1) the apparent negligible indirect effects of β_2-AAs in the endocrine-altered animal models, and 2) the direct effects of β_2-AAs monitored using the hind-limb perfusion, point towards a direct mechanism of action independent of other hormones or factors.

As noted briefly above, a characteristic of many hormone-receptor signaling systems is the mechanism of feedback regulation and this too can be used as an indicator of direct β_2-AA-mediated effects on muscle. This feedback regulation mechanism, referred to as agonist-induced desensitization, is well documented for mammalian β_2-ARs (Section 1.5.2.1). Multiple mechanisms contribute to desensitization and they are divided into acute "uncoupling" and chronic "down-regulation" responses to agonist stimulation. The latter, which is relevant to chronic treatments with agonists as in the present study, is defined as a decline in the total number of receptors and contributes to cellular desensitization. It occurs more slowly than uncoupling and may be subject to recovery. This decline in receptor numbers is a result of both decreased receptor protein synthesis and increased degradation, mediated by processes of endocytosis and hydrolysis in lysosomes (Lefkowitz, 1998). Chronic exposure has been shown to decrease receptor mRNA abundance for β_1- and β_2-ARs likely contributing to the decline in receptor protein (Liggett and Lefkowitz, 1994). Furthermore, studies indicate that the growth promoting actions of β_2-AA are effective for a limited time period corresponding to the period of time needed for down-regulation (rats, Rothwell *et al.*, 1987; Kim *et al.*, 1992; pigs, Smith, 1989).

The effect of β_2-AAs on skeletal muscle, as mentioned, is an increase in muscle mass relative to body weight; this increase is attained by pure hypertrophy (increase size of the muscle fibers), not hyperplasia (increase in number of muscle fibers) [Beermann, 2002]. This hypertrophy is due to modulation of muscle protein turnover, indicating that protein synthesis, degradation, or both are impacted. Both sides of the equation have been implicated as contributing to increased muscle gain (Kim and Sainz, 1992). Protein degradation is not typically measured directly, but rather as the difference between measured rates of synthesis and gain. If increased rates of synthesis are not observed then protein degradation is assumed to be responsible (Reeds *et al.*, 1986). Protein synthesis as shown using amino acid tracer experiments is increased in several species, but responses tend to be transient (Emery *et al.*, 1984; Bergen *et al.*, 1989; Maltin *et al.*, 1989; Byrem *et al.*, 1998). Alternatives to the measurement of protein synthesis and degradation are to measure mRNA expression of specific genes or enzymes important in protein synthesis and degradation. Increases in mRNA abundance for myofibrillar proteins (α-actin, myosin) and calpastatin (endogenous inhibitor of calpains, proteases important in protein degradation) are observed (Moody *et al.*, 2000; Mills, 2002; Beermann, 2002), which would favor a role for both increased synthesis and decreased degradation rates. Furthermore, a direct link is reported between cAMP and transcriptional regulation of the genes for myosin heavy chains in cardiomyocytes (Gupta *et al.*, 1996) and cattle calpastatin (Cong *et al.*, 1998). Increases in mRNA can be interpreted as evidence for increased rates of transcription or increased stability of mRNA encoding for such proteins.

To date, β_2-AA studies in fish have been restricted to reports on residue distribution (Brambilla *et al.*, 1994) and effects on body composition (Mustin and Lovell, 1993; Webster *et al.*, 1995; Vandenberg and Moccia, 1998). One study assessed the indirect mechanism of β_2-AA using GH, but found slight decreases in GH without evidence for an anabolic response (Vandenberg *et al.*, 1998). The study by Vandenberg and Moccia (1998) reported an increase in the percent protein of moisture free muscle with β_2-AA treatments, which is at the moment the best evidence for a modulation of the muscle protein turnover (Mustin and Lovell, 1993). No direct measurements of protein turnover have been reported. Of the few studies on β-AR down-regulation in fish, only one was able to show this in rainbow trout red blood cells following chronically elevated catecholamine levels (Gilmour *et al.*, 1994). However no such studies are reported for skeletal muscle.

3.2 Hypothesis

Feeding rainbow trout, the β_2-AAs clenbuterol and ractopamine will enhance muscle growth by increasing muscle protein synthesis. However, sustained stimulation of β_2-ARs by β_2-AAs will cause down-regulation of the receptors and decrease β_2-AR mRNA expression.

3.3 Objectives

The main objectives of this study are to determine the impact of feeding rainbow trout β_2-AAs on 1) muscle β_2-ARs binding characteristics and 2) muscle β_2-AR mRNA expression, and 3) muscle fractional protein synthesis rates.

3.4 Materials and Methods

3.4.1 Animals, β_2-Adrenergic Agonist Diets and Experimental Design

Female juvenile rainbow trout (*Oncorhynchus mykiss*), weighing approximately 75-80 g, were obtained from Linwood Acres Trout Farm (Campellcroft, ON). Fish were transported to the University of Ottawa Aquatic Care Facility and were maintained in fibreglass holding tanks (110-115 L) of well aerated, dechloraminated City of Ottawa tap water at 13.0 ± 1.0 °C. Fish were subjected to a constant 12L:12D photoperiod and fed five times weekly with commercial trout pellets [(Martin Mills 5 PT, 5 mm in size and composed of: 41.0% crude protein (min); 11.0% crude fat (min); 3.5% crude fibre (max); 1.0% calcium (actual); 0.85% phosphorus (actual); 0.45% sodium (actual); 6,800 IU • kg^{-1} vitamin A (min); 2,100 IU • kg^{-1} vitamin D (min); 80 IU • kg^{-1} vitamin E (min); 200 IU • kg^{-1} vitamin C (min)].

Following an acclimation period of approximately one month, fish were anaesthetized in MS-222 and tagged with VIalpha alphanumerically coded tags (Northwest Marine Technology Inc., WA) into the periocular transparent tissue. Body weight (BW in g) and length (L in cm) as well as any individual distinguishing external features were recorded (to help individual identification in the event a tag was lost). Fish were separated evenly into three groups of 20-21 fish per 110-115 L tank (Fig. 3.1). Fish were fed twice daily (total of 1.5% BW • day^{-1}) with one of three experimental diets: SHAM (carrier), CLEN (40 ppm clenbuterol; Sigma) or RACT (40 ppm ractopamine Eli-Lilly). Commercial trout pellets (Martin Mills 5 PT, size 5 mm) (2 kg) in a Pyrex bowl (37 x 25 x 6 cm in size) were carefully and evenly sprayed with 25 mL of 95% EtOH solution containing the antioxidant ascorbic acid (0.1 mg • mL^{-1}) and 96 mg of clenbuterol (CLEN) or 96 mg of ractopamine (RACT). The EtOH solution contained 20% more clenbuterol or ractopamine to compensate for loss during spraying.

Five fish from each group (4 average weight and length for the particular group plus 1 that was less than average to act as a control) were taken after 30 days of treatment and used for the protein synthesis experiments (Fig. 3.1) and the remaining fish were fed for an additional 7 days after which they were used for the receptor experiments. The day of the experiment, 4 h after the morning meal, fish were sacrificed by a sharp blow to the head. Weight and length were recorded, blood was immediately removed by caudal puncture using a heparinized syringe and kept on ice to be subsequently centrifuged at 12,000 x g (Eppendorf 5414c) to isolate plasma. Plasma samples were aliquoted and frozen at -80 °C until used. Tissues (liver, heart, red and white muscles) were isolated (liver and heart weight were recorded), freeze clamped between aluminum blocks cooled in liquid N_2 and stored at -80 °C. Small pieces of liver (approx. 500 mg), white and red muscles were kept for RNA isolation. Fish were acclimated in May, feeding started in June and ended at the beginning of July; receptor characterization

Figure 3.1: Diagram representing the experimental design in which 20-21 fish were kept in three groups, SHAM, CLEN and RACT receiving control diet, 40 ppm clenbuterol diet and 40 ppm ractopamine diet, respectively. Groups received a ration of 1.5% BW • day^{-1}, which was separated into two rations fed morning and afternoon. Five fish from each group (4 average, 1 small) were used for protein synthesis experiments after a treatment period of 30 days, while the remaining (15-16 fish) were kept under treatment conditions for 7 more days until sacrificed and tissues isolated. Fish used for protein synthesis were injected with ^{3}H-Phe and kept in a separate tank further subdivided in three where fish could be isolated according to treatment group for the incorporation period of 4 h after which the fish were sacrificed and tissues isolated.

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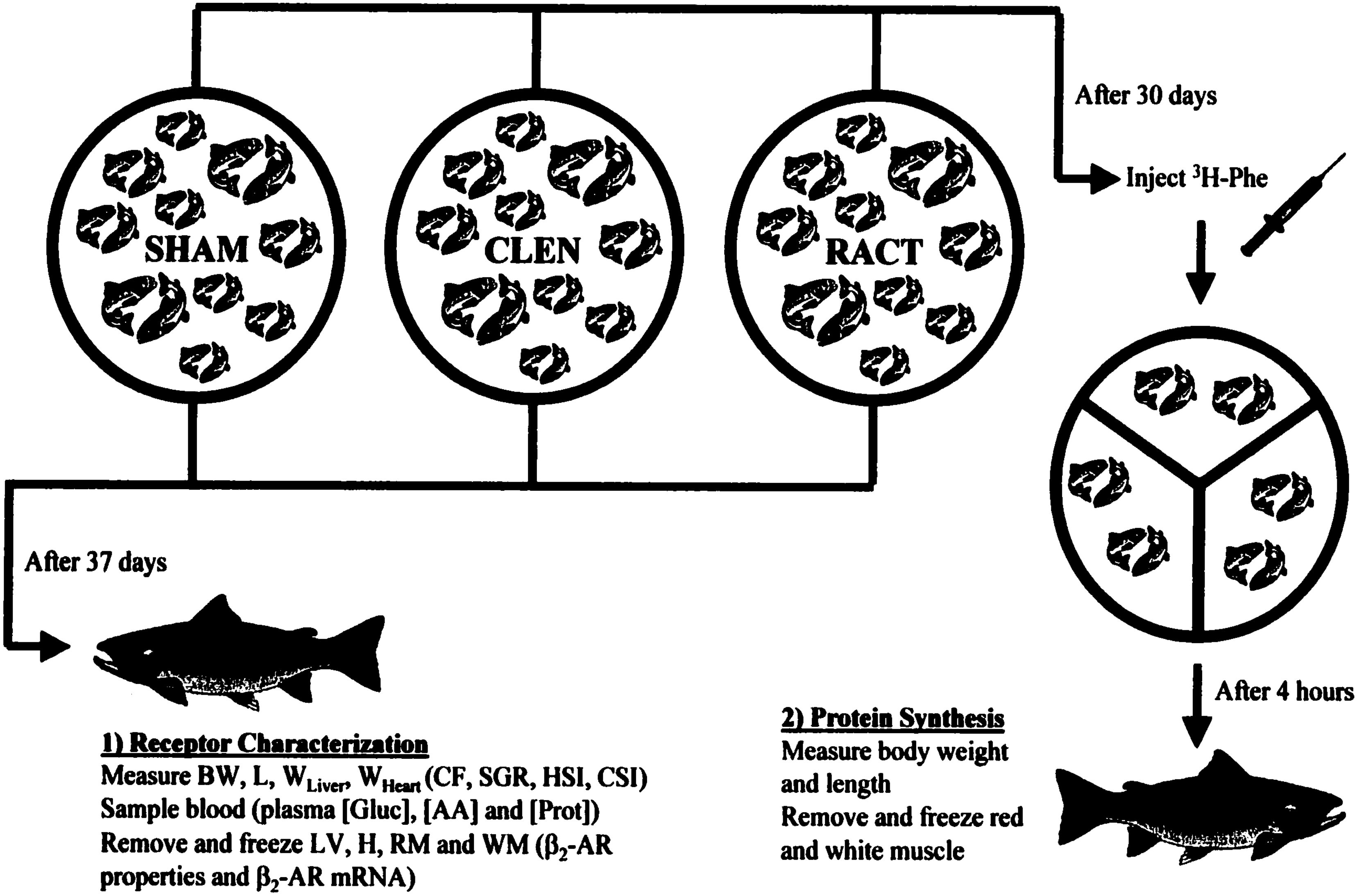

1) Receptor Characterization
Measure BW, L, W_{Liver}, W_{Heart} (CF, SGR, HSI, CSI)
Sample blood (plasma [Gluc], [AA] and [Prot])
Remove and freeze LV, H, RM and WM (β_2-AR properties and β_2-AR mRNA)

2) Protein Synthesis
Measure body weight and length
Remove and freeze red and white muscle

experiments were conducted between July and August, while molecular experiments and blood parameter measurements were carried out between September and November on frozen samples.

3.4.2 Body and Physiological Parameters and Calculations

Using the recorded initial and final body weights (BW_i and BW_f) and lengths (L_i and L_f) of each individual fish, the initial and final condition factors (CF_i and CF_f), were calculated (Busacker *et al.*, 1990) as follows:

$$CF_i = 100 \bullet \frac{BW_i}{L_i^3} \qquad \text{and} \qquad CF_f = 100 \bullet \frac{BW_f}{L_f^3}$$

Where the weight is in g and the length in cm. Specific growth rates (SGR_{exp}, in % • day^{-1}) were also calculated from the recorded BW_i and BW_f of each individual fish according to Ricker (1979):

$$SGR_{exp} = 100 \bullet (Ln(BW_i) - Ln(BW_f)) \bullet t^{-1}$$

Where t is 37 days. Hepato-somatic index (% BW) and cardio-somatic index were also calculated using the liver weight (W_{Liver}, in g) and heart weight (W_{Heart}, in g) and the BW_f (g):

$$HSI = \frac{W_{Liver}}{BW_f} \bullet 100 \qquad \text{and} \qquad CSI = \frac{W_{Heart}}{BW_f} \bullet 100$$

Plasma samples were assayed for clenbuterol using an EIA kit for CLEN (Neogen, Lexington, KY; see Appendix for details); no commercial estimates for RACT are available. Plasma glucose estimates were obtained using the enzymatic Hk/G6PDH assay modified for microplates as reported by Mommsen and Moon (1990). Plasma samples were also assayed for amino acids by the method of Troll and Cannan (1953). Plasma protein estimates were obtained as previously described for membrane protein (Section 2.4.2) using the Bicinchoninic Acid (BCA) assay (Sigma) with bovine serum albumin (BSA) as a standard. All of the above assays were monitored using a SPECTRAmax PLUS 384 (Molecular Device, Sunnyvale, CA) spectrophotometer.

3.4.3 β_2-Adrenoceptor Binding Characteristics

3.4.3.1 Muscle Membrane Preparation

Muscle membrane preparation was identical to that described in Section 2.4.2, with the exception that specific binding assays were done within 2 weeks of membrane preparation.

3.4.3.2 Specific Binding Assays

Specific binding assays were performed as described in Section 2.4.3.1.

3.4.4 β_2-Adrenoceptor mRNA Expression

3.4.4.1 Total RNA Isolation

Total cellular RNA was isolated from frozen tissues of the rainbow trout using Trizol reagent (GibcoBRL, Burlington, ON, Canada) according to the protocol provided by the manufacturer. RNA concentrations and quality were verified using spectrophotometry and gel electrophoresis. Aliquots of RNA to be used in RNase protection assays were treated with DNase 1, DNA Free™ (Ambion Austin, Tx, USA) before use.

3.4.4.2 RNase Protection Assay

Levels of expression of the rainbow trout β_2-AR gene in red and white muscles were determined using the RNase protection assay, RPAIII™ (Ambion) as described in Nickerson *et al.* (2001). The RPA was chosen over regular Northern blot analysis for its better resolution of the level of expression of the β_2-AR gene. The templates used to synthesize the probes for the RNase protection assay experiments were kindly provided by James Nickerson and were obtained by PCR amplification of the 3rd intracellular loop region from a plasmid clone of the rainbow trout β_2-AR (bases 778 to 880) and by PCR amplification of a portion of the rainbow trout β-actin (bases 275 to 610). The primers used to amplify both probe templates incorporated the promoter sequences for T7 and SP6 RNA polymerases (see Appendix, Table A.1) so that antisense or sense RNA probes could be transcribed. Radiolabeled antisense RNA probes were transcribed using MAXIscript™ (Ambion) with T7 RNA polymerase and ^{32}P-UTP (Amersham Pharmacia, Baie d'Urfé, QC, Canada). Full-length probes were purified from a denaturing 4% polyacrylamide, 8 M urea gel. Approximately 4.2 x 10^4 cpm of the β_2-AR probe and 4.2 x 10^3 cpm of the β-actin probe were co-hybridized to 20 μg of total RNA for approximately 16 h at 42 °C. Nonhybridized transcripts were digested with approximately 0.4 units of RNase A and 15 units of RNase T1 (cloned) at 37 °C for 90 min. Protected fragments were resolved on a denaturing 6% polyacrylamide, 8 M urea gel that was dried and subjected to autoradiography.

3.4.4.3 Semi-Quantitative Measurements of β_2-Adrenoceptor mRNA

The 8 M urea gels, on which the protected fragments were resolved, were dried onto chromatography paper in a gel drier (BIORAD, Model 583) for 2 h. The dried gels were then placed into the cassette with a phosphor screen for 18-28 h. Following exposure the phosphor screen was scanned using a phosphor-imager (BIORAD Molecular Imager FX) and density of β_2-AR and β-actin bands was quantified using Quantity One 4.1.1 software (BIORAD). β_2-AR mRNA expression was expressed as a ratio of β_2-AR band density/β-actin band density.

3.4.5 Fractional Protein Synthesis Rates

3.4.5.1 Injections of L-[2,6-^{3}H]-Phenylalanine

Protein synthesis studies, using the phenylalanine flooding method, were undertaken on 5 fish taken from each of the three groups after exactly 30 days of feeding β_2-AAs (see Section 3.4.1). Each fish was anesthetized in MS-222, then received a single intravenous injection of L-[2,6-^{3}H]-phenylalanine (^{3}H-Phe; Amersham Canada Ltd, Oakville, ON; specific activity 55 Ci • mmol^{-1}) solution: 1 mL • 100 g^{-1} BW of a 150 mM solution of L-phenylalanine in 0.9 %

NaCl (specific activity approx. 50 μCi • mL^{-1}). All 15 fish (5 from each of the 3 treatments) were then placed into a holding tank (110-115 L) equipped with a tripartite separator keeping the fish with their respective groups for the remaining 4 h incorporation period. The fish were then killed by a sharp blow to the head and weight and length as well as identification were recorded. The tissues, liver, heart, red and white muscles (muscle was taken from the same location directly below dorsal fin), were quickly dissected and frozen in liquid N_2.

3.4.5.2 Tissue Treatments and Protein Fractionation (see Fig. 3.2)

Samples (100 mg) of red and white muscles were crushed using a porcelain mortar and pestle kept at liquid N_2 temperatures. The powder was resuspended in 2.0 mL 0.5 M perchloric acid (PCA) and immediately sonicated for 10-15 sec (Fig. 3.2). The homogenate was centrifuged at 6,000 x g for 10 min in a Sorvall RC-5B Plus (SS 34 rotor) at 4 °C (all subsequent centrifugations are identical). The supernatant was collected, the pellet was discarded and 0.6 mL saturated tri-potassium-citrate was added to the supernatant. The resulting solution was centrifuged at 6,000 x g for 10 min. The pellet was once again discarded and the supernatant was labeled as the amino acid free pool (FP) and kept at -4 °C until used.

Samples (200 mg) of red and white muscles were crushed as noted and the resulting powder was resuspended in 4.0 mL buffer A (in mM: 100 KCl, 5 Tris-HCl, 1 DL-dithiothreitol (DTT), at pH 7.0) and sonicated for 10-15 sec (Fig. 3.2). The protein was precipitated with a 20% perchloric acid (PCA) solution added drop-wise, and centrifuged at 6,000 x g for 10 min. The supernatant was discarded and the pellet was washed twice by adding 4.0 mL 0.5 M PCA and centrifuged at 6,000 x g for 10 min. The supernatant was once again discarded and the pellet solubilized in 10 mL 0.3 M NaOH. The solution rested at room temperature (~19.0°C) for 1 h. The protein was then precipitated by adding 2.0 mL 20% PCA and centrifuging at 6,000 x g for 10 min. The supernatant was discarded and the pellet was washed twice by

Figure 3.2: Diagramatic representation of tissue treatment and fractionation. Desired fractions are in bold and are (from left to right): free pool (FP), whole protein fraction (WP), sarcoplasmic soluble protein fraction (SSP), myofibrillar protein fraction (MyofP), mitochondrial protein fraction (MitoP) and post-mitochondrial protein fraction (post-MitoP).

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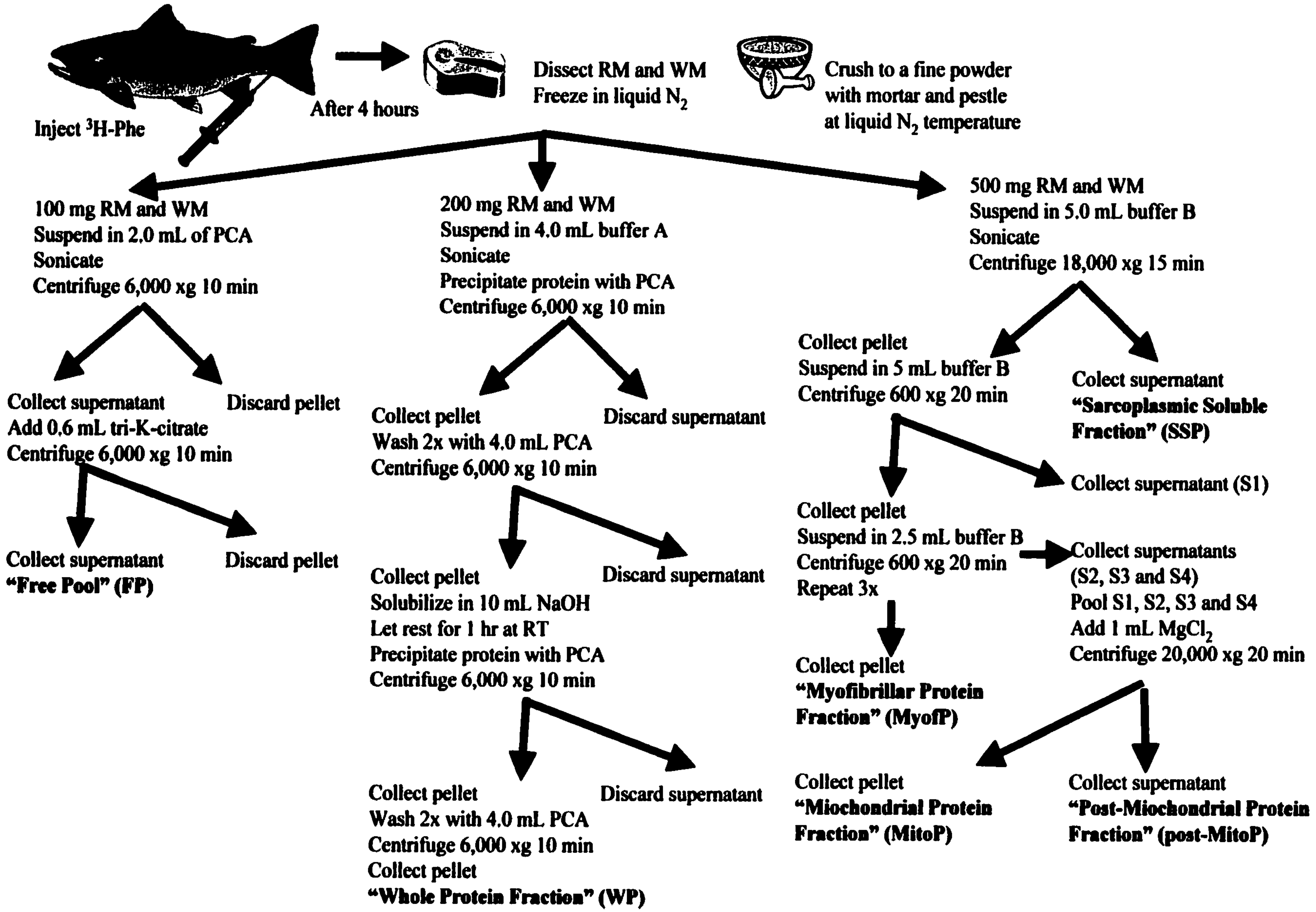
Inject ^{3}H-Phe
After 4 hours
Dissect RM and WM
Freeze in liquid N_2
Crush to a fine powder
with mortar and pestle
at liquid N_2 temperature
100 mg RM and WM
Suspend in 2.0 mL of PCA
Sonicate
Centrifuge 6,000 xg 10 min
Collect supernatant
Add 0.6 mL tri-K-citrate
Centrifuge 6,000 xg 10 min
Discard pellet
Collect supernatant
"Free Pool" (FP)
Discard pellet
200 mg RM and WM
Suspend in 4.0 mL buffer A
Sonicate
Precipitate protein with PCA
Centrifuge 6,000 xg 10 min
Collect pellet
Wash 2x with 4.0 mL PCA
Centrifuge 6,000 xg 10 min
Discard supernatant
Collect pellet
Solubilize in 10 mL NaOH
Let rest for 1 hr at RT
Precipitate protein with PCA
Centrifuge 6,000 xg 10 min
Discard supernatant
Collect pellet
Wash 2x with 4.0 mL PCA
Centrifuge 6,000 xg 10 min
Collect pellet
"Whole Protein Fraction" (WP)
Discard supernatant
500 mg RM and WM
Suspend in 5.0 mL buffer B
Sonicate
Centrifuge 18,000 xg 15 min
Collect pellet
Suspend in 5 mL buffer B
Centrifuge 600 xg 20 min
Colect supernatant
"Sarcoplasmic Soluble Fraction" (SSP)
Collect supernatant (S1)
Collect pellet
Suspend in 2.5 mL buffer B
Centrifuge 600 xg 20 min
Repeat 3x
Collect supernatants
(S2, S3 and S4)
Pool S1, S2, S3 and S4
Add 1 mL $MgCl_2$
Centrifuge 20,000 xg 20 min
Collect pellet
"Myofibrillar Protein Fraction" (MyofP)
Collect pellet
"Miochondrial Protein Fraction" (MitoP)
Collect supernatant
"Post-Miochondrial Protein Fraction" (post-MitoP)

adding 4.0 mL 0.5 M PCA and centrifuging at 6,000 x g for 10 min. The pellet was labeled as the whole protein fraction (WP) and was kept at -4 °C until hydrolyzed.

The fractionation of muscle protein was performed using 500 mg of red and white muscles according to modified procedures of Fauconneau *et al.* (1995) and Etlinger *et al.* (1975). Samples were crushed as noted and the resulting powder was resuspended in 5.0 mL buffer B (in mM: 100 KCl, 5 Tris-HCl, 1 DTT, 2 chloramphenicol and 10 mg • mL^{-1} cycloheximide, at pH 7.0) and sonicated for 10-15 sec (Fig. 3.2). The homogenate was centrifuged at 18,000 x g for 15 min. The supernatant was collected, labeled sarcoplasmic soluble protein fraction (SSP) and kept at 4 °C until the remaining fractions were obtained. The pellet was resuspended in 5 mL buffer B and then centrifuged at 600 x g for 20 min. The supernatant was collected and labeled S1, the pellet was resuspended in 2.5 mL buffer B and centrifuged at the same settings. The resulting supernatant, S2, was collected and pooled with S1. The pellet was resuspended again in 2.5 mL buffer B, sonicated for 10-15 sec and centrifuged once more. The supernatant, S3, was collected and pooled with S1 and S2. The previous step was repeated and S4 was pooled with the previous supernatants. The precipitate contained the myofibrillar proteins and therefore was labeled myofibrillar protein fraction (MyofP) and was kept at 4 °C. One mL 3 mM $MgCl_2$ was added to the pooled supernatants (S1, S2, S3 and S4), and centrifuged at 20,000 x g for 20 min. The pellet contained organelle membranes and mainly mitochondrial membranes, and thus is referred to as the mitochondrial protein fraction (MitoP); the supernatant is referred to as the post-mitochondrial protein fraction (post-MitoP) according to Etlinger *et al.* (1975) and McMillan and Houlihan (1990).

To each of the four protein fraction solutions, SSP, MyofP, MitoP and post-MitoP, 10 mL 0.3 M NaOH was added and the solution was rested for 1 h at room temperature (approx. 19.0 °C). Two mL 20% PCA was added to the solutions to precipitate the protein and the fractionations were then centrifuged at 6,000 x g for 10 min. The pellet was then washed twice with 4.0 mL 0.5 M PCA and centrifuged at 6,000 x g, discarding the supernatants. The pellets were then flash frozen in liquid N_2 and kept at -80 °C until analyzed.

3.4.5.3 Protein Hydrolysis

The frozen pellets were transferred into hydrolysis cap tubes (Kimax, VWR), 0.3 mL of high purity 36-38% HCl (OmniTrace HCl, VWR) was added and the samples were sonicated until the pellet was completely dispersed. The tubes were then sealed and incubated at 110 °C for 24 h. The hydrochloric acid was then removed by evaporation in a vacuum dessicator in the presence of NaOH and P_2O_5 until dry. The amino acid residue was resuspended in 1 mL 0.5 M sodium citrate (pH 6.3), transferred into 2.0 mL screw cap plastic centrifuge tubes and stored at -80 °C until analyzed.

3.4.5.4 Analysis of Fractional Protein Synthesis Rates

The phenylalanine in the free pool (0.5 mL) and in the hydrosylate of the protein fraction (0.5 mL) was converted into β-phenylethylamine (β-PEA) by incubation at 50 °C for 24 h in 0.25 mL of a L-tyrosine-decarboxylase solution (0.7 and 1.4 units • mL^{-1} for the free pool and protein fractions, respectively, plus 0.5 M sodium citrate, 0.5 mg • mL^{-1} pyridoxal phosphate, at pH 6.3). The reaction was stopped by cooling on ice and adding 0.5 mL 3 M NaOH.

The remaining phenylalanine was separated from the β-PEA by successive extraction with heptane and chloroform. The sample (1.25 mL) was transferred to a 15 mL polypropylene conical tube and 5 mL of a 3:1 heptane:chloroform solution was added and vortexed vigorously. The upper organic phase (approx 4.5 mL) was transferred to another 15 mL tube, 2.5 mL chloroform plus 2 mL 0.01 M H_2SO_4 were added and the solution was vortexed vigorously. The pH of the upper aqueous phase was tested using litmus paper and the pH was corrected to approximately 1-2 pH units by adding H_2SO_4 drop-wise. Each sample of aqueous phase was aliquoted, 1 mL for radioactivity measurements of labeled β-PEA and 0.5 mL for fluorometric measurements of total β-PEA (labeled and unlabelled).

The 1 mL aliquot for radioactive β-PEA measurements was placed in polyethylene scintillation vials containing 5 mL scintillation cocktail (Safety-Solve; Research Products International, Mount Prospect, IL). The vials were left in the dark for at least 24 h and the radioactivity was determined using a Beckman Coulter LS6500 multi-scintillation counter. The radioactivity was expressed as dpm • mL^{-1} to be used later in calculations of fractional rate of protein synthesis (k_s). The 0.5 mL aliquot for fluorometric measurements was assayed for β-PEA content using a ninhydrin reagent (10 mM ninhydrin and 0.2 mM L-leucylalanine in 0.5 M KH_2PO_4 at pH 8.0) and β-PEA in 0.01 M H_2SO_4 as standards (Suzuki and Yagi, 1976; Garlick *et al.*, 1980; Houlihan *et al.*, 1986; Fauconneau *et al.*, 1995). The reaction was carried out at 60 °C for 1 h in darkness. The samples were then left to cool at room temperature (approx. 19.0 °C) for 15 min and 0.2 mL was transferred to a 96 well microplate. The fluorescence was measured with excitation at 390 nm and emission at 495 nm using a SPECTRAmax GEMINI XS microplate fluorometer. Measurements of β-PEA content were corrected for dilution and expressed as nmol • mL^{-1} to be used later in calculations of k_s.

3.4.5.5 Calculations of Fractional Rate of Protein Synthesis

The fractional rate of protein synthesis k_s (in % • day^{-1}) was calculated according to Garlick *et al.* (1980) from the specific activities of phenylalanine in the free pool S_A and in the protein fraction S_B (in dpm • $nmol^{-1}$) using the following equations:

$$k_s = \frac{S_B}{S_A} \bullet \frac{100}{t}$$

where t is the incorporation time (in days). The specific activities of the free pool or protein fractions were obtained by dividing the value obtained from the radioactivity measurements (dpm • mL^{-1}) by the value obtained from the fluorometric measurements (nmol • mL^{-1}).

3.4.6 Statistics

Receptor saturation and competition data were analyzed by the EBDA and LIGAND computer programs (Munson and Rodhard, 1980). All further data conversions used Microsoft Excel 2000, graphs were plotted using SigmaPlot 2000 (Jandel Scientific) and statistical differences were evaluated using appropriate tests with SigmaStat 2.0 (Jandel Scientific). A value of $P < 0.05$ was accepted to indicate significant differences.

3.5 Results

3.5.1 β_2-Adrenoceptor Binding Characteristics/mRNA Expression

3.5.1.1 Body and Physiological Parameters and Calculations

To assess individual SGR, fish were tagged at the beginning of the experiment. However, some of the fish lost their tags and their identity was assessed using the assumption that the biggest fish at the beginning would still be the biggest at the end of the experiment. This assumption was tested using all experimental fish that retained their identification tags (Fig. 3.3). A statistically significant positive correlation (Spearman's rank correlation coefficient, $P < 0.05$) was obtained when comparing BW_i and BW_f. This relation was then used to identify the few fish that had lost their tags and that did not possess any noticeable anatomical differences that would permit identification. The number of fish of each group identified using this method is reported on Table 3.1 (in square brackets).

The impact of feeding β_2-AA on various body and physiological parameters was assessed for fish fed treated diets for 37 days; these fish were also used for receptor assays. All

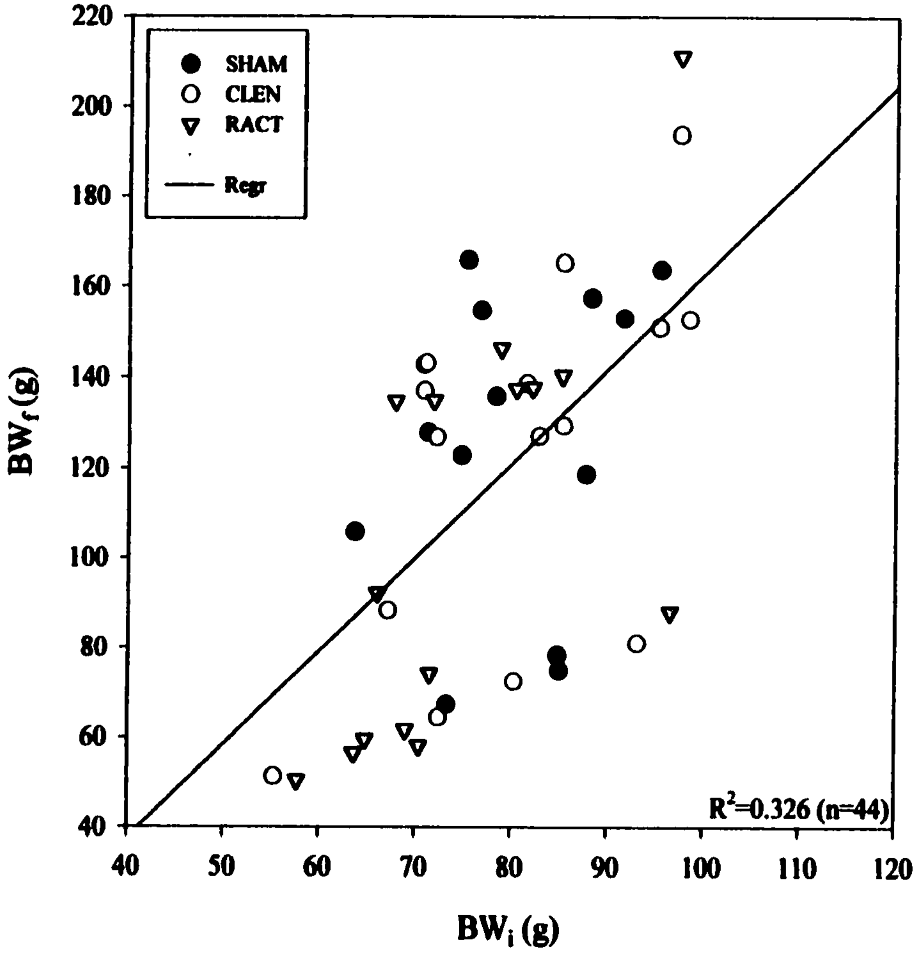

Figure 3.3: Relationship between individual known initial and final body weight (BW_i and BW_f, respectively; in g) of rainbow trout fed SHAM (solid circles), CLEN (open circles) and RACT (gray triangles, down) diets. A significant positive correlation was obtained (Spearman's rank correlation coefficient, $P < 0.05$) and subsequent calculations (Table 3.1) for trout with lost identification tags was assessed using this correlation.

Table 3.1: Body and physiological parameters for SHAM, CLEN and RACT treated rainbow trout for the receptor characterization experiments.

	SHAM		CLEN		RACT	
	Mean ± SEM	n	Mean ± SEM	n	Mean ± SEM	n
BW_i (g)	78.3 ± 2.3	15	78.4 ± 2.5	16	79.2 ± 2.8	15
BW_f (g)	122.8 ± 11.0^a	15	124.0 ± 10.4^a	16	122.8 ± 13.6^a	15
L_i (cm)	19.9 ± 0.3	15	19.7 ± 0.2	16	20.1 ± 0.2	15
L_f (cm)	21.7 ± 0.4^b	15	22.1 ± 0.3^b	16	22.0 ± 0.5^b	15
CF_i	0.99 ± 0.02	15	1.01 ± 0.02	16	0.98 ± 0.02	15
CF_f	1.15 ± 0.06^c	15	1.11 ± 0.05	16	1.09 ± 0.07	15
SGR_{exp} (% • day^{-1})	1.12 ± 0.28	15 [6]	1.16 ± 0.23	16 [5]	1.08 ± 0.30	15 [5]
W_{Liver} (g)	1.23 ± 0.20	15	1.40 ± 0.18	15 (1)	1.64 ± 0.34	14 (1)
HSI (% BW_f)	0.89 ± 0.10	15	1.04 ± 0.10	15 (1)	1.10 ± 0.17	14 (1)
W_{Heart} (g)	0.18 ± 0.02	14 (1)	0.16 ± 0.01	16	0.19 ± 0.02	14 (1)
CSI (% BW_f)	0.16 ± 0.02	14 (1)	0.14 ± 0.01	16	0.17 ± 0.01	14 (1)

$[\text{Gluc}]_{\text{Plasma}}$ (mM)	4.20 ± 0.37	10 (5)	4.74 ± 0.40	16	4.95 ± 0.60	15
$[\text{Prot}]_{\text{Plasma}}$ ($\text{mg} \bullet \text{mL}^{-1}$)	21.5 ± 2.5	10 (5)	22.6 ± 1.7	16	24.2 ± 2.5	15
$[\text{AA}]_{\text{Plasma}}$ ($\mu\text{g} \bullet \text{mL}^{-1}$)	29.1 ± 3.1	10 (5)	24.7 ± 2.4	16	30.9 ± 3.1	15
$[\text{CLEN}]_{\text{Plasma}}$ (nM)	BD	15	11.5 ± 3.5*	16	-	-

Values are means ± SEM: initial and final body weights (BW_i and BW_f); initial and final lengths (L_i and L_f); initial and final condition factors (CF_i and CF_f); specific growth rate (SGR_{exp}); liver weight (W_{Liver}); hepato-somatic index (HSI); heart weight (W_{Heart}); cardio-somatic index (CSI); plasma glucose, protein, amino acid and clenbuterol concentrations ($[\text{Gluc}]_{\text{Plasma}}$, $[\text{Prot}]_{\text{Plasma}}$, $[\text{AA}]_{\text{Plasma}}$ and $[\text{CLEN}]_{\text{Plasma}}$). There are no significant differences between treated vs. SHAM for any of the parameters measured (one-way ANOVA, $P < 0.05$) with the exception of the $[\text{CLEN}]_{\text{Plasma}}$ that were higher in CLEN compared with SHAM which were below detection (BD), represented by * (t-test, $P < 0.05$); there are significant differences when comparing BW_f with BW_i, L_f with L_i and CF_f with CF_i within the same treatment groups, represented by ^{a}, ^{b} and ^{c}, respectively (paired t-test, $P < 0.05$). Sample size is indicated and includes the number of missing samples represented in round brackets and data added using the BW_i vs BW_f correlation (Fig. 3.3) represented in square brackets. A dash represents no data available.

of the measured and calculated parameters from fish used in this feeding experiment are reported in Table 3.1. No statistically significant differences are noted among groups fed the different diets in any parameter measured and calculated with the exception of $[\text{CLEN}]_{\text{Plasma}}$ which was significantly higher in CLEN-fed compared with the SHAM (t-test, $P < 0.05$) group. For statistical analysis, a value of the lowest level detectable by the kit was given to below detection results (5 fish in SHAM, others were within the non-linear range and most likely do not contain any CLEN) and a value of the highest level detectable by the kit was given to those values above detection limits (6 fish in CLEN; refer to Appendix for details on the clenbuterol EIA kit). As noted on Table 3.1, there were subtle trends towards an increase in W_{Liver}, HSI and $[\text{Gluc}]_{\text{Plasma}}$ in β_2-AA-treated fish. When comparing initial and final parameters and calculations within the same group, there were significant statistical differences. Significant increases were observed in BW and L in all three groups, while CF increased during the treatment period in the SHAM group only with trends towards an increase in the two treatment groups.

3.5.1.2 Specific Binding Assays

Specific binding assays were performed to assess the affects of treatment on the affinity (K_d) and number (B_{max}) of β-adrenergic binding sites on red and white muscle membranes (Figs 3.4, 3.5, 3.6 and 3.7). Specific binding increased as the concentration of radiolabelled ligand increased to eventually saturate at approximately 1.5 nM. Specific binding was higher than non-specific binding up to concentrations near 3 nM. Scatchard analysis (Fig 3.5B RM; Fig 3.7B WM) was linear (EBDA; Munson and Rodhard, 1980) and indicated the presence of a homogeneous class of binding sites in both tissues and treatment groups (LIGAND, at $P < 0.05$; Munson and Rodhard, 1980).

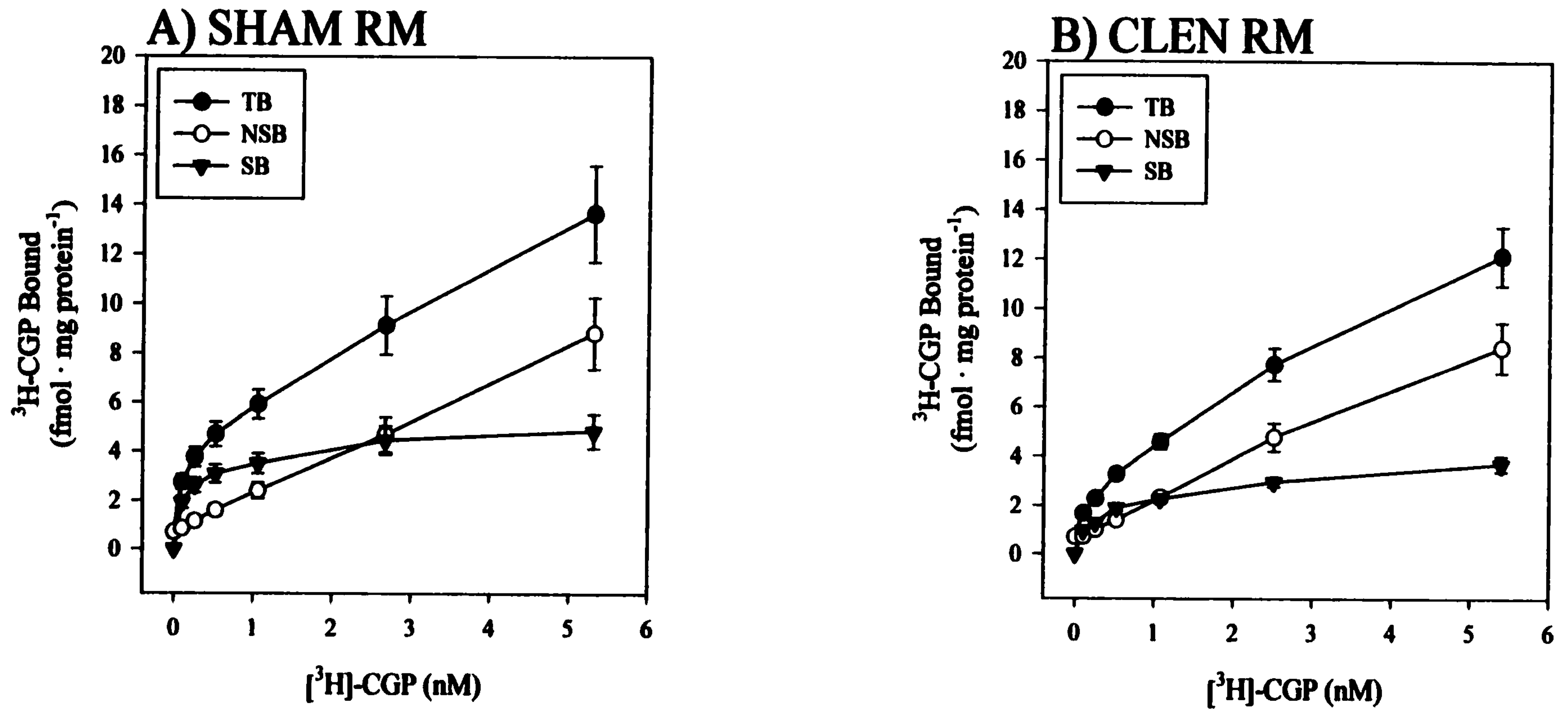

Figure 3.4: Radiolabelled saturation curves for ^{3}H-CGP binding to red muscle (RM) membranes prepared from rainbow trout fed SHAM (A) and CLEN (B) diets. Assays contained between 250-350 μg protein • 50 μL^{-1} and incubations were for 60 min with increasing concentrations of ^{3}H-CGP in the absence (total binding, TB; solid circles) and in the presence of 10 μM (±)-CGP 12177A (non-specific binding, NSB; open circles). Specific binding (SB; solid triangles, down) was assessed by subtracting the NSB from the TB. Values represent means ± SEM of 15-16 experiments (each experiment from an individual animal) done in duplicates.

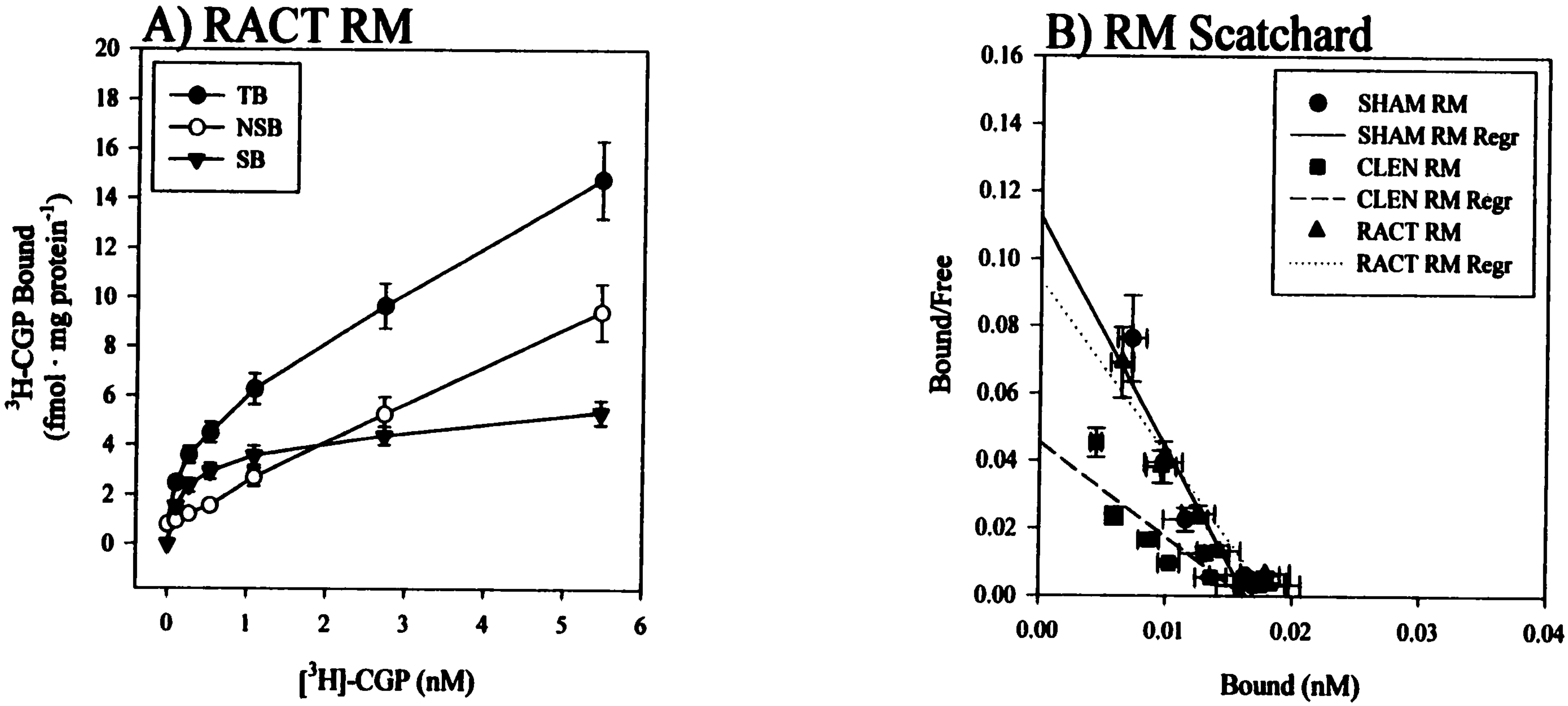

Figure 3.5: Radiolabelled saturation curves for ^{3}H-CGP binding to red muscle (RM) membranes prepared from rainbow trout fed RACT (A) diet. Conditions are as indicated in Fig. 3.4. Scatchard plots (B) of specific binding to RM membranes are shown for all RM data.

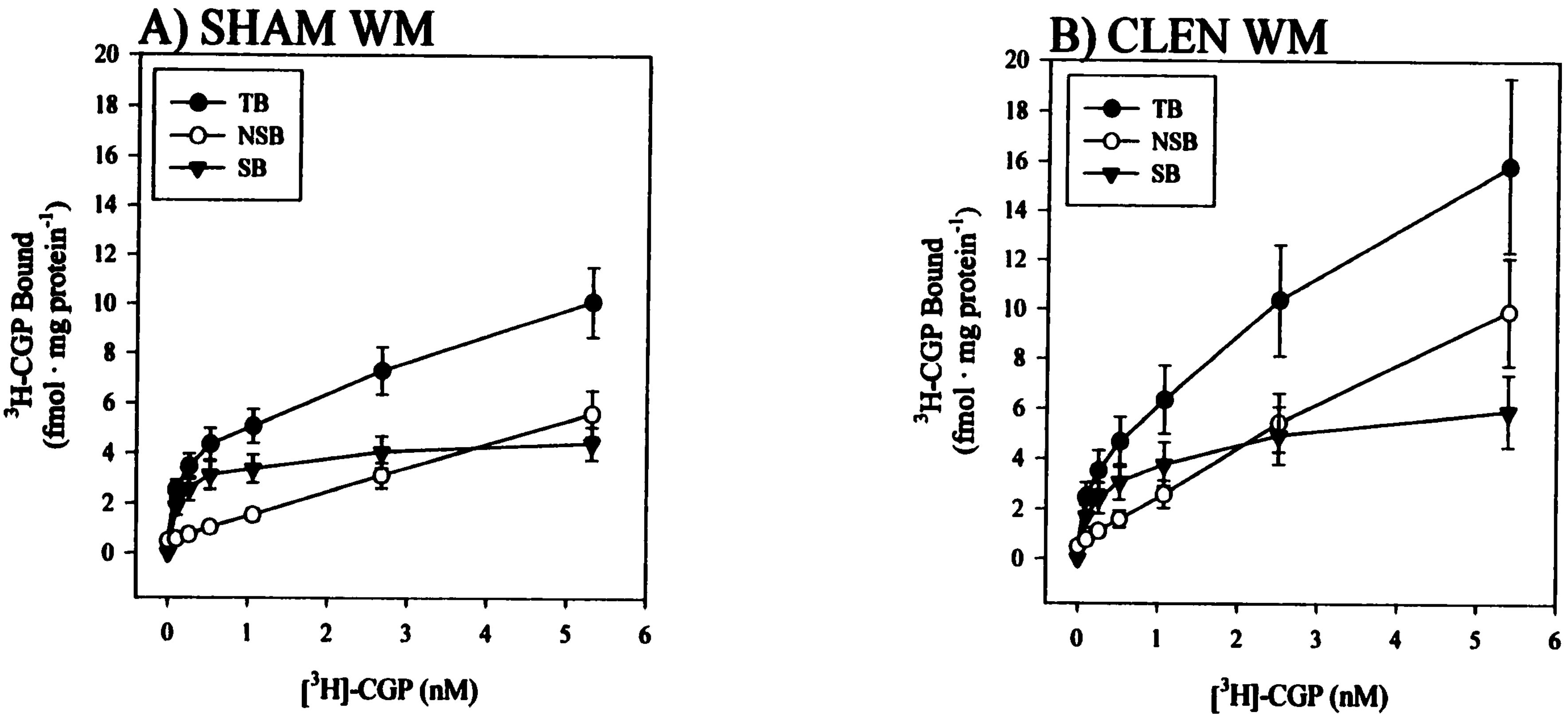

Figure 3.6: Radiolabelled saturation curves for ^{3}H-CGP binding to white muscle (WM) membranes prepared from rainbow trout fed SHAM (A) and CLEN (B) diets. Conditions are as indicated in Fig. 3.4.

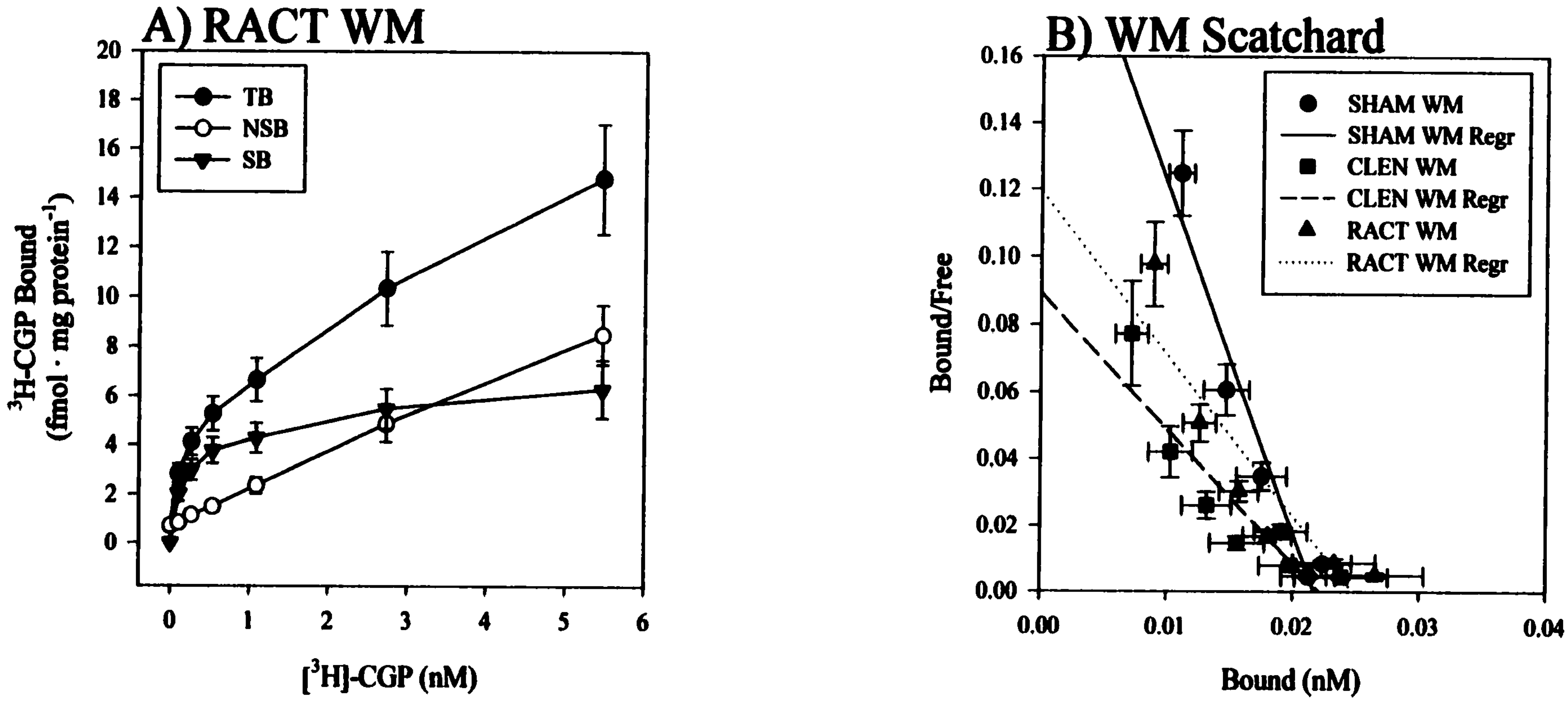

Figure 3.7: Radiolabelled saturation curves for ^{3}H-CGP binding to white muscle (WM) membranes prepared from rainbow trout fed RACT (A) diet. Scatchard plots (B) of specific binding to WM membrane are shown for all WM data. Conditions are as stated in Fig. 3.4 and 3.5.

Scatchard analyses values are summarized on Table 3.2. A one-way ANOVA ($P < 0.05$) revealed a significant increase in K_d values between red muscle membranes prepared from CLEN and RACT trout when compared with SHAM membranes and between white muscle membranes for CLEN compared with the SHAM (but not RACT). No significant changes in B_{max} were found among treatments in either red or white muscle membranes, although values in membranes from the CLEN and RACT trout were generally higher than for the SHAM treatment. No difference existed in B_{max} values between red and white muscle membranes as was previously reported (Table 2.1).

3.5.1.3 Specific Growth Rates

Within a range of $[CLEN]_{Plasma}$ between 0 and 6 nM, a significant positive correlation (Spearman's rank correlation coefficient, $P < 0.05$) was observed between $[CLEN]_{Plasma}$ and SGR_{exp}, $[CLEN]_{Plasma}$ and CF_f, but not between $[CLEN]_{Plasma}$ and BW_f in rainbow trout fed CLEN diets (Fig 3.8A, B and C respectively). These correlations imply the simple relationship between fish eating well, acquiring more β_2-AA, and increasing body parameters compared with fish eating less.

3.5.1.4 β_2-Adrenoceptor mRNA Expression

Not all individuals from each group could be analyzed for β_2-AR mRNA expression, so only the rainbow trout with the highest values of plasma CLEN and the highest CF_f were used. This is based upon the relationships noted on Fig. 3.8 and in Section 3.5.1.3. To control for possible effects of food consumption on β_2-AR mRNA expression, fish with similar SGR_{exp} and CF_f were taken from the SHAM group.

Semi-quantitative measurements of β_2-AR gene expression relative to β-actin expression in red and white muscles of rainbow trout were examined using the RNase protection assay (RPA). Measurements were quantified using a phosphor-imager and the ratios

Table 3.2: Binding affinities (K_d) and maximum number of binding sites (B_{max}) for ^{3}H-CGP (β-adrenoceptors) in red and white muscle membranes prepared from SHAM, CLEN and RACT treated rainbow trout.

		K_d (nM)	B_{max} (fmol • mg protein^{-1})
Red Muscle	SHAM	0.21 ± 0.04	1.39 ± 0.21
	CLEN	0.51 ± 0.09*	1.81 ± 0.42
	RACT	0.48 ± 0.12*	2.05 ± 0.30
White Muscle	SHAM	0.18 ± 0.04	1.47 ± 0.21
	CLEN	0.31 ± 0.04*	1.91 ± 0.46
	RACT	0.28 ± 0.05	2.14 ± 0.37

All values are mean ± SEM (n = 15-16), calculated from Scatchard analysis of the binding data (Fig 3.5B and 3.7B). Significant difference between treated vs. SHAM of the respective tissue is represented by * (one-way ANOVA, $P < 0.05$).

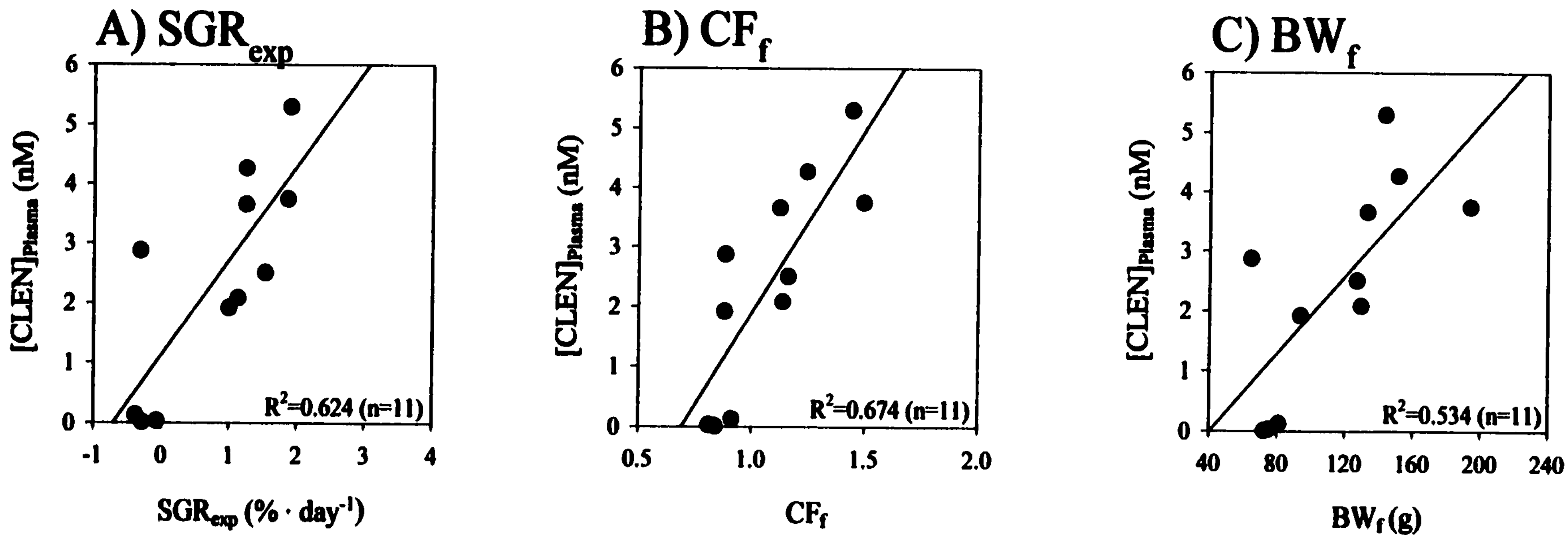

Figure 3.8: Relationship between plasma clenbuterol levels ([CLEN]$_{Plasma}$, in nM) and specific growth rate (SGR$_{exp}$, in % • day^{-1}; in A), [CLEN]$_{Plasma}$, and final condition factor (CF$_f$; in B) and [CLEN]$_{Plasma}$ and final body weight (BW$_f$ in g; in C) of rainbow trout fed the CLEN diet. Within a given range of [CLEN]$_{Plasma}$, there is a significant positive correlation (Spearman's rank correlation coefficient, $P < 0.05$) between [CLEN]$_{Plasma}$ and SGR$_{exp}$ and CF$_f$ but not BW$_f$.

of β_2-AR/β-actin densities were recorded. Neither CLEN nor RACT significantly changed the β_2-AR mRNA expression levels in either muscle type (Fig. 3.9 and 3.10). However, the large error values may be masking an increase in β_2-AR mRNA expression with treatments of β_2-AA.

3.5.2 Fractional Protein Synthesis Rates

The impact of feeding β_2-AAs for 30 days on fractional protein synthesis rates (k_s, in % • day^{-1}) was assessed in red and white muscle fractions using the ^{3}H-Phe technique (Fig 3.11). Results for the MitoP and post-MitoP fractions (see Fig. 3.2) were inconclusive in both muscle types as the level of radioactivity present was at background. In the WP fraction (Fig 3.8A) of red and white muscle k_s, values of the treated fish all show a trend towards an increase when compared with the respective SHAM group but only the CLEN red muscle k_s values are significantly increased. Similar results are observed for the MyofP fraction (Fig. 3.8B), except red muscle from both CLEN and RACT treated fish show a significant increase in k_s values. In the SSP fraction (Fig. 3.8C), no red muscle effects were observed. However, white muscle k_s values are significantly increased in fish treated with RACT when compared to SHAM. When k_s values are compared between red and white muscles of each respective treatment, k_s values were consistently and significantly higher in red muscle compared with white in all three protein fractions, at least for the SHAM and CLEN treated fish. As noted, some fish simply did not thrive on these diets. These individuals had fractional protein synthesis rates for all three protein fractions between 2-10% that of the fish in each group used to report data in Fig. 3.11.

3.6 Discussion

The main objectives of this study were to assess the impact of feeding β_2-AAs on muscle β_2-ARs binding characteristics and fractional protein synthesis rates. Increases in

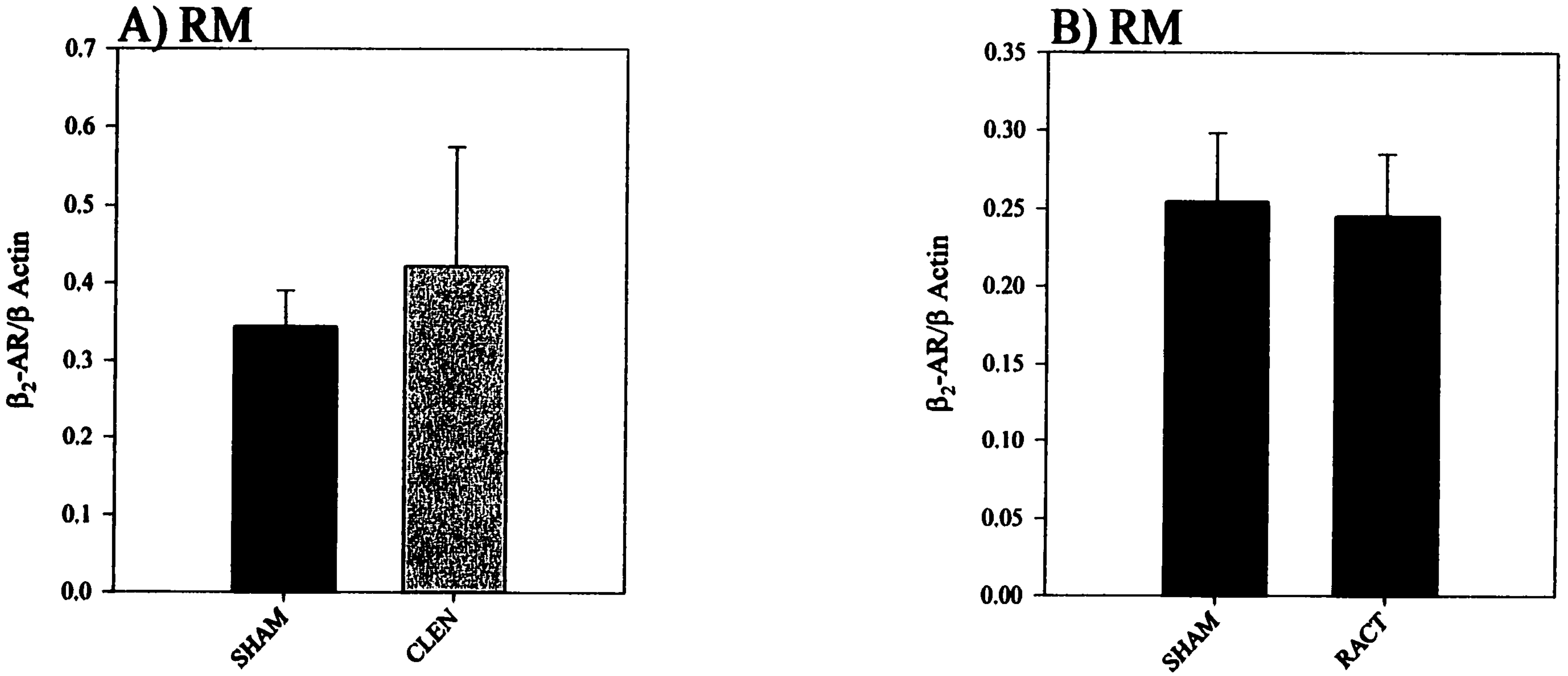

Figure 3.9: β_2-AR mRNA expression measured by the RNase protection assay (RPA) in red muscle (RM) from rainbow trout fed SHAM, CLEN (A) and RACT (B) diets. Each graph represents individually prepared RPAs, which were resolved on separate gels. Values represent means ± SEM (n = 4-8) of ratios of β_2-AR band density/β-actin band density. No significant differences were observed between SHAM and treatments (Mann Withney rank sum test, $P < 0.05$).

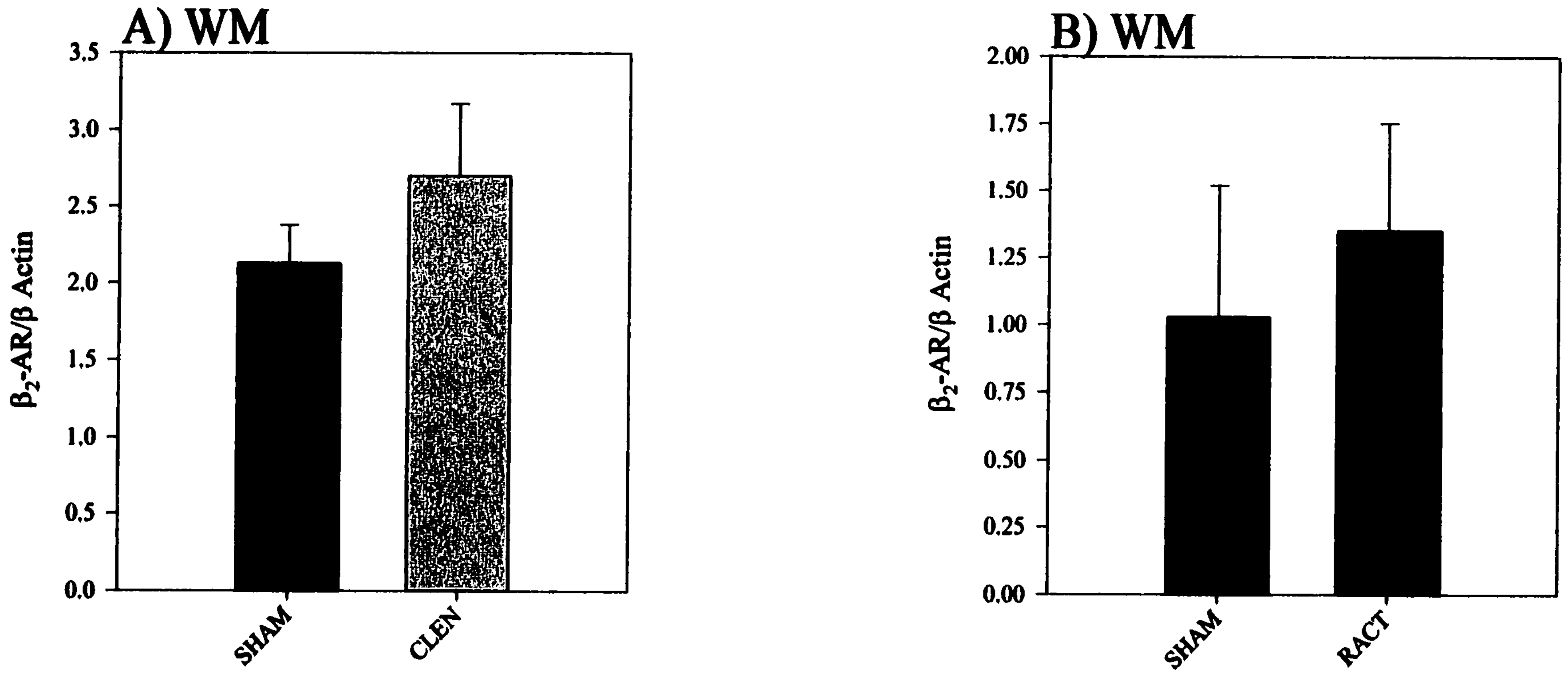

Figure 3.10: β_2-AR mRNA expression measured by the RNase protection assay (RPA) in white muscle (WM) from rainbow trout fed SHAM, CLEN (A) and RACT (B) diets. Conditions are as noted in Fig. 3.9.

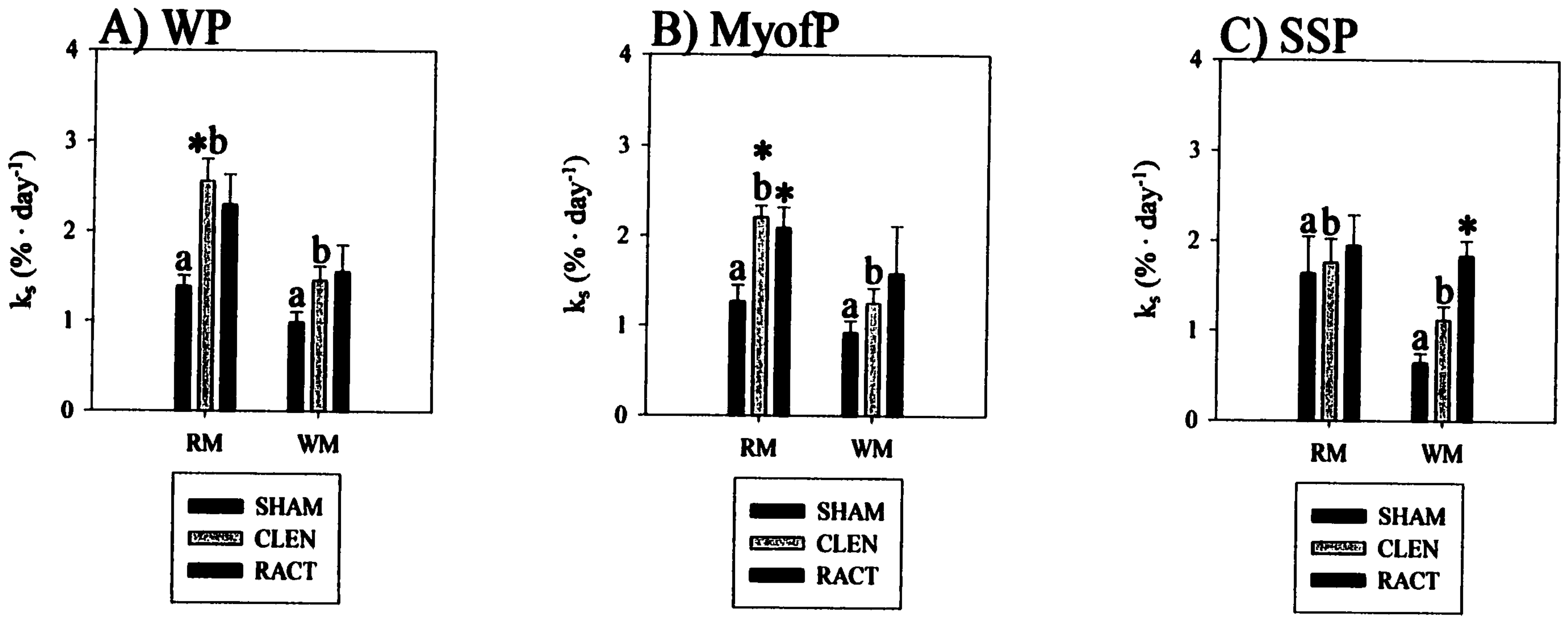

Figure 3.11: Fractional protein synthesis rates (k_s, in % • day^{-1}) in whole (A) myofibrillar (B) and sarcoplasmic soluble (C) protein fractions of red and white muscles prepared form rainbow trout fed SHAM (black), CLEN (light gray) or RACT (dark gray) diets. Measurements were made 4 h after injection of a large dose of ^{3}H-Phe (see Section 3.4.5.2). Values represent means ± SEM (n = 4) and significant differences between treatment and SHAM are indicated by * (one-way ANOVA, $P < 0.05$). Significant differences between red and white muscle tissue within the same treatment group are indicated by ^{a} (SHAM) and ^{b} (CLEN); there were no differences between tissues in the RACT group (t-test or Mann-Whitney rank sum test if normality test failed, $P < 0.05$).

muscle fractional protein synthesis rates, down-regulation of β_2-ARs and decreased β_2-AR mRNA expression following treatment with β_2-AAs have been previously demonstrated in mammals. However this is the first experiment examining these parameters in fish fed β_2-AAs.

3.6.1 Body Weight and Physiological Parameters

The anabolic effects of β_2-AAs have been reported for many mammalian species (including rodents, ruminants and swine), avian species (chicken, turkey and duck) and three teleost species (rainbow trout: Vandenberg and Moccia, 1998; Vandenberg *et al.*, 1998; channel catfish: Mustin and Lovell, 1993; blue catfish: Webster *et al.*, 1995). In general, substantially larger anabolic responses are obtained in rodents and ruminants, with average responses in swine, turkey and duck and lower responses in chicken (Beermann, 1993; Mersmann, 1998; Moody *et al.*, 2000) and more modest responses in fish (Mustin and Lovell, 1993; Vandenberg and Moccia, 1998; Vandenberg *et al.*, 1998). However comparisons across studies, not including species-specific differences, are extremely difficult to make due to differences in the compound studied, dosage, duration, and especially response parameters measured (Yang and McElligott, 1989; Kim and Sainz, 1991; Reeds and Mersmann, 1991; Beerman, 1993; Mersmann, 1998; Moody *et al.*, 2000). The following discussion is restricted to studies using the same β_2-AAs (CLEN, RACT) for a prolonged treatment period and similar response parameters as those used in the present study.

No anabolic effects of feeding CLEN or RACT for 30 days to rainbow trout were observed (Table 3.1). The absence of a body weight increase is contradictory to most studies done in rats (CLEN, 2 ppm; Emery *et al.*, 1984), pigs (RACT, 2.5-30 ppm; Nelson *et al.*, 1987; Watkins *et al.*, 1990; Anderson *et al.*, 1991), cattle (RACT, 10-80 ppm; Anderson *et al.*, 1989; Preston *et al.*, 1990), turkey (RACT, 22-44 ppm; Wellenreiter and Tonkinson, 1990) and channel catfish (RACT, 20-100 ppm; Mustin and Lovell, 1993) where weight gain was reported to be 17%, 7-13%, 20-25%, 15% and 17%, respectively. Other studies report no weight gain but repartitioning effects in rat (CLEN, 3 ppm; Sneddon *et al.*, 2000), pigs (CLEN, 1 ppm; Beermann, 1993), cattle (CLEN, 10-100 ppm; Beermann, 1993), rainbow trout (RACT, 5-40 ppm; Vandenberg and Moccia, 1998; Vandenberg *et al.*, 1998) and blue catfish (RACT, 20-100 ppm; Webster *el al.*, 1995). No change in length with treatment is contradictory to one study on channel catfish (RACT, 20-100 ppm; Mustin and Lovell, 1993) but similar to reports on rainbow trout (RACT, 5-40 ppm; Vandenberg and Moccia, 1998; Vandenberg *et al.*, 1998). Condition factor, which incorporates both weight and length and is used to describe fish growth (Busacker *et al.*, 1990), was measured by Vandenberg and Moccia (1998) following treatments with RACT and no change was observed since they reported no change in weight or length. Specific growth rates may have increased in the previously mentioned studies that reported increased weight gain with β_2-AA treatments; however, none reported SGR specifically. It is important to point out that anabolic responses can be obtained without a significant change in body weight, length, condition factor and specific growth rates in mammals, poultry and fish, as body stores can be repartitioned from fat to muscle resulting in no noticeable change in body weight.

β_2-AA treatments did not change liver or heart weights or their proportion to body weight (HSI, CSI). In mammals, there is a general trend to decrease liver and increase heart weights (Reeds and Mersmann, 1991). Reduced liver weight in rats (CLEN; Reeds *et al.*, 1988) and pigs (RACT; Aalhus *et al.*, 1990) are thought to be a direct result of glycogen depletion (Wariss *et al.*, 1990). Heart weight is increased in studies with rats (Emery *et al.*, 1984; Polla *et al.*, 2000; Sneddon *et al.*, 2000) in a manner similar to the hypertrophic response of skeletal muscle (Yang and McElligott, 1989; Moody *et al.*, 2000). The study on rainbow trout by Vandenberg and Moccia (1998) also found no change in liver weight or HSI following β_2-AA treatments.

β_2-AA treatment did not alter plasma protein, amino acid or glucose values (Table 3.1). Plasma protein and total amino acids have not been measured in any previous studies. One study in rats fed a low-protein diet did report an hypoalbuminemia (Sawaya and Lunn, 1998), however the rainbow trout in the present experiment most likely had sufficient protein in the diet (41.0%) to prevent such an effect on plasma protein content. The same reasoning applies to plasma amino acid content, which was sufficiently high to withstand any increase in amino acid demand by increased skeletal muscle protein synthesis. Byrem *et al.* (1998) found increased amino acid uptake into muscle using the closed hindlimb perfusion model, but in well fed conditions it is unlikely that the whole body level plasma amino acid concentration would change. Interestingly, trout plasma glucose levels were not changed by β_2-AA treatments, but this has also been observed previously in mammals. Upon infusion of β_2-AA in hind-limb perfusion studies, there was an acute and transient increase in plasma glucose concentration, which eventually returned to normal levels (Reeds and Mersmann, 1991; Byrem *et al.*, 1998; Moody *et al.*, 2000; Beermann, 2002). However in the rainbow trout study of Vandenberg *et al.* (1998), there were significantly increased plasma glucose concentrations only after 12 weeks, but not after 8 weeks or less of treatments with RACT.

Plasma clenbuterol levels reached 11.5 nM (Table 3.1) 4 h after the last CLEN (40 ppm) feeding; levels in the SHAM group were below the detectable limit or within the lower non-linear portion of the standard curve (see Appendix, Fig. A.1). The CLEN-treated group had values within the range observed over an 11 day pre-trial study, and 10 days after feeding stopped (see Appendix, Fig. A.2). In human studies β_2-AAs generally reached peak plasma levels 1 to 3 h after oral administration (Morgan, 1990). Multiple studies in cattle (CLEN, 5-10 ppm; Meyer and Rinke, 1991; Stoffel and Meyer, 1993) reported plasma CLEN levels reached peak plasma concentration of 0.2-2.5 nM following a single oral dose, while prolonged oral administration of the same dose resulted in doubling of the plasma levels. One similar study in cattle monitoring CLEN levels throughout the treatment period (15 days) showed maximal plasma concentrations after 10 days and further accumulation in the plasma over time (Sauer *et al.*, 1995). The experimental setting for studies in mammals, such as humans and cattle, is more controlled than in a study using rainbow trout. Individual dosage of the drug cannot be easily monitored or assured in the fish study. Many studies report fish social interactions in aquaculture rearing conditions, and it is well documented that rainbow trout kept in groups develop dominance hierarchies, and more importantly with regards to this study, feeding hierarchies (McCarthy *et al.*, 1991). A hierarchy was demonstrated in this study where increases in plasma clenbuterol were significantly correlated with increases in specific growth rates and final condition factor (Fig 3.6A and C, respectively). This simple correlation indicates an uneven distribution of the dose or food among individual fish of the same treatment group as previously demonstrated for rainbow trout (Carter *et al.*, 1995). This same study also showed a significant positive correlation between SGR_{exp} and food consumption. It is apparent that studies on fish kept in groups must consider the impact of differences in food consumed, in other words the dose that each individual fish receives. This adds an additional variable to the study and may contribute to differences obtained if one compares results with mammalian studies.

3.6.2 β_2-Adrenoceptor Binding

Many mammalian studies have reported desensitization by down-regulation of muscle tissue β_2-ARs following chronic exposure to β_2-AAs (Elfellah *et al.*, 1988; Smith, 1989; Spurlock *et al.*, 1994; Summers *et al.*, 1995; Roberts and Summers, 1998; and reviewed by:

Kim and Sainz, 1992; Beermann, 2002; Mills, 2002). Down-regulation is measured as a decrease in the number of specific β_2-AR binding sites (B_{max}). In rat soleus (red) and plantaris (white) muscles, receptor numbers declined by 40-45% after a 3 day treatment with cimaterol, a β_2-AA, without any change in affinity (Kd) (Kim and Sainz, 1990). Similar results were obtained in rat soleus (red) muscle, after a 14 days treatment with ISO (Summers *et al.*, 1995) and in rabbit gastrocnemius (white) muscle treated with ADR (Elfallah *et al.*, 1988). Contrary to these results, the present study (Table 3.2; Fig 3.4 RM; Fig 3.5 WM) found no change in B_{max} values, but similar results have also been reported in pig skeletal muscle (RACT; Smith, 1989). The pig study suggested an adaptive response to chronically elevated levels of β_2-AA. To explain the lack of down-regulation, others hypothesize that β_2-AAs might stimulate the synthesis of new β_2-AR protein and effectively mask down-regulation (Mills, 2002). Finally, despite the absence of down-regulation or desensitization, post-receptor desensitization (e.g. cAMP production) cannot be ruled out, as it was not assessed in this study.

Interestingly, chronic feeding of β_2-AAs to rainbow trout decreased the affinity of the receptors (i.e., increased K_d values) in red muscle for both the CLEN and RACT groups, and in white muscle for the CLEN group only (Table 3.2). This result is contrary to studies previously cited in mammals where no change in affinity was observed even in cases where B_{max} did not change. On the other hand, changes in binding of antagonists to β-ARs are reported to be affected by the fluidity of the cell membrane (Shinitzky, 1984). Thus changes in K_d may reflect changes in lipid and/or cholesterol metabolism or mobilization as these β_2-AAs cause decreased fat content in mammals, birds and fish. Another explanation could be the interference of β_2-AA residues in the tissues and ultimately in the membrane preparation, as this was observed in a study on β_2-ARs in lung and skeletal muscle of rats treated with CLEN with and without the presence of dexamethasone (Huang *et al.*, 2000) and in a study on agonist-induced desensitization of the β_2-AR (Waldo *et al.*, 1983). Waldo *et al.* (1983) refer to an "altered membrane form of the β_2-AR" in which the receptor achieved an equilibrium between an active (high affinity) and an inactive (lower affinity) state. The active form has a higher affinity for agonists while the inactive form has a higher affinity for antagonists. This in a sense renders the competitive interaction between agonists and antagonists not a true competition, as the two do not compete for the same binding site (Johnson, 2001). In this study, I can hypothesize that significant residual agonist could bind the high affinity active state of the β_2-AR and the ^{3}H-CGP the low affinity inactive state, thus decreasing receptor affinity (i.e., increased K_d values). Residues of CLEN in rainbow trout treated with 5 ppm reached 75 ppb in white muscle (440 ppb in liver) after 21 days. Even with these data, there is no way to confirm this hypothesis.

Another possibility is that there is more than one subtype of β-AR in skeletal muscle of rainbow trout (J.G. Nickerson, pers. commun.). These subtypes may have similar affinities since LIGAND analysis indicated a single receptor population (Figs 3.5D and 3.7D), yet they may not be identical. It is then conceivable that a change in proportion of the various subtypes by treatments with β_2-AAs could change affinity without a change in receptor numbers. However, this interpretation could not be tested or confirmed even with data obtained from the mRNA expression experiments, specifically targeting the β_2-AR mRNA, and will be further discussed in that Section.

3.6.3 β_2-Adrenoceptor mRNA Expression

To establish the impact of feeding β_2-AAs on β_2-AR gene expression in red and white muscles of rainbow trout, mRNA levels were determined using a RNase protection assay. It has been well established in mammals that β_2-AR mRNA levels decrease substantially following acute and chronic exposure to β_2-AAs (Perkins *et al.*, 1991; Strosberg *et al.*, 1992;

Summers *et al.*, 1997). No such decrease in β_2-AR mRNA expression was observed in trout muscles, and if any change, a slight increase occurred (Fig. 3.7). Studies in mammals reported decreases in both receptor numbers and receptor mRNA levels which was not the case in rainbow trout. However, a study using pig adipocytes exposed to RACT reported a decline in receptor protein levels was not matched by a decline in receptor mRNA levels (Ding *et al.*, 2000). The authors provided two possible explanations. The first is an increase in β_2-AR mRNA synthesis by β_2-AAs, similar to the arguments used by Mills (2002) for the number of receptors. The second explanation is simply a time delay between decreased β_2-AR mRNA synthesis that is not matched by a decrease in receptor numbers. One must not rule out the possible modulation of β-actin mRNA levels by β_2-AAs, as it was the gene used to normalize β_2-AR mRNA expression. The use of other genes, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), to normalize expression levels could be tested and used.

3.6.4 Fractional Protein Synthesis Rates

A flooding dose of ^{3}H-phenylalanine was used to establish the impact of feeding β_2-AA on fractional protein synthesis rates (k_s, in % • day^{-1}) in red and white muscles of rainbow trout. In general, the k_s values (Fig 3.8) obtained in the SHAM group for the same protein fraction compare well with results obtained by Fauconneau *et al.* (1995) in rainbow trout, especially in red muscle. The values reported here for white muscle are 3-4 times higher than reported by Fanconneau *et al.* (1995), but this difference may relate to the size of the fish used. Smaller fish used in the present study would be expected to have higher k_s values than the larger fish used by Fanconneau *et al.* (>250 g) (Fauconneau *et al.*, 1986). There could also be differences in commercial strains of fish used between the two studies. Selection processes may be different but this cannot be assessed.

Many reports in mammals, both *in vivo* and *in vitro* have focused on assessing the anabolic effects of β_2-AA on muscle protein turnover and there is evidence to support increased protein synthesis, decreased protein degradation and a combination of both. These studies have been reviewed extensively (Yang and McElligott, 1989; Kim and Sainz, 1991; Reeds and Mersmann, 1991; Beerman, 1993; Mersmann, 1998; Moody *et al.*, 2000). Emery *et al.* (1984) reported a 26% increase in rat muscle (gastrocnemius, whole protein, no fractionation) k_s values *in vivo*, following a 2 ppm CLEN treatment for 16 days also using the Phe-flooding method. In our study, the white muscle whole protein fraction (Fig 3.8A) showed a similar trend towards an increase of k_s by both CLEN and RACT (not statistically significant). In red muscle, CLEN significantly increased k_s by almost 2-fold while a trend towards an increase was observed in the RACT-treated group. Helferich *et al.* (1987) reported a 50% increase in the rate of α-actin synthesis in pig muscle (longissimus dorsi) following a 20 ppm RACT treatment for 21 days using incorporation of ^{14}C-lysine. The same study also measured a 2-fold increase in α-actin mRNA using dot blot hybridization. In the myofibrillar protein fraction (Fig 3.8B), CLEN and RACT significantly increased protein synthesis by about 30% in red muscle, but only trends towards an increase were observed in white muscle. No studies were found reporting effects of any β_2-AA on sarcoplasmic soluble protein fraction synthesis (Fig 3.8C). Like the WP and MP fractions, no significant changes were observed in the SSP fraction in red muscle, but increases were observed in the white muscle. This sarcoplasmic soluble protein fraction is composed primarily of enzymes (Fauconneau *et al.*, 1995; Etlinger *et al.*, 1975), including metabolically important enzymes such as glycolytic enzymes. An increase in metabolic enzyme protein as a result of β_2-AA treatment may result in increased white muscle metabolic activity. A study with Atlantic cod reported a positive correlation between increased metabolic enzyme activity, namely pyruvate kinase and lactate dehydrogenase, and individual growth rates (SGR) (Pelletier *et al.*, 1995). This may point to an increased (Johnston, 1982) anaerobic metabolism capacity, the main source of energy production in white muscle. Unfortunately, without comparable results for the mitochondrial fraction of red muscle, it is not possible to speculate on whether a similar increase in aerobic metabolic capacity occurs in this muscle subtype.

There was also a significant difference between muscle types and within their respective treatment groups with respect to protein synthesis rates. The higher rates in red compared to white muscle remain unchanged with CLEN treatments but not with RACT treatments where this relationship appears to be abolished by the treatment, implying muscle type specificity of the drugs. Certainly the higher rates of protein synthesis by both CLEN and RACT in red compared with white muscles may indicate differences in delivery of the drug to the tissue. Studies on circulation and blood volumes of various organs and tissues in rainbow trout find that red compared with white muscles contain more capillaries, which in turn means exposure to higher blood volumes (Bushnell *et al.*, 1998) and necessarily exposure to more β_2-AAs than white muscle. Another possibility is that in the holding conditions of the present study, rainbow trout are confined to low current holding tanks and swim exclusively in a light and sustained manner in which only the slow-twitch oxidative red muscle is used. Two studies in *mdx* mice showed an increased CLEN hypertrophy response (or decreased atrophy from muscular dystrophy) when mice were subjected to combined CLEN and running and swimming exercise regimes (Dupont-Versteegden, 1996; Hayes and Williams, 1997).

Finally, reasonable levels of variability accompanied by low n-numbers diminish the sensitivity of determining subtle yet true modulation of protein synthesis rates by β_2-AAs. However, this study was successful in showing increased protein synthesis rates in whole, myofibrillar and sarcoplasmic soluble protein fractions by β_2-AA.

3.7 Conclusions

This study did not detect any change in body or physiological parameters measured or calculated following chronic oral administration of the β_2-AAs CLEN or RACT to rainbow trout. In contrast, observations support positive increases in protein synthesis in red muscle whole and myofibrillar protein fractions and white muscle sarcoplasmic soluble protein fraction. Rainbow trout red and white muscle β_2-AR were not desensitized or down-regulated and β_2-ARs mRNA levels were not changed upon sustained stimulation by β_2-AAs. This study speculates possible changes in β-AR subtypes proportions in rainbow trout red and white muscles by treatments with β_2-AA. This study is the first to report β_2-AA impacts on protein synthesis and β_2-AR properties and confirm important mechanistic clues to the function of β_2-AAs in skeletal muscle of a non-mammalian, non-avian species. The results obtained, including the results of Chapter 2, support a direct mechanism but cannot eliminate involvement of an indirect mechanism.

CHAPTER 4. General Conclusions

In most mammals and a few birds, the β_2-adrenoceptor (β_2-AR) system has been well characterized. This includes a basic mechanistic handle of the underlying pathway in addition to an observed enhancement of muscle growth upon muscle β_2-AR stimulation with β_2-adrenergic agonists (β_2-AAs). However, when considering this system on a wider scope, there is a considerable knowledge gap regarding the use of such compounds in the vertebrates; there have been numerous studies on mammals, fewer on birds, none on reptiles and amphibians and even fewer on fish. Thus, this thesis does make a significant contribution to basic knowledge of this system in fish and provides evidence for a conserved muscle β_2-AR system in the vertebrate lineage.

In short, this thesis examined the presence and functionality of β-ARs in red and white muscles of the rainbow trout (*Oncorhynchus mykiss*). Specific binding assays performed on red and white muscle membrane preparations revealed the presence of a single class of binding sites with similar affinities (K_d) in both red and white muscles with significantly higher numbers of binding sites (B_{max}) in red compared with white muscle. Competition assays with various selective and non-selective β-adrenergic agonists and antagonists revealed an "atypical" β-AR pharmacology which could simply represent a non-mammalian β-AR classification or the presence of more than one β-AR subtype with similar affinities that could not be resolved using specific binding assays. These radioligand binding assays are the first evidence of the presence of an "atypical" β-AR or multiple β-AR subtypes with similar affinity in both red and white muscles. Furthermore, the functionality of these receptors was tested using red and white muscle membrane preparations by means of assessment of adenylyl cyclase (ACase) activity. ACase/cAMP assays showed a dose-dependent increase in cAMP production in the presence of increasing concentrations of β_2-AAs in both red and white muscle membranes with significantly higher basal production in the red compared with the white muscle, consistent with a higher number of bindings sites in the red muscle. Moreover, this agonist-induced increase in cAMP production was blocked by the β-adrenergic antagonist propranolol (PROP). Forskolin (FSK), a direct ACase activator markedly increased cAMP production 7-14-fold above basal and 3-fold more than the most effective β-AAs tested (i.e., isoproterenol). In addition, there was a subtle difference in basal and β_2-AA sensitivity between red and white muscle; red muscle membranes had higher basal ACase activities but required higher concentrations of β_2-AAs to produce significantly higher ACase activities compared with basal. The combination of subtle differences in β-AR numbers, β-AR subtype(s), basal ACase activity and β_2-AA sensitivity provide the first evidence of specific differences between skeletal muscle types in the physiological modulation or control exerted by the adrenergic system.

The impact of feeding β_2-AAs on rainbow trout red and white muscle, β_2-AR binding characteristics and mRNA expression was assessed in parallel with red and white muscle fractional protein synthetic rates. Feeding 40 ppm of CLEN or RACT for 30-37 days had no significant effects or apparent anabolic effects on specific body and physiological parameters. The β_2-AAs did not significantly change the number of binding sites, but did significantly decrease β-AR affinity. These changes in affinity by feeding β_2-AAs may reflect a change in the β-AR subtype present in trout muscle. Further studies must be performed to confirm this hypothesis. Similarly, no change in β_2-AR mRNA levels was observed with β_2-AA treatments. However, treatments did significantly increase red and white muscle fractional protein synthesis rates in whole protein, myofibrillar protein and sarcoplasmic soluble protein fractions. This is the first report demonstrating changes in skeletal muscle protein metabolism upon treatment with β_2-AAs in a non-mammalian or a non-avian species. Differences were noted between muscle types with respect to fractional protein synthesis rates further supporting the existence of subtle differences in the modulation of the adrenergic system of the two muscle types. Finally, in combination with the muscle β-adrenergic system characterization experiments, this study is the first to report important mechanistic clues on the underlying mechanisms behind β_2-AA modulation of protein accretion in skeletal muscle of fish.

Figure 4.1 summarizes the fish components of the system determined primarily from this thesis. When comparing Figure 4.1 (fish model) with Figure 1.5 (mammalian model; section 1.5.2.3), we find that the fish β_2-AR system is very similar to the mammalian system. Both have functional muscle β_2-ARs (atypical or with presence of other subtypes in fish) that are stimulated by β_2-AAs (and blocked by β-adrenergic antagonists), resulting in elevation of muscle intracellular cAMP levels, ultimately modulating muscle protein accretion (i.e., increasing protein synthesis in mammals and fish, decreasing protein degradation at least in the mammalian model). However, the two differ in the scope of the β_2-AA-induced muscle growth, as no clear anatomical responses, despite increased muscle protein synthesis, were observed in the muscles of treated fish. Also, no agonist-induced desensitization of the receptors was observed in the fish system. This considered and despite the information gathered, many questions remain and more questions can now be asked on two different levels, A) at the level of the fish model and B) at the level of the vertebrate model. Such questions include: A1) What is the exact β-AR subtype or subtypes present in fish muscle? A2) What is the exact role of the muscle β-AR subtype or subtypes? A3) If there is more than one subtype in muscle, is the coupling to ACase different? And if so does the stimulation of the different subtypes result in the same cellular response? B1) Are there differences in the muscle β-AR system within the vertebrate lineage (i.e., receptors, underlying pathway and response to β_2-

Figure 4.1: Cartoon summarizing the rainbow trout muscle adrenergic system and various effects of β_2-adrenergic agonists (β_2-AAs). Included are skeletal muscle cell and blood vessel. Arrows and components with a solid line indicate confirmed interaction and presence while a dashed line represents indications but not conclusive interactions or presence. A triangle arrow end indicates a positive effect on the indicated parameter while a dotted arrow end represents inhibition. Note the letters "no DR" on the β_2-AR indicating no down-regulation of the receptor upon chronic exposure to agonist. Abbreviations are from top to bottom, left to right: Prot, protein; AA, amino acids; Gluc, glucose; β_2-AA, β_2-adrenergic agonist; FSK, forskolin; β-Ant, β-adrenergic antagonist; β_2- and $\beta_?$-AR, β_2- and $\beta_?$-adrenoceptor subtypes; G_s, G_s-protein; AC, adenylyl cyclase; no DR, no down-regulation; WP, MP and SSP, whole, myofibrillar and sarcoplasmic soluble protein fractions; PKA, protein kinase A. Indicated by ^{1}, effects were observed only in red muscle with CLEN; ^{2}, only in red muscle with both CLEN and RACT; ^{3}, only in white muscle with RACT. Diagram is based on results obtained in this study.

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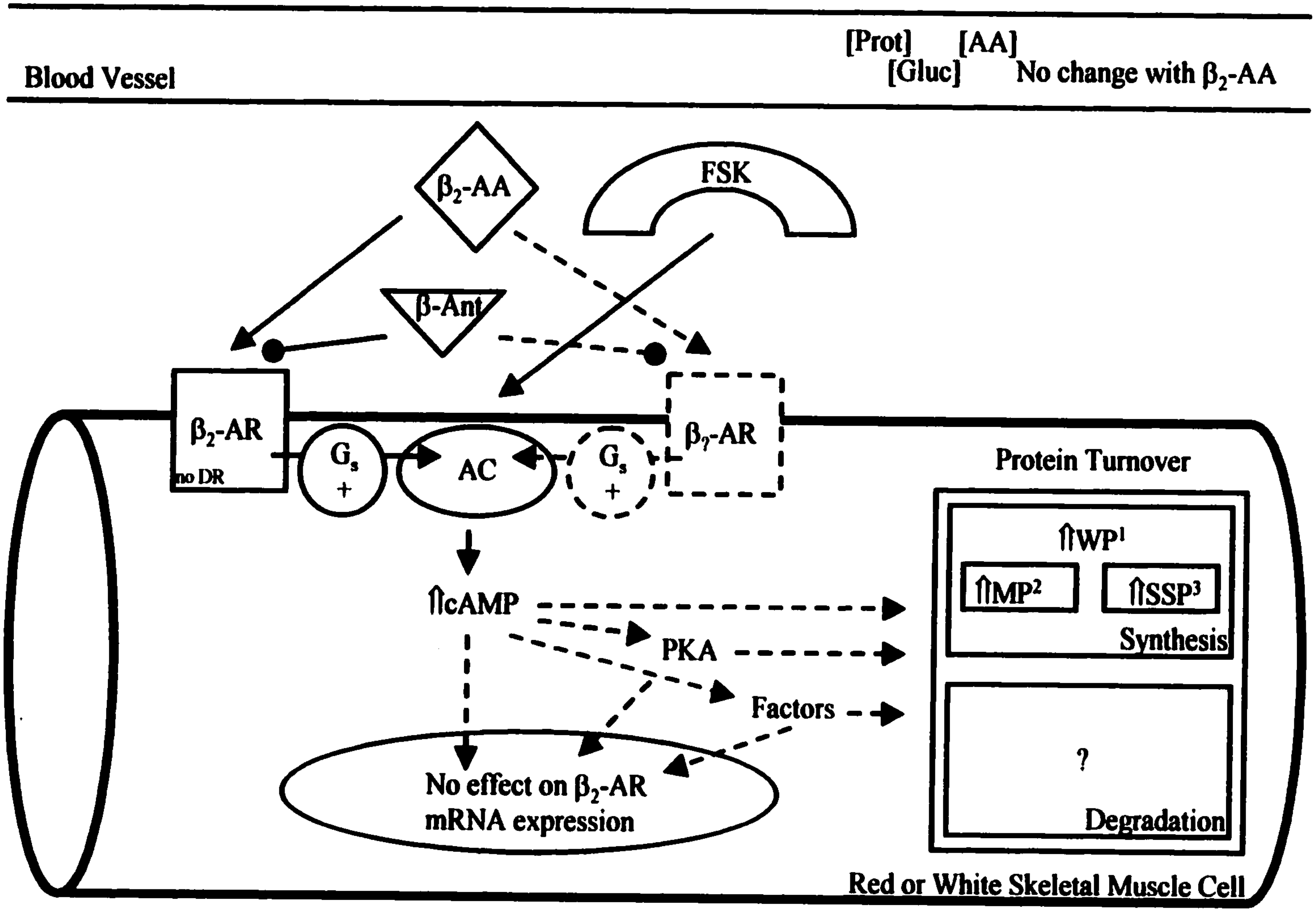
Blood Vessel
[Prot]
[AA]
[Gluc]
No change with β_2-AA
β_2-AA
FSK
β-Ant
β_2-AR
no DR
G_s
+
AC
G_s
+
$\beta_?$-AR
Protein Turnover
⇑WP[1]
⇑MP[2]
⇑SSP[3]
Synthesis
?
Degradation
⇑cAMP
PKA
Factors
No effect on β_2-AR mRNA expression
Red or White Skeletal Muscle Cell

AAs)? B2) If there are differences, what are they exactly and how did they evolve or why do they differ? B3) What is the role of this system and how did it evolve?

To answer these questions at the level of the vertebrate model, many more basic characterization studies need to be performed on muscle of non-mammalian organisms such as reptiles and amphibians in order to bridge the knowledge gap within this lineage. In parallel, at the level of the fish model, studies investigating the muscle β-AR system under various environmental and physiological conditions (such as sex of the animal, age, season, exercise, stress, satiated or starved, etc...) could provide important clues leading to answers to the previously mentioned questions at the level of the fish model. Also using molecular techniques (such as cloning, sequencing and expressing the various fish β-AR subtypes in cell-lines) could provide the ideal conditions for a thorough investigation of the specific non-mammalian pharmacology of the various subtypes. Furthermore, such a system could help in the discovery of more potent agonists already available in mammals but ideal for a specific fish β-AR. Potentially, such an agonist could prove to be considerably more potent as a fish specific growth promoter to be used in aquaculture.

On the same note, more research needs to focus on gathering much needed information into the exact mechanisms underlying the β_2-AA-induced increase in protein synthesis observed in this study. Such studies could benefit from the development of novel *in vivo* techniques similar to the "hind-limb perfusion technique" used in mammals. One could potentially perfuse the caudal artery, thereby creating a localized increase in β_2-AAs within the caudal musculature of the fish or using mini osmotic pumps with localized delivery. Samples of blood could be collected from the caudal vein permitting a "real-time" monitoring of the plasma amino acid, protein and glucose concentrations. Also, the development of new *in vitro* fish muscle models such as myosatellite cells or myotubes (formed from the fusion of myosatellite cells) as potential model systems will permit further investigation of the mechanisms of action and cellular or physiological responses following stimulation of the muscle β-AR system by β_2-AAs.

In conclusion, it is obvious that the fish muscle β-AR system and the impact of β_2-AA treatments in relation to fish muscle growth needs further attention - despite the modest responses in comparison to mammals - as it provides very important basic knowledge and potentially beneficial science applicable in aquaculture.

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APPENDIX

A.1 Clenbuterol EIA Validation

In order to validate many aspects of the β_2-adrenergic agonist (β_2-AA) experiments a method was needed to quantify the amount of agonist in both the food and the fish. No commercial method was available for the quantification of ractopamine (RACT), but Neogen Corp. (Lexington, KY) provides an enzyme immuno-assay (EIA) kit (with 96 well microplate) to measure clenbuterol (CLEN) in the nM range. The kit is not sold as a true CLEN quantification kit, however standards are available for CLEN to generate a dose-dependent curve and provide a reasonably accurate quantification tool. Using the kit as directed, four different standard curves were performed and measured in duplicates in different media (Fig. A.1): water, assay buffer, fish plasma and plasma:buffer (1:1). No obvious differences were noted in the values between media or interference by components found in fish plasma. The standard curve is linear between 0.001 and approximately 0.1 nM. In addition, adrenaline (ADR) and noradrenaline (NOR) the two endogenous catecholamines (CA), at concentrations of 10 µM did not interfere with CLEN detection by the kit. Plasma from 5 different fish was taken and the same amount of CLEN added to each. All 5 measurements were within duplicate variation and differences in plasma viscosity did not impede quantification. For measurements of plasma CLEN, sufficient fresh plasma (containing heparin) was obtained from individual fish and used to prepare CLEN standards. To estimate levels of CLEN in the CLEN-treated fish, plasma from an average fish in the CLEN group was taken and a serial dilution (using control plasma containing heparin) was done to estimate the appropriate dilution needed for measurements to fall within the linear portion of the standard curve. However, 6 fish from the CLEN group had plasma levels over the linear portion of the curve and for statistical purposes

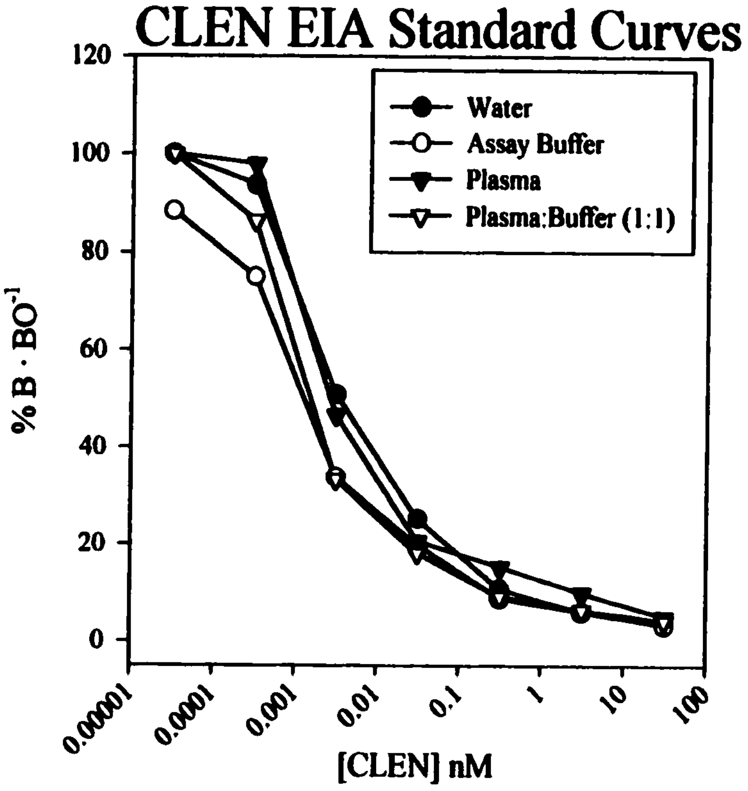

Figure A.1: Clenbuterol EIA kit validation. Standard curves from clenbuterol EIA performed in different media (n=1, done in duplicates): water (solid circles), assay buffer (open circles), plasma (solid triangles down) and plasma:buffer (1:1) (open trianbles down). There are no differences between standard curves obtained using the different media. Note the linear portion of the curve extends from approximately 0.001 to 1.0 nM [CLEN]. Internal positive and negative horse urine controls (data not shown), provided as part of the kit, were always measured to confirm any possible problems between assays.

were assigned a concentration equivalent to the highest concentration of standards (31.4 nM). We did not order another kit for measurements of 6 samples as the goal was mainly to show the presences of nM amounts of CLEN in the treated fish.

Quantification in food was done by solubilizing the CLEN in water. Samples of food pellets, 5 g, were added to 5 mL ddH$_2$O in a centrifuge tube, sealed with parafilm, wrapped in aluminum foil and rested at RT (approx. 19.0 °C) until soft. The solution was sonicated thoroughly for 1 min and centrifuged for 10 min at 500 x g. An aliquot of the supernatant was taken and frozen at -80 °C until analysis. Levels were measured in comparison to CLEN standards prepared in water. Measured CLEN diets in the experiment contained approximately 38 ppm.

A.2 β_2-AA Delivery: Silastic Implants and Food

Silastic implants containing CLEN (estimated to reach approximately 50 nM levels for a period of 20 days; V.L. Trudeau, pers. commun.) were implanted intraperitonealy by making a very small incision ventrally. For this experiment, fish were anaesthetized and surgically equipped with a dorsal aorta cannula for blood sampling. The implants were inserted during the same surgery after the cannula was successfully inserted and secured. Also, before inserting the implant a 0.1 mL blood sample was removed (control levels for CLEN measurements). This delivery method was found to be unsuccessful for chronic administration of CLEN as levels in plasma quickly peaked at very high levels (close to 300 nM!!!) returning to near baseline by 2 days (Fig. A.2A). This is unfortunate since the dosage using this technique would have been identical in all individual animals whereas feeding does not ensure even distribution of the dose among animals kept in a group.

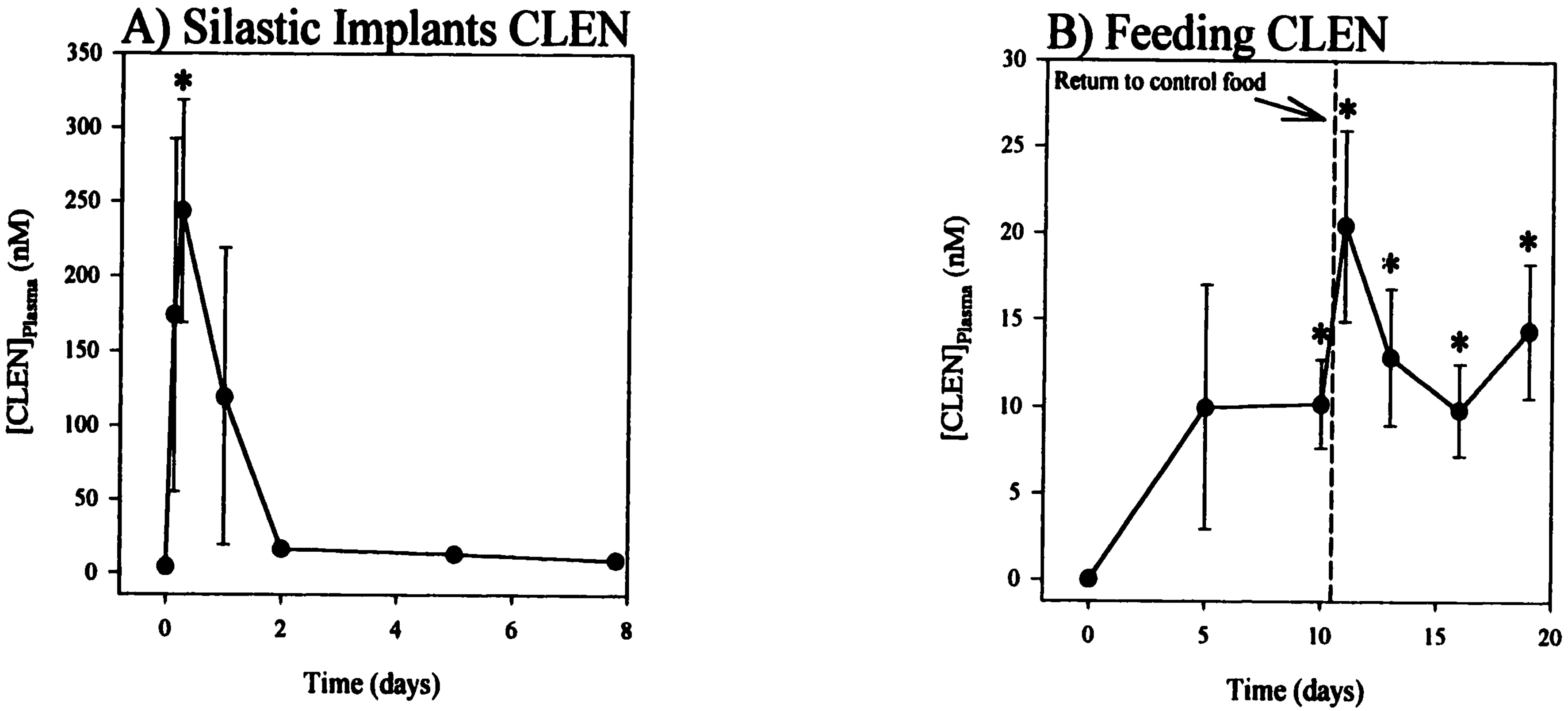

Figure A.2: Clenbuterol delivery method validation. Clenbuterol plasma levels in fish, equipped with a dorsal aorta cannula, treated with silastic implants containing CLEN (A; mean ± SEM, n=3). Clenbuterol plasma levels in fish fed 2.0% BW • day^{-1} with CLEN sprayed food (10 ppm); dashed line indicates return to control food (B; mean ± SEM, n=5). Statistically significant differences from time zero (A; paired t-test, $P < 0.05$) and between control fed fish (B, data not shown; t-test, $P < 0.05$) is indicated by *.

In contrast, using food as a method of delivery proved to be considerably more suitable for chronic treatments and is a common method of choice for β_2-AA delivery. Figure A.2.B shows levels of CLEN in plasma of fish fed 10 ppm CLEN diets (2.0% BW • day^{-1}) for a period of 10 days. A significantly higher level of plasma CLEN was obtained after 10 days of feeding (compared with control group fed normal diet) and stayed high for at least 10 more days after treatment was stopped and fish were returned to the control diet.

A.3 β_2-AA Loss to Water

One particular aspect of using food as a vehicle is the loss of CLEN to the water before the pellets are consumed. This was tested by submerging 5 g of food pellets in water for up to a minute and the rate of loss was estimated to be approximately 0.5% of total CLEN • sec^{-1} (Fig. A.3). At this rate, the estimated time to complete disappearance is approximately 3.5 min, which is reasonably long considering the consumption rate of the fish. Also to prevent such a problem and to sustain more constant levels of CLEN, the fish were fed half their daily feed intake; twice daily and fish were fed a few pellets at a time to ensure minimal loss to the water.

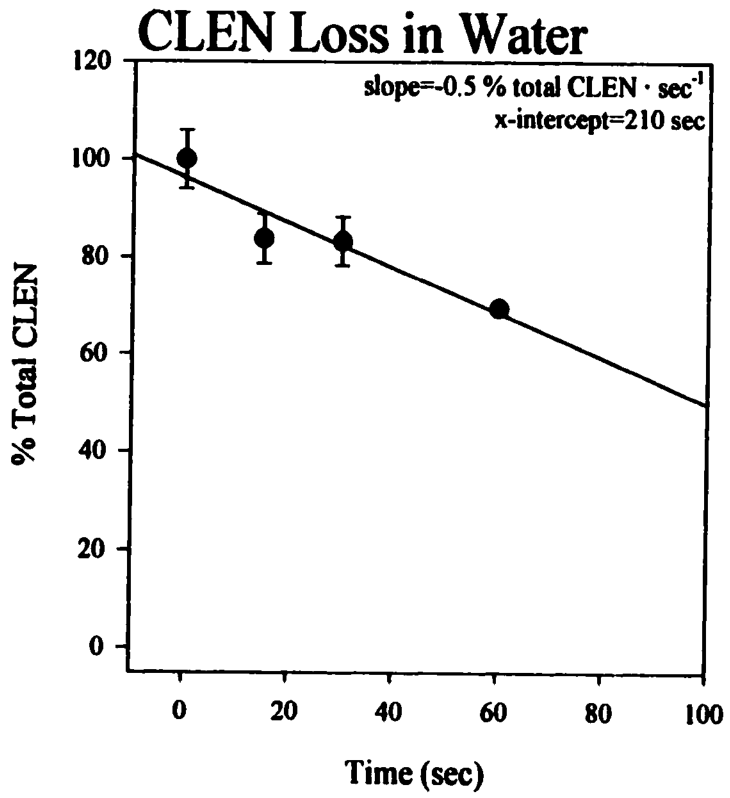

Figure A.3: Clenbuterol delivery method validation. Clenbuterol left in food pellets submerged in dechloraminated City of Ottawa tap water; rate at room temperature (approx. 19.0 °C) of clenbuterol loss is approximately 0.5% of total clenbuterol • sec^{-1}. The approximated time to complete loss, assuming a constant rate of disappearance, is approximately 210 sec or 3.5 min. Values represent mean ± SEM of 3 individual experiments done in duplicates.

Table A.1: Primers used to amplify probe templates of the rainbow trout β_2-AR and β-actin to be used for RPA (Section 3.4.4.2).

Rainbow trout gene (Accession Number)	5' Primers	Pos.	3' Primers	Pos.	Temp. (°C)
β_2-AR (AY044093)	*ATTTAGGTGACACTATAGAAGVC* GGGGACGGGAGCGGCGGAGG	778-798	*TAATACGACTCACTATAGGGAGA* CAGAGGSARAAGRYGCCCAT	860-880	50
β-actin (AJ438158)	*ATTTAGGTGACACTATAGAAGVC* CGAGCACGGCATCGTCACC	275-294	*TAATACGACTCACTATAGGGAGA* CGCGGCCGGCCAGATCCAG	591-610	66

Accession number refers the GenBank nucleotide database (http://www.ncbi.nlm.nih.gov). Primer DNA sequence is given according to the IUPAC code. Sequences in *italic* represent the promoter sequences for T7 and SP6 RNA polymerases. Position represents the position relative to the nucleotide sequence. Temperature refers to annealing temperature used in PCR.

Table A.2: Body parameters for SHAM, CLEN and RACT treated rainbow trout for the protein synthesis experiments.

	SHAM	CLEN	RACT
W_i (g)	82.3 ± 5.6	82.5 ± 5.8	74.1 ± 4.2
W_f (g)	133.3 ± 10.3[a]	130.7 ± 9.9[a]	125.7 ± 11.1[a]
L_i (cm)	20.4 ± 0.4	20.6 ± 0.4	19.5 ± 0.3
L_f (cm)	22.7 ± 0.5[b]	22.1 ± 0.3[b]	21.9 ± 0.3[b]
CF_i	0.96 ± 0.02	0.94 ± 0.03	1.00 ± 0.02
CF_f	1.14 ± 0.03[c]	1.20 ± 0.05[c]	1.19 ± 0.08
SGR_{exp} (% • day^{-1})	1.61 ± 0.21	1.53 ± 0.25	1.73 ± 0.24

Values are means ± SEM (n = 4): initial and final weights (W_i and W_f); initial and final lengths (L_i and L_f); initial and final condition factors (CF_i and CF_f); specific growth rate (SGR_{exp}). There are no significant differences between treated and SHAM for any body parameters measured (repeated measures one-way ANOVA, $P < 0.05$). There are significant differences when comparing BW_f with BW_i, L_f with L_i and CF_f with CF_i within the same treatment group represented by [a], [b] and [c], respectively (paired t-test, $P < 0.05$). These data do not include the small fish (control for fractional protein synthesis rates).