THE EFFECT OF THE *NEUROTENSIN* GENE ON GROWTH AND CARCASS TRAITS IN BEEF CATTLE

A Thesis Submitted to the College of

Graduate Studies and Research

In Partial Fulfillment of the Requirements

For the Degree of Master of Science

In the Department of Animal and Poultry Science

University of Saskatchewan

Saskatoon, SK

By

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ABSTRACT

Neurotensin (*NTS*) is a tridecapeptide which is widely distributed in the central nervous system and digestive tract. It is highly expressed in neurons of the hypothalamus, a region of the brain known to control feeding behavior. Several studies have shown that intracerebroventricular injection of *NTS* decreased food intake in rats. *NTS* was therefore characterized and analyzed for associations with growth and carcass traits in beef cattle.

NTS mRNA was successfully isolated from brain, spinal cord, abomasum, rumen wall, small intestine and skin samples. The complete bovine mRNA sequence was obtained from skin, along with partial genomic sequence. Three single nucleotide polymorphisms (SNPs) in the 3' untranslated region (3'UTR) and six intronic SNPs were identified. The three SNPs in the 3'UTR were not in linkage disequilibrium. Of the three SNPs in the 3'UTR, two had minor allele frequencies of 2% and therefore were not analyzed further. The minor allele frequencies for the third SNP (*419G>A) ranged between 0% and 23% for four major beef breeds. The *419G>A SNP was also used to map *NTS* to bovine chromosome five between markers *BM6026* (13 cM, LOD=4.03) and *RM103* (4 cM, LOD=3.63).

No significant associations between the *419G>A SNP and growth traits were identified. Statistical analysis revealed significant genotype associations for rib eye area (REA), grade fat and moisture in the Canadian Beef Reference Herd (CBRH). These associations were not verified in a second group of purebred yearling bulls. However, significant associations with end of trial fat, %fat and fat deviation were associations for

marbling and quality grade. In all cases the *AA* genotype was associated with increased fat.

Although significant associations between carcass measurements and genotype at the *491G>A SNP were present in some populations, none of these associations were found in more than one population. It was therefore concluded that the *419G>A SNP on the bovine *NTS* gene does not prove to have an economic advantage to the beef cattle industry.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Sheila Schmutz, for her vast support, encouragement and expertise. I would also like to thank my committee members, Dr. Fiona Buchanan, Dr. John McKinnon, the committee chair, Dr. Andrew Van Kessel and my external examiner, Dr. Phyllis Shand.

Many thanks also go out to Tom Berryere, for his help within the laboratory. Lab members, Kim Mclean, Dayna Dreger, Sarah Helgeson, Julie Goodall, Kaley Pugh and Janel Dickin were also helpful for their vast amount of understanding and encouragement.

I would also like to thank my mom and dad for their support throughout all of my University years. My Grandfather, Winston Goodhew, also played a major role on my University career and without it, I may not have made it this far. Thank you also to Steven Doig for his support and understanding.

Thank you also for the funding of this project, which was provided by the Natural Science and Engineering Research Council of Canada through the Collaborative Research and Development program, and a contribution from Quantum Genetics, Inc.

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LIST OF ABBREVIATIONS

aa	Amino Acid
APD	Antipsychotic Drug
bp	Base Pair
CBRH	Canadian Beef Reference Herd
cM	Centimorgan
CNS	Central Nervous System
cDNA	Complementary DNA
r	Correlation Coefficient
dNTP	Deoxyribonucleotide triphosphate
dH_2O	Distilled water
DNA	Deoxyribonucleic Acid
DOF	Days on feed
EoT	End of Trial
Fat Dev.	Fat Deviation
GI	Gastrointestinal
ICV	Intracerebroventricular
IC	Intracisternal
IP	Intraperitonial
IV	Intravenous
kb	Kilo Bases
LD	Linkage Disequilibrium

LOD	Logarithm of the Odds
$MgCl_2$	Magnesium Chloride
mRNA	Messenger RNA
µl	Microliter
µg	Microgram
mM	Millimolar
NN	Neuromedin N
NTS	Neurotensin
NTR	Neurotensin Receptor
PVN	Paraventricular Nucleus
PCR	Polymerase Chain Reaction
QTL	Quantitative Trait Locus
RFLP	Restriction Fragment Length Polymorphism
REA	Rib Eye Area
RNA	Ribonucleic Acid
SNP	Single Nucleotide Polymorphism
SoT	Start of Trial
SAS	Statistical Analysis System
UTR	Untranslated Region

1.0 GENERAL INTRODUCTION

Neurotensin (NTS) is a 13 amino acid (aa) peptide (Gln-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH) which was first isolated from bovine hypothalamus by Carraway and Leeman in 1973. In rats, the injection of *NTS* has been shown to decrease food intake and body weight (Luttinger *et al.* 1982, Levine *et al.* 1983, Stanley *et al.* 1985, Sahu 1998, Beck *et al.* 1998). The injection of *NTS* has also been shown to influence gastrointestinal function (Evers 2002).

Several polymorphisms have been identified in the *neurotensin* gene in humans and analyzed for associations with neuropsychiatric disorders, alcohol dependence and restless leg syndrome, however, no studies have focused on polymorphisms within the cattle *NTS* gene. *NTS* was therefore studied as a candidate gene in this study for optimizing growth traits in beef cattle based on the evidence that *NTS* decreases food intake and body weight in rodent species. In turn, potential *NTS* genotype associations with growth traits may prove to be an economic benefit to the beef cattle industry. Therefore, this thesis focuses on the characterization of *NTS* in cattle and its potential associations with growth traits.

2.0 LITERATURE REVIEW

2.1 *NTS* Gene Structure

Cloning and sequence analysis of cDNA for canine *NTS* revealed the primary structure of the 170 aa precursor protein (Figure 2.1) that encodes both *NTS* and *neuromedin N* (*NN*) (Dobner *et al.* 1987). The *NN/NTS* coding domains are tandemly positioned on exon 4 with *NN* proceeding *NTS* in the precursor sequence (Dobner *et al.* 1987). The *NTS* and *NN* sequences are both flanked and separated by three Lys-Arg sequences (Figure 2.1; Dobner *et al.* 1987).

Neuromedins are a novel family of peptides isolated from porcine spinal cord in the 1980's based on their ability to contract smooth muscle (Brighton *et al*. 2004). This family of peptides includes the neuromedins B and C (bombesin-like), K (neurokinin B) and L (neurokinin A, substance K, or neurokinin ∝), N (neurotensin-like), and U (Brighton *et al*. 2004). Neuromedins are found in many tissues including the central nervous system and have been shown to influence a wide variety of physiological functions (Brighton *et al*. 2004). Only *neuromedin N* is encoded by the gene studied here.

NN is a 6 aa peptide (Lys-Ile-Pro-Tyr-Ile-Leu-OH) that shares strong homology with the C-terminal of *NTS* with the four carboxyl-terminal aa's of *NTS* being identical (Minamino *et al.* 1984). *Neuromedin N* produces a contractile activity on

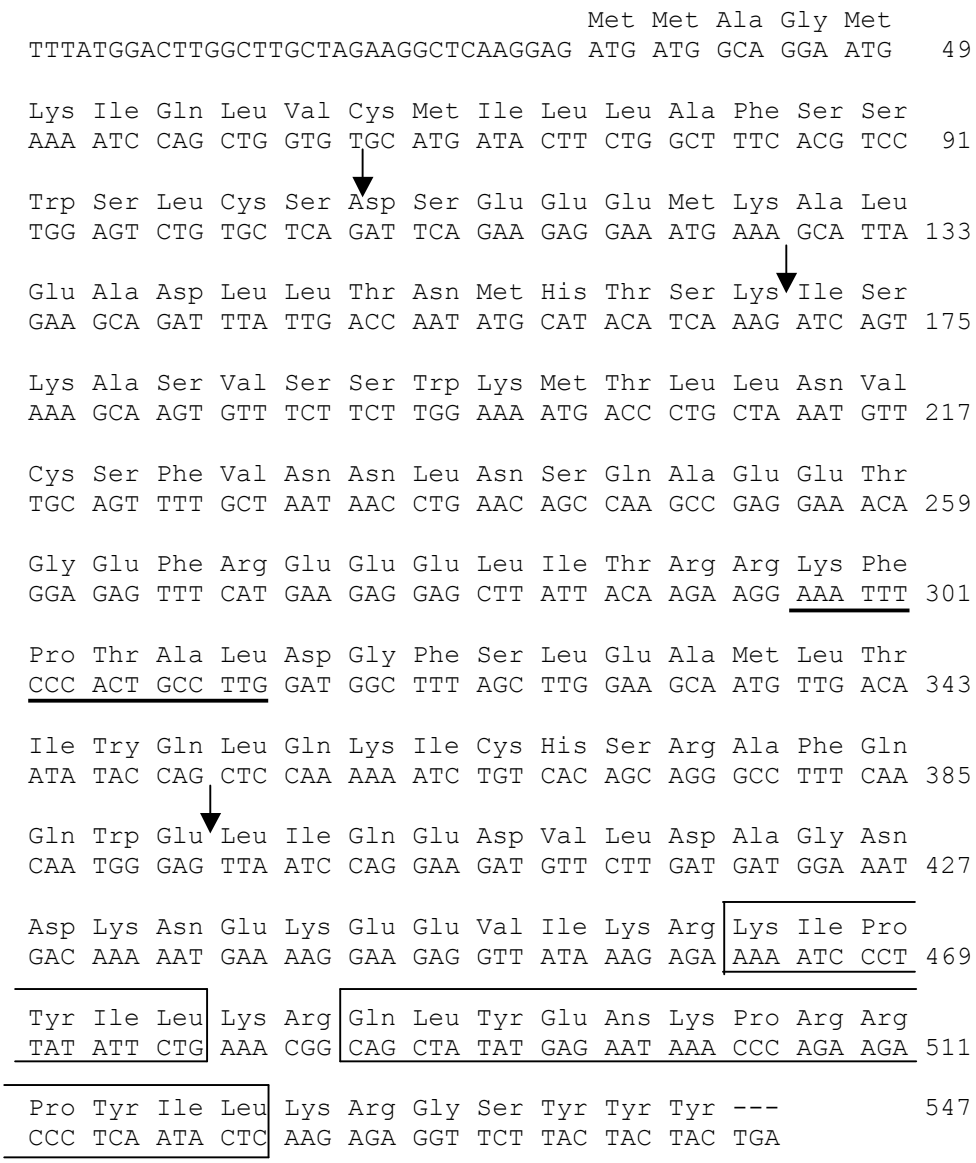

Figure 2.1: Predicted amino acid sequence from DNA sequence of canine preproneurotensin/neuromedin N. The *NN* and *NTS* coding regions are boxed by solid lines and a region similar to *NN* is underlined (adapted from Dobner *et al.* 1987). Numbers to the right indicate the final nucleotide position in each row. Arrows indicate exon boundaries.

the ileum in guinea pigs and induces a hypotensive response that is similar to *neurotensin.*

A study by Kislauskis *et al.* (1988) identified that the rat *NTS* gene spans approximately 10.2 kilo bases (kb) and is divided into four exons as revealed by comparison with two heterologous cDNA sequences from the dog and cow. The cattle cDNA sequence for *NTS* was found to span 1166 bps, which contained the four exons as well as the 5' and 3' untranslated regions (UTRs) (Figure 2.2). The cattle cDNA sequence was significantly longer than the dog sequence which consisted of 759 bp. This was due to the shortened 3' UTR of the dog cDNA (Kislauskis *et al*. 1988).

Full-length human *NN/NTS* cDNA was first cloned by Dong *et al.* (1998). The *NTS* cDNA in the human consists of 1241bp (GenBank NM 006183). This difference in nucleotide length compared to cattle also occurs in the 3' and 5' untranslated regions.

2.2 Expression

Like many polypeptide hormones or neuropeptide precursors, *NN/NTS* precursor undergoes differential processing in tissues, which gives rise to different combinations of peptides (Friry *et al*. 2002). A study by Kislauskis *et al*. (1988) stated that RNA blot analysis revealed that the rat *NN/NTS* gene is transcribed to yield two distinct mRNAs, 1.0 kb and 1.5 kb in size. These two mRNAs result from the differential utilization of two consensus poly (A) addition signals and differ in the length of their 3' untranslated region (Kislauskis *et al.* 1988). They also identified that there was a striking variation in the relevant levels of these two mRNAs between the brain and intestine. They

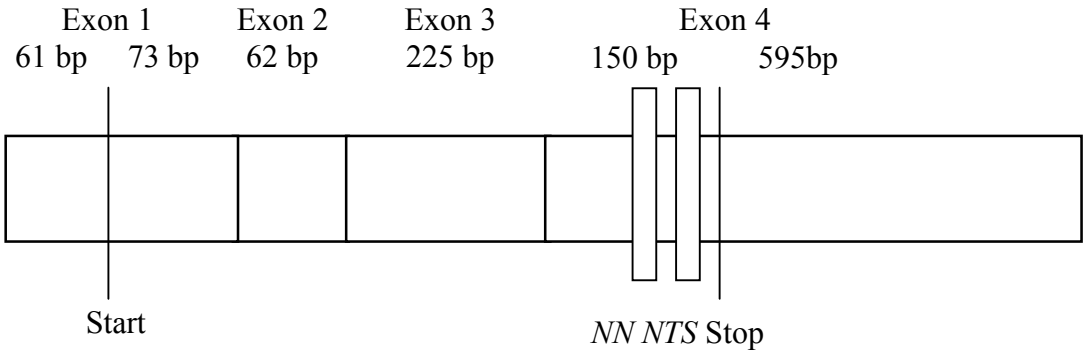

Figure 2.2: A schematic diagram of the exon sizes in the cattle *NTS* gene.

concluded that the smaller (1.0 kb) mRNA greatly predominates in the intestine while both mRNA species are almost equally abundant in the hypothalamus.

These results were supported by Rostene and Alexander (1997), who indicated that the *NN/NTS* precursor protein contained four potential sites of proteolytic cleavage by which *NTS*, *NN* and extended forms of each could be generated. De Nadia *et al.* (1994) identified that the posttranslational processing of the *NN/NTS* precursor yields *NTS*. This basic pattern of *NN/NTS* processing appears to be similar throughout the brain (De Nadia *et al.* 1994). However, the predominant molecular form of *NN* in the gastrointestinal tract differs from that in the brain, with mammalian intestine containing predominantly extended forms of *NN* (Carraway and Mitra 1990).

A more recent study by Friry *et al.* (2002) also supported these findings and stated that in the brain, *NN/NTS* processing gives rise to *NTS* and *NN* whereas in the gut, processing leads mainly to the formation of *NTS* and a large peptide ending with the *NN* sequence at its C-terminus.

In the rat hypothalamus, neurosecretory cells capable of *NTS* synthesis are located in specialized groups of nerve cells in the brain (arcuate nucleus) and the paraventricular nucleus (PVN) (large neurons of the front half of the hypothalamus) (Rostene and Alexander 1997). In some of these cells, *NTS* coexists with other neurohormones or transmitter-synthesizing enzymes (Rostene and Alexander 1997).

A study by Evers *et al.* (1991) found that *NTS* was expressed throughout the small intestine of the adult rat, with the greatest abundance in the mid and distal intestine. In contrast, *NTS* was not expressed in the adult pancreas, stomach or colon.

NTS is localized in the brain and specialized endocrine cells (N-cells) dispersed throughout the mucosa of the small intestine (Reinecke 1985). In most brain regions, including the hypothalamus, *NTS* mRNA is first detected in the perinatal period and levels gradually increase to reach a plateau by adulthood (Rostene and Alexander 1997) (Figure 2.3).

The study by Evers *et al.* (1993) characterized the development pattern of *NN/NTS* expression in the gut of both humans and rats. They found that *NTS* expression in the small intestine is initially low in the fetal rat, however, the expression is dramatically increased three days postnatal. After postnatal day 28, *NTS* mRNA levels remained relatively constant in the ileum but were decreased in the jejunum. Furthermore, similar to the rat, *NTS* expression was low in the human fetal ileum but was dramatically increased in the adult. The jejunum and ileum show a similar pattern of expression until the end of the fourth postnatal week. *NN/NTS* is transiently expressed in the colon of rats and humans during a development stage similar to the small intestine.

2.3 Receptors

NTS mediates its effects through cell surface receptors, which are distributed heterogeneously in the CNS with both high and low affinity sites (Goedert *et al.* 1984). In the rat brain, Goedert *et al.* (1984) found that the highest density of binding sites were located in the hypothalamus and the frontal cortex, with intermediate sites in the striatum, thalamus and midbrain and low levels in the cerebellum. The review by

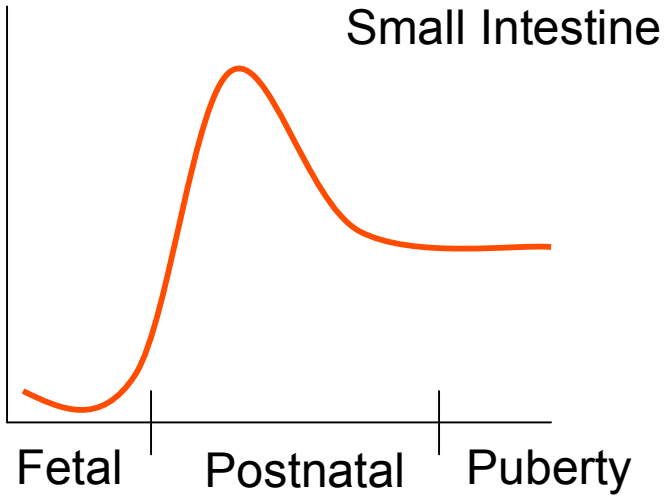

Figure 2.3: *NTS* expression in the small intestine.

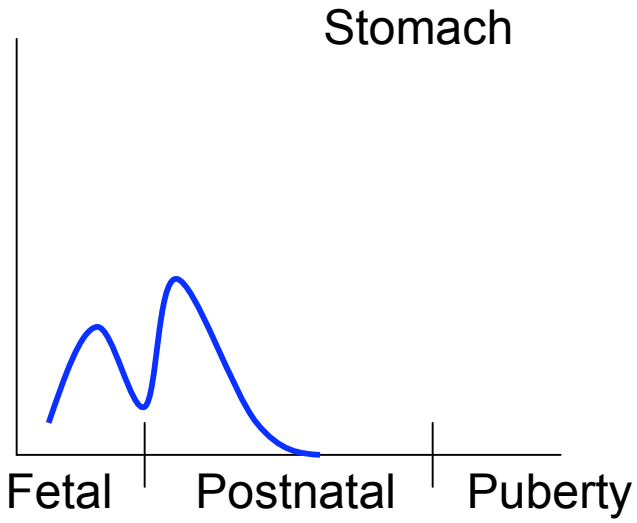

Figure 2.4: *NTS* expression in the stomach.

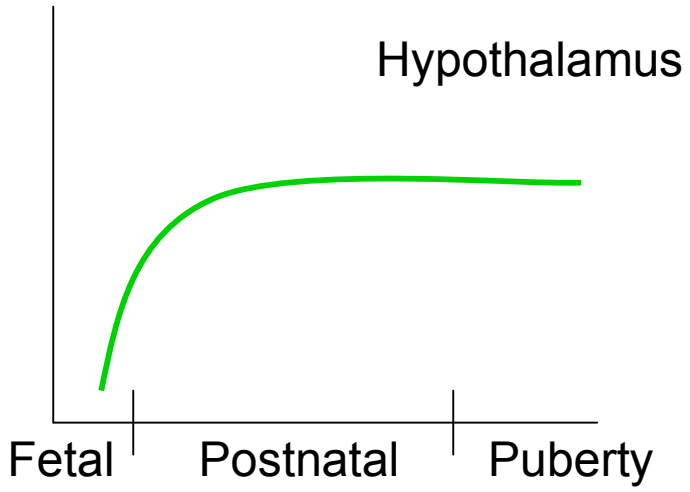

Figure 2.5: *NTS* expression in the hypothalamus.

Dobner (2005) states that *NTS* and *NN* appear to have largely overlapping functions and bind to a common group of receptors that include two G protein-coupled receptors (*NTR1* and *NTR2*) and two predominately intracellular receptors (*NTR3/sortilin* and *NTR4/SorLA*).

NTR1 was the first *NTS* receptor to be cloned (Tanaka *et al.* 1990). It was identified as a G protein-coupled protein which showed a selective and high affinity binding to *NTS* peptides. This receptor appears to mediate most of the pharmacological and physiological effects of *NTS* (Dobner 2005).

The second *NTS* receptor (*NTR2*), cloned by Mazella *et al.* in 1996, was shown to share substantial sequence identity with *NTR1*, but was pharmacologically distinct. The pharmacological properties of *NTR2* correspond to those of a low-affinity, levocabastine-sensitive *NTS* binding site, which is highly expressed in the cerebellum and hippocampus of the adult mouse brain. Levocabastine is a selective second-generation H_1 receptor antagonist which is more selective for peripheral H_1 receptors. The physiological functions of *NTR2* however, remain uncertain. However, there is increasing evidence that *NTR2* may mediate certain analgesic effects of *NTS* (Dobner 2005).

Mazella *et al.* (1998) cloned *NTR3* which corresponds to the protein designated gp95/sortilin previously purified and cloned. This receptor was then designated as the *NTR3/sortilin* receptor and was described as the first neuropeptide receptor that did not belong to the family of G-protein-coupled receptors.

Jacobsen *et al.* (2001) isolated and sequenced *NTR4* also known as *SorLA/LR11*, a mosaic protein. *SorLA* was shown to have similarities with

NTR3/sortilin. Both are synthesized as a proreceptor that is later cleaved. *NTR3* and *NTR4* receptors appear to function primarily in receptor internalization and ligand degradation (Dobner 2005). Although these two receptors may mediate some functions of *NTS*, the available evidence suggests that most of the physiological functions and pharmacological effects of *NTS* are mediated by *NTR1* (Dobner 2005).

2.4 Functional Aspects of *NTS*

2.4.1 Central Nervous System

Different functions of *NTS* have been studied in a vast number of systems within varying organisms. One of the first reported effects of *NTS* was observed after direct injection into the brain, where it induced hypothermia (Bissette *et al*. 1976). The study by Bissette *et al.* (1976) looked at hypothermia and intolerance to cold induced by intracisternal (IC) and intravenous (IV) administration of *NTS* in mice. The IC administration of *NTS* decreased the core temperature in a dose related manner. In contrast, there were no significant effects on cold tolerance after IV administration. Therefore, Bissette *et al.* (1974) concluded that *NTS* can be included in the list of peptides which seem to directly influence the CNS.

A study by Nemeroff *et al*. (1977) also identified that centrally administered *NTS* lowers body temperature in rats and mice, in a dose-related fashion. They also showed a reduction in locomotor activity after *NTS* administration. The hypothermic effects of *NTS* were not present after peripheral administration, suggesting that the

peptide is not able to cross the blood-brain barrier in sufficient quantities to exert direct brain effects.

The next major CNS effect of centrally administered *NTS* described was its pain reducing effects (Clineschmidt *et al.* 1979). This study identified that *NTS* administered IC in mice increased the reaction time in a hot plate test and decreased writhing induced by acetic acid. The antinociception (increased tolerance to pain) effect of *NTS* lasted about an hour. The mice were also identified to be hypothermic and have reduced locomotor activity. These effects were not identified after the IV administration of *NTS*. This reduction in sensitivity is normally produced when an opium containing substance (opioid) combines with a receptor. Importantly, *NTS*-induced antinociception is not mediated through the opioid system, because *NTS* was not antagonized by naloxone (a narcotic analgesic antagonist used in the reversal of acute narcotic analgesic respiratory depression) (Clineschmidt *et al.* 1979). Therefore, it was suggested that the *NTS* effects result from an interaction of the peptide with *NTS*-specific receptor sites in the CNS.

2.4.1.1 Antipsychotic Drug

NTS was formally proposed to be a possible endogenous neuroleptic (a tranquilizing drug used to treat mental disorders) 27 years ago based on the fact that central *NTS* administration produced a spectrum of effects that were similar to those of antipsychotic drugs (APD) (Nemeroff 1980). Nemeroff (1980) identified that IC or intracerebroventricular (ICV) injected *NTS* produced: potentiation of barbiturate sedation, hypothermia, diminished locomotor activity and muscle relaxation in rats, all

of which are also caused by neuroleptics. In addition, bilateral nucleus accumbens injections of *NTS*, like haloperidol (a major antipsychotic agent), antagonized increased locomotor activity and rearing induced by d-*amphetamine.* Hypothermia and muscle relaxation which is induced by either *NTS* or neuroleptics is antagonized by IC injection of thyroid-releasing hormone. These findings, therefore, support the hypothesis that *NTS* is a neuromodulator as shown by its similarity to neuroleptics.

Decreased *NTS* signaling has been shown to contribute to defects in sensorimotor gating in animal models of schizophrenia in a study by Binder *et al*. (2001b). Sensorimotor gating was assessed in rats by measuring the acquisition of latent inhibition and the prepulse inhibition of the acoustic startle reflex. APD-induced enhancement of latent inhibition and disrupted prepulse inhibition has been shown to have predictive validity for APD activity. The disruption of *NTS* neurotransmission, using a *NTS* receptor antagonist, blocked the behavioral effects of APD in these two distinct tests for sensorimotor gating. These results suggest that the development of direct *NTS* agonists may provide novel therapeutic approaches toward the treatment of schizophrenia in humans (Binder *et al*. 2001b).

The review by Binder *et al.* (2001a) identified that both typical and atypical APD increase *NTS* mRNA expression, *NTS* peptide concentrations and cause *NTS* release into the nucleus accumbens. Typical APD are those which are effective in reducing the positive symptoms of schizophrenia (e.g. delusions and hallucinations) whereas atypical APD improve both positive and negative symptoms (e.g. social withdrawl and amotivation) (Kinkead *et al.* 1999). This suggests that the mechanisms

underlying both the therapeutic and side effects of APD treatment might involve increased *NTS* signaling in the nucleus accumbens (Binder *et al*. 2001a).

2.4.1.2 Appetite Control

Several peptides have been found to be present in both the gut and brain. Suggestions indicate that within the CNS, these peptides may be involved in digestive functions that are complementary to their function in the periphery. Bombesin is one example of a peptide which is located in the gut and the brain and has been shown to cause a reduction in food intake after both peripheral and central administration in rats. Because *NTS* is also found in both the brain and gut, Luttinger *et al.* (1982) decided to study the effects on *NTS* on food consumption.

Luttinger *et al.* (1982) looked at the effect of *NTS* on food consumption through the use of Sprague-Dawley albino rats which were deprived of food for 24 hours. The experimental group was administered 10 µl of a 0.9% saline solution containing 30 µg of *NTS* through ICV injections. Intracerebroventricular injection of *NTS* produced a dose-related decrease in food intake. This decrease was augmented at one hour post administrations, with recovery of intake occurring around three hours. ICV injection of *NTS* after the ingestion of a novel flavor did not produce a flavor aversion.

A similar study by Levine *et al.* (1983) also looked at the effect on *NTS* on feed intake, but did so through the examination of *NTS* on multiple feeding paradigms in rats. ICV injection of *NTS* was once again shown to decrease food intake after 30 hrs of starvation compared to the controls. Norepinephrine and insulin induced feeding was

also decreased after *NTS* injection, however, muscimol (chemical substance which acts on the CNS to alter behavior) induced feeding was not. The study also showed that an intact vagus nerve is not necessary for *NTS* effects, for *NTS* decreased food intake in vagomatized rats (Levine *et al.* 1983).

NTS extended the suppression of feeding elicited by norepinephrine when injected into the PVN of rats (Stanley *et al.* 1985). *NTS* injected into the PVN ipsilateral to norepinephrine was more effective than contralateral, suggesting that *NTS* and norepinephrine interact in an anatomically site-specific manner (Stanley *et al.* 1985). These authors suggested that the reduction in feed intake may be partially behavior specific.

Hawkins (1986) examined the injections of *NTS* into the nucleus accumbens, ventromedial hypothalamus and cerebroventriculum of rats. Although no effect was seen in the nucleus accumbens, a reduction in feed intake was observed from injections into two other brain areas; with a maximum response occurring at approximately 90 min after the highest dose of *NTS* was administered. This time may reflect the ability of *NTS* to be degraded by peptidases in the CNS, which supports the contention that endogenous *NTS* may act as a satiety factor via its effects on the CNS.

In humans, meal stimulation caused *NTS* concentrations in the blood to increase 100-300% within 30 min following the meal (Melchior *et al.* 1994). They looked at three experimental sessions to determine their influence on *NTS* concentrations. In the first session, subjects were fasted after their usual breakfast; in the second session subjects received a warm palatable meal following breakfast and in the third session participants received a cold, poorly acceptable meal following breakfast. *NTS*

concentrations were significantly higher after both meals than during the fasting session. The *NTS* increase was significantly higher following the palatable meal, than during the fasting session or the cold meal session, when measured thirty minutes after consumption.

Leptin is one of the major peripheral signals that controls food intake and body weight (Sahu 1998). It is defined as a blood borne factor that is generated from the *Obese* gene which has been shown to decrease food intake and body weight in association with an increase in *NTS* (Sahu 1998). Sahu (1998) also identified that leptin inhibits the gene expression of neurons which are excitatory (e.g. neuropeptide Y) and stimulates expression of those which are inhibitory (e.g. *NTS*).

The interaction between leptin and *NTS* was studied by Beck *et al.* (1998). They focused on *NTS* for several reasons. First, it is present in brain areas such as the PVN where the existence of leptin receptors have been detected. Second, it inhibits food intake when it is injected in these areas of the brain or in the brain ventricles. Finally, its secretion is triggered by fat digestion and hypothalamic contents are sensitive to diet composition. ICV injection of *NTS* in rats caused a short lasting (30 min) inhibitory effect of *NTS* on food intake (Beck *et al.* 1998). The effect of ICV injection of leptin was observed after 24 hours. Co-injection with leptin, increased *NTS* effects at 30 minutes and prolonged it for an additional 30 minutes, while *NTS* increased the effect of leptin at 30 and 60 minutes, but not at 24 hours.

Beck *et al.* (1998) also identified that *NTS* concentrations were augmented in selective brain areas in fat-preferring rats. This increase was observed in the parvocellular part of the PVN and was associated with an increase in circulating leptin

levels. These results suggest that the short-term anorexigenic effects of leptin in normal rats are partly mediated by changes in *NTS* processing or release. Therefore, the *NTS* increase observed in fat-preferring rats would limit the over consumption of energy, a physiological mechanism translated by leptin.

Another study by Sahu *et al.* (2001) looked at the relative role of *NTS* in mediating leptin's action by examining the effects of *NTS* antiserum and *NTS* receptor antagonist on the satiety action of leptin in the rat. Administration of 4 μg of leptin decreased relative food intake, while prior administration of *NTS* antiserum (5 μl) completely blocked the effects of leptin on food intake. The effect of leptin on food intake was also completely abolished by prior intraperitoneal (IP) administration of *NTS* receptor antagonist at 2, 4 and 6 hr periods. They conclude that this neuropeptide is a quantitatively important component of the leptin sensitive neural circuity.

The potential involvement of *NTS* in food intake and body weight regulation is evident from the findings that the central administration of *NTS* inhibits food intake in the rat (Beck *et al.* 1998, Levine *et al.* 1983, Luttinger *et al.* 1982, Sahu 1998, Stanley *et al.* 1985). The *NTS* gene expression and peptide levels are decreased in obese rats and the *NTS* expression has also been shown to increase in response to leptin (Sahu 1998).

Tritos *et al.* (1998) suggest that a number of peptides expressed in the mammalian hypothalamus are involved in the regulation of food intake and energy balance. These authors suggested that melanin-concentrating hormone and neuropeptide Y are appetite stimulants, whereas melanocyte-stimulating hormone and *NTS* have appetite suppressing properties. The functional interactions between these orexigenic

and anorexigenic peptides, after ICV administration in rats, were described. In particular, they found that administration of *NTS* prevents only the orexigenic effect of melanin-concentrating hormone, but does not prevent the appetite-stimulating effect of neuropeptide Y. They concluded that the interactions between these neuropeptides are specific, although the underlying mechanisms are yet to be explored.

2.4.2 Neuroendocrine System

Involvement of *NTS* in neuroendocrine regulation was supported by its distribution, *NTS*-like immunoreactivity was found in the hypothalamus and anterior pituitary (Tyler-McMahon *et al.* 2000). Figure 2.4 shows that *NTS* has been identified to dose-dependently increase adrenocorticotropic hormone secretion, decrease thyroid stimulating hormone and lutenizing hormone (Fuxe *et al.* 1984) and cause an increase in blood glucose (Wolfe et al. 1978).

A study by Fuxe *et al.* (1984) identified that ICV injections of *NTS* increased adrenocorticotropic hormone secretion in male rats. This secretion was dose-dependently increased, especially in the absence, but also in the presence of tyrosine hydroxylase (an enzyme which catalyzes the first step in the biosynthesis of catecholamines). Serum thyroid stimulating hormone levels were dose-dependently and significantly decreased in the absence and presence of tyrosine hydroxylase inhibition. In the case of lutenizing hormone secretion, however, *NTS* could only reduce serum lutenizing hormone levels in the presence of tyrosine hydroxylase inhibition.

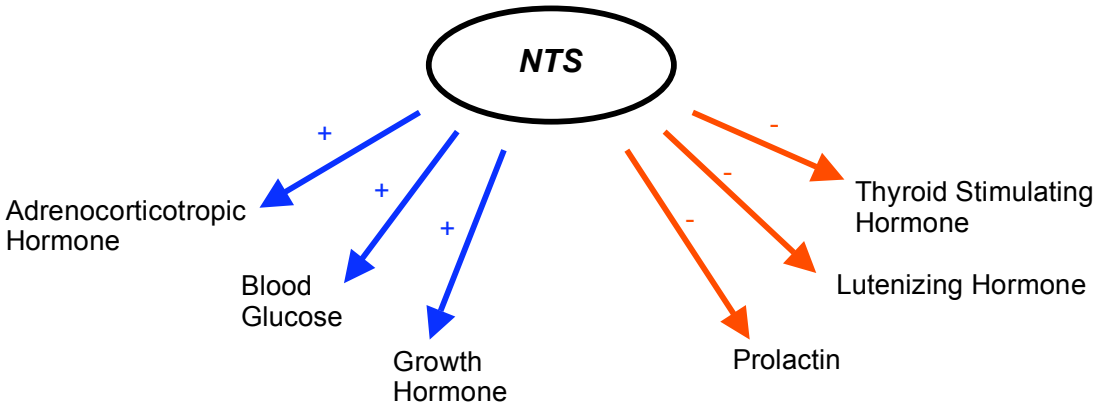

Figure 2.6: Effects on *NTS* on the neuroendocrine system.

NTS has also been shown to have a hyperglycemic effect when injected into rats. Wolfe *et al*. (1978) expanded on this finding and looked at the effect of *NTS* on the rate of glucose production and uptake. Results showed a progressive rise in plasma glucose concentration after IV injection of *NTS*, with glucose production being elevated 35-40% throughout the first 30 minutes after administration. Wolfe *et al.* (1978) therefore concluded that the rise in blood glucose after *NTS* injection was entirely due to an increased rate of glucose production.

Under all conditions reviewed by Rostene and Alexander (1997), ICV administration of *NTS* was shown to decrease plasma prolactin levels, whereas IV administration of *NTS* increased prolactin levels. This conclusion is also supported in the study by Fuxe *et al.* (1984) who identified that prolactin secretion was dose-dependently reduced after ICV *NTS* administration, but only in the presence of tyrosine hydroxylase inhibition, which produces a hypersecretion of prolactin.

The effect of exogenous *NTS* on growth hormone secretion appears to be dependent upon the route of administration as well as the animal model (Rostene and Alexander 1997). ICV administration of *NTS* was identified to increase growth hormone levels of hypophysial portal blood (Abe *et al.* 1981). *NTS* appears to alter growth hormone secretion by acting at the hypothalamic level since no evidence indicates that *NTS* can act alone at the pituitary level to influence growth hormone secretion (Rostene and Alexander 1997).

2.4.3 Gastrointestinal Tract

NTS appears to cause different effects in different portions of the gastrointestinal tract. These effects on gastrointestinal motility clearly depend on the animal species and also differ according to the mode of application (Reinecke 1985). Some of the physiological effects of *NTS* on the gastrointestinal (GI) tract identified by Assimakopoulos *et al.* (2005) include: stimulation of pancreatic secretion, stimulation of colonic motility and inhibition of small intestine and gastric motility.

Assimakopoulos *et al.* (2005) investigated the effect of IP injections of *NTS*, in partial hepatectomy rats, on intestinal barrier function in partially anesthetized rats. Results indicated that *NTS* improved intestinal barrier function and reduced endotoxemia, with the effect being mediated, at least in part, by the trophic, mitogenic and antioxidant effect on the intestinal epithelium.

Subcutaneous injections of *NTS* were also shown to stimulate the growth of various GI tissues, including the pancreas and gastric antrum (portion of the stomach lined with mucosa which does not produce acid) (Feurle *et al.* 1987). Chronic subcutaneous injections of *NTS* increased pancreatic weight and thickness of the muscular and mucosal layers of the antrum without showing any effects on the stomach.

NTS has also been shown to have a stimulatory effect on the gallbladder in some species (Fujimura *et al.* 1994). Gui *et al.* (2001) examined the effects of *NTS* on hepatic bile acid output and showed that intestinal *NTS* mRNA expression and blood levels were altered in rats fed chow containing bile acid and the bile acid chelator. Final

results indicated that enhancement of enterohepatic bile acid cycling is yet another mechanism by which *NTS* could facilitate the digestion and absorption of lipids.

NTS has been closely associated with fat intake and utilization, with the ingestion of fat being the strongest stimulus for *NTS* release in animals and humans (Ferris *et al.* 1981). Perfusion of the small intestine with a lipid solution resulted in elevated *NTS* levels. Therefore, the intraintestinal lipid was shown to be an effective and specific stimulus for the release of *NTS* from the small intestine into the portal circulation.

Piatek *et al*. (2005) used lipid mobilizing activity and serum lipolytic activity as screening parameters indicative of changes in lipid metabolism induced by *NTS*. The main effect of *NTS* administration observed was the inhibition of lipolytic processes in the adipose tissue, causing inhibition of lipid mobilizing activity and serum lipolytic activity. It also appears that *NTR2* receptor played a key role in changes in the lipid and cholesterol metabolism parameters observed.

2.5 Polymorphisms

Given that *NTS* can selectively modulate dopaminergic neurotransmission, Austin (2000) hypothesized that *NTS* dysfunction might contribute to the pathogenesis of neuropsychiatric disorders, particularly schizophrenia. This study identified three sequence variants in the human *NTS* gene that might alter *NTS* structure and function. Denaturing high performance liquid chromatography was used to screen 1381 bases of the neurotensin gene including all four exons and 283 bases of the 5' flanking region.

Denaturing high performance liquid chromatography analysis revealed heteroduplexes in three PCR fragments. Sequencing identified three heteroduplexes as a C>G (-166C>G), an A>G (-3A>G) and a C>A (+187C>A). Polymorphisms -166C>G and -3A>G are located in the 5' flanking region and 5' UTR respectively. The third variant, +187C>A, results in a leucine to isoleucine change in the precursor protein at residue number 64 from the amino terminus. Using case-control study design and a novel genotyping system based upon a primer extension protocol and HPLC detection, no evidence supported the hypothesis that the variation in the proneurotensin gene contributed to susceptibility to schizophrenia.

Restless leg syndrome has been mapped to chromosome 12 in humans, in a region that also contains *NTS*. Since, *NTS* is an important modulator of the dopaminergic transmission, it represents a strong functional and positional candidate in the context of restless leg syndrome (Desautels *et al.* 2003). Nineteen individuals were investigated using a combined denaturing high performance liquid chromatography and direct sequencing method. Analysis of the genomic sequence revealed two intronic polymorphisms and one variant in the 5' UTR. The 5' UTR variant resulted in an *A* to *G* transition at position –3 from the start codon. The first intronic variant resulted in an *A* to *G* transition while the second consisted of a *T* to *A* transition. None of the polymorphisms resulted in an amino acid change. None of the observed variants co-segregated with restless leg syndrome and no disease-associated polymorphisms were detected in any of the analyzed individuals, indicating that it is unlikely that *NTS* is the gene responsible for restless leg syndrome.

NTS receptors in the neurons of the ventral segmental area facilitate dopamine release, making the *NTS* gene an excellent candidate gene for alcohol dependence and for other behaviors that involve reinforcement (Vanakoski *et al.* 2000). Vanakoski *et al.* (2000) looked at the *neurotensin* 479A>G promoter variant in order to determine the frequency of the variant and to study the association between *NTS* and alcohol dependence in a Finnish population. They compared 229 unrelated healthy controls to 134 unrelated alcohol-dependent subjects. In addition, 276 relatives of the alcohol-dependent and control subjects were genotyped. The frequency of the rarer 479*G* allele was 0.07 in controls and 0.06 in alcohol-dependent subjects, and this difference was not statistically significant. The study therefore revealed no association between alcohol dependence and this marker.

3.0 HYPOTHESIS

Based on previous evidence that *NTS* has decreased food intake, body weight and influenced gastrointestinal function in rodent species *NTS* was therefore studied in beef cattle. *NTS* was chosen as a candidate gene for optimizing growth traits in beef cattle, for beef cattle producers seek to maximize profitability. In turn, potential *NTS* genotype associations with growth traits may prove to be an economic benefit to the beef cattle industry.

Therefore, I hypothesized that potential polymorphisms within the bovine *NTS* gene may be associated with growth traits in cattle. In beef cattle particularly, I hypothesized that genotype variants may be associated with weaning weight and in turn yearling weight and hot carcass weight.

Additional objectives included tissue expression, comparison of cattle sequence to various species for amino acid homology, linkage mapping, linkage disequilibrium and haplotype analysis of polymorphisms if more than one was discovered.

4.0 MATERIALS AND METHODS

4.1 Animals

4.1.1 Canadian Beef Reference Herd

The Canadian Beef Reference Herd (CBRH) was developed by multiple ovulation embryo transfer in order to produce a reference herd for gene and QTL (quantitative trait locus) mapping (Schmutz *et al.* 2001a). The herd consisted of five purebred bulls (Angus, Hereford, Simmental, Charolais and Limousin) which were mated to 13 purebred heifers (Angus, Hereford, Simmental, Charolais, Limousin and Belgian Blue). These matings resulted in 17 full-sib families, five of which were purebred families and the remaining 12 were crossbred. The families ranged in size from two to seventeen calves per family producing a total of 136 offspring.

These calves were born in the spring of 1997 either at the Termuende Research farm in Lanigan, SK or at the recipient cooperator's farm. Calves born at the recipient cooperator's farm were relocated to the Termuende Research farm within one month of calving. The calves were then weaned at the University of Saskatchewan feedlot where they were backgrounded and finished until slaughter, which occurred at 18-20 months of age. Growth and carcass traits were obtained for all offspring as well as the parents.

4.1.2 Bull Population

The bull population consisted of 168 yearling bulls representing four beef breeds (Charolais, Angus, Hereford and Simmental). These animals were part of a previous study which used real-time ultrasound to predict carcass traits (Bergen *et al.* 1996). These bulls were housed at the Beef Research Unit at the University of Saskatchewan for a 112 day performance test in which they were fed a moderate energy ration. Animals were slaughtered within two weeks of the end of trial with carcass measurements collected on the chilled carcasses (Bergen *et al.* 1996).

4.1.3 Steer Population

This population of steers was raised in a commercial feedlot setting, but with additional data collected as a validation group for potential DNA tests related to traits of commercial significance (Quantum Genetics, Inc. unpublished data).The steer population consisted of 492 crossbred steers which were purchased across western Canada and shipped to the Lakeside Research facility in Brooks, AB for a slaughter trial. Steers were classified as either British or Continental type cattle based on visual observation and ranged in age from 12 to 16 months. These cattle were fed the same diet and slaughtered either at 119 or 140 days on feed (DOF). Growth characteristics were measured throughout the trial with carcass measurements taken post slaughter.

4.2 DNA and RNA Extraction

DNA was extracted from all animals in the three populations outlined above, prior to the beginning of this research. Genomic DNA was obtained through blood extractions from the CBRH following the procedures of Montgomery and Sise (1990) while the tissue extractions were following those of Sambrook *et al.* (1989). Genomic DNA was extracted from the bull population from whole blood via a phenol/chloroform extraction (Fitzsimmons *et al.* 1998). Extracted genomic DNA from the steer population was supplied by an industry collaborator. RNA was also isolated using the trizol method (Gibco, Gaithersburg, MD), from four purebred animals (Charolais, Belgian Blue, Galloway and Simmental), in order to synthesize cDNA using Superscript^{TM} (Gibco, Gaithersburg, MD).

4.3 Sequencing

NTS bovine genomic (GenBank NW_197475) and mRNA (GenBank M18621) sequences were available. These sequences were then used to design primers for the *NTS* gene. Forward (5'- CTCTGTCACATTTCCACAC -3') and reverse (5'- GCCTGT TCCTGACGTTTATG -3') primers were designed to amplify the complete *NTS* gene from cDNA in order to determine tissue expression using gel electrophoresis. This primer set isolated 640 bp, which contained the majority of the 5'UTR and all of the coding sequence. *NTS* amplification was attempted using cDNA from twelve different tissue samples (kidney, muscle, lung, liver, iris, abomasum, brain, spinal cord, small

intestine, rumen wall, fat tissue and skin from three random animals) in order to determine the location of *NTS* expression.

To identify polymorphisms in mRNA, we obtained cDNA from the skin of four purebred beef cattle: a Charolais, Belgian Blue, Galloway and Simmental. The majority of the 3'untranslated region (UTR) and part of exon 4 was also isolated through the use of forward (5'- GAGAATAAACCCAGAAGACCC-3') and reverse (5'-GATCATTCATTAAGACACGC-3') primers. This primer set isolated a fragment containing 592 bp. The same four purebred beef cattle (Charolais, Belgian Blue, Galloway and Simmental) were also used to isolate the 3'UTR of the *NTS* gene, along with the five sires and 13 dams of the CBRH.

Two sets of primers were used to isolate intron 1 as a result of its large size. Forward (5'- CAGCTGGTGTGCATGATTC -3') and reverse (5'- CCACCTTGAT GTGATCATTC -3') primers were designed to isolate the first section of intron 1 and part of exon 1. This primer set isolated a fragment containing 910 bp. Forward (5'-GAA TGATCACATCAAGGTGG -3') and reverse (5'-GA TGTATG CATATTGGTCAG -3') primers were designed for the second section of intron 1 and part of exon 2 consisting of 952 bp from genomic DNA. The five sires of the CBRH were used to isolate intron 1.

A single reaction contained 1.5 µl of 10X PCR buffer (Fermentas, Burlington, ON), 0.3 µl of 10mM dNTPs, 0.45 µl of 50mM $MgCl_2$ (Fermentas, Burlington, ON), 10 pmol of forward and reverse primers, 0.1 µl of Taq DNA polymerase (Fermentas, Burlington, ON) and 9.65 µl of sterile dH_2O. This cocktail was added to 1 µl of DNA per sample to obtain a total volume of 15 µl.

The amplification consisted of an initial denaturation at 94° C for four minutes, followed by a cycle that ran 35 times: 94° C for 50s, 55s at an annealing temperature specific to the primer set and 72° C for 50s. A final extension of 72° C was maintained for four minutes. Annealing temperature for the mRNA primer set was 53° C, the 3'UTR set was 58° C, the first section of intron 1 was 53° C and the second section was 51° C. All reactions were performed using a Stratagene® Robocycler 40. Bromophenol blue marker dye (2.5 µl) (Gibco, Gaithersburg, MD) was then added to the samples which were then loaded onto a 2% agarose gel which ran for approximately one hour at 80 W.

PCR products from all three primer sets were purified using the QIAquick® Gel extraction kit (QIAGEN, Mississauga, ON). The purified PCR product was quantitated on a 2% agarose gel with a DNA Mass™ ladder (Gibco, Gaithersburg, MD) and sent for sequencing at the Canadian National Research Council Plant Biotechnology Institute, Saskatoon, SK. Sequencing results were aligned and analyzed using the Sequencher™ program (Version 4.1, Gene Codes Corporation).

4.4 Genotyping

PCR-RFLPs were designed for the three SNPs in the 3'UTR. SNP *287G>T was genotyped using new forward (5'- GTCACATAGAATGTT TGTC -3') and reverse (5'- GATCATTCATTCCGCACAGA -3') primers. The PCR product was 313 bp in length and was amplified as described above, with an annealing temperature of 48° C. The restriction enzyme *Apo*1 (Fermentas, Burlington, ON) cut the *T* allele at 58 bp in

order to distinguish between the two alleles of the SNP. A single digestion reaction consisted of 1.5 µl of tango buffer (Fermentas, Burlington, ON) and 1.0 µl of enzyme which was incubated for three hours at 37° C and loaded onto a 3% agarose gel.

SNP *383T>C was genotyped using a mismatch forward primer (5'- AGAACA AAGTAACACC TGTC -3') and reverse (5'- GATCATTCATTCCGCACAGA -3') primer. The amplification of this 177 bp product was the same as that described above with the exception of the annealing temperature (57° C). The restriction enzyme *BsmA*1 (Fermentas, Burlington, ON) cut the *C* allele at 18 bp, with the digest being the same as described above.

SNP *419G>A was genotyped using the forward (5'- GCCTACCTGTCATTAA CCC -3') and reverse (5'- CATTCTTTGAAGATGGTCC -3') primers. This primer set amplified a 196 bp product. Once again the amplification procedure was similar with the annealing temperature differing (51° C). This digest was also similar to the previous, with the restriction enzyme (*Hha*1) (Fermentas) cutting the *G* allele at 77 bp, however this digest also contained 1.0 µl of BSA (bovine serum albumin) (New England Bio Labs, Pickering, ON).

4.5 Mapping

Neurotensin was linkage mapped using the CriMap program (Green *et al.* 1990). Genotypes for the sires, dams and offspring within the CBRH were obtained from the *419G>A SNP in order to map the gene.

4.6 Statistical Analysis

The StatView SE+ Graphics™ statistical program (©1998 Abacus Concepts, Inc.) was used for preliminary analysis of the data. Simple regression was performed, employing the *419G>A SNP genotype as the X variable (genotype = 0, 1 or 2 guanine alleles) and a specific growth/carcass measurement as the Y variable. An association was termed significant if the P-value was ≤ 0.05. If an association was significant, an unpaired *t* test was then used to identify if a dominant/recessive genotypic effect was present.

The SAS (Statistical Analysis System) (Version 9.1.3) was also used for statistical analysis. The MIXED model procedure in the SAS program was used to analyze a factorial design in order to determine if a significant interaction, between *NTS* genotype and breed type (British or Continental), had an effect on growth/carcass measurements. In the steer population, *NTS* genotype and days on feed were also analyzed for an interaction effect because animals were slaughtered at two different end points. If an interaction effect was significant, then days on feed or British versus Continental type cattle were analyzed separately for genotype effect in a completely randomized design.

5.0 RESULTS

5.1 Tissue Expression

In order to determine the location of *NTS* tissue expression, tissue samples from a random crossbred steer, Holstein bull calf and a two day old crossbred calf were analyzed. *NTS* mRNA was successfully isolated from the skin, abomasum, rumen wall, small intestine, spinal cord and brain. Expression was not identified in the kidney, muscle, lung, liver, iris or fat tissue.

5.2 Sequence Analysis and Polymorphisms

The complete coding sequence of *NTS* in cattle was amplified. Figure 5.1 shows a comparison of our cattle *NTS* mRNA to previously amplified human, dog, rat and mouse coding sequences. When comparing the coding sequence, the dog and cattle sequences are closely conserved with 94% of the amino acids identical. The rat sequence is identified as being more divergent when compared to dog and cattle sequence with 75 and 77% of amino acids being identical.

Analysis of the 5'UTR and complete coding sequence, containing both *NN* and *NTS*, did not reveal any SNPs in the cDNA from a Charolais, Belgian Blue, Galloway and Simmental or the two sequences in GenBank (NM_173945, BC102381). This does not preclude additional SNPs which may be present in dairy or *Bos indicus* breeds but is

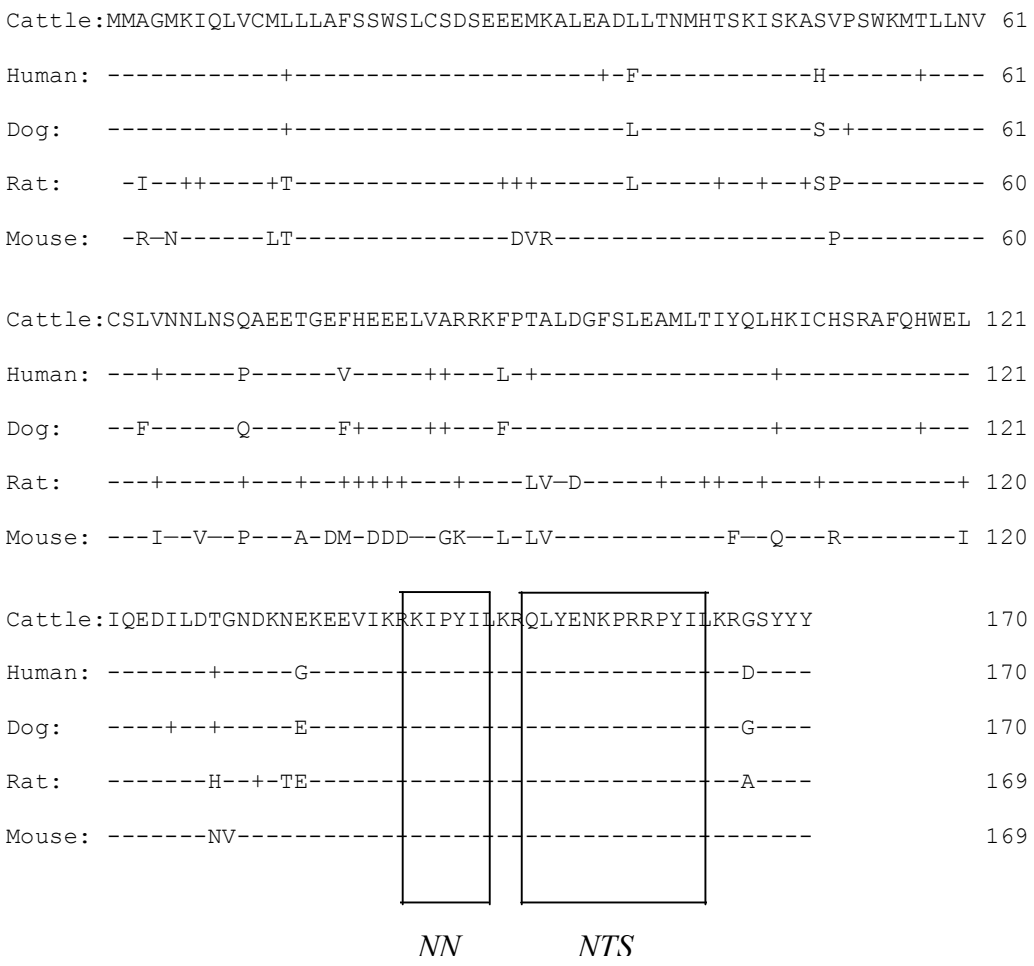

Figure 5.1: Comparison of the *NTS* coding region in various species. Figure adapted from Dong *et al.* (1998) and GenBank sequences (NM_173945, NM_006183, NM_001102381, NM_024435). Dog sequence adapted from Kislauskis *et al.* 1988.

a useful representative sample of beef breeds. Analysis of the 3'UTR in these four purebred beef cattle, as well as the sires and dams of the CBRH, revealed three SNPs (*287G>T, *383T>C, *419G>A).

Sequence analysis of intron 1 in the five CBRH sires revealed six intronic SNPs (c.73+369T>C, c.74-580C>T, c.74-495T>C, c.74-343T>C, c.74-224C>A, c.74-214G>A). The approximate locations of the SNPs are represented in Figure 5.2. None of the SNPs identified changed an amino acid, since they were not located in the coding sequence of the gene.

5.3 Linkage Mapping

A PCR-RFLP was designed to detect the *419G>A SNP in the 3'UTR of exon 4 (Figures 5.2 and 5.3). *NTS* was mapped to cattle chromosome 5 (Figure 5.4) by genotyping the parents and offspring of the CBRH for the *419G>A SNP. Genotypes from eight microsatellite markers were available from a previous study (Schmutz *et al*. 2001b). *NTS* was mapped between markers *BM6026* (13 cM, LOD=4.03) and *RM103* (4 cM, LOD=3.63). A QTL for carcass fat (Stone *et al.* 2005) was reported in this region.

5.4 Linkage Disequilibrium and Haplotypes

PCR-RFLPs were also designed to detect the *287G>T (Figure 5.5) and *383T>C SNPs (Figure 5.6) in the 3'UTR. The *287G>T and *383T>C SNPs along with the *419G>A SNP were genotyped for all the animals in the CBRH in order to

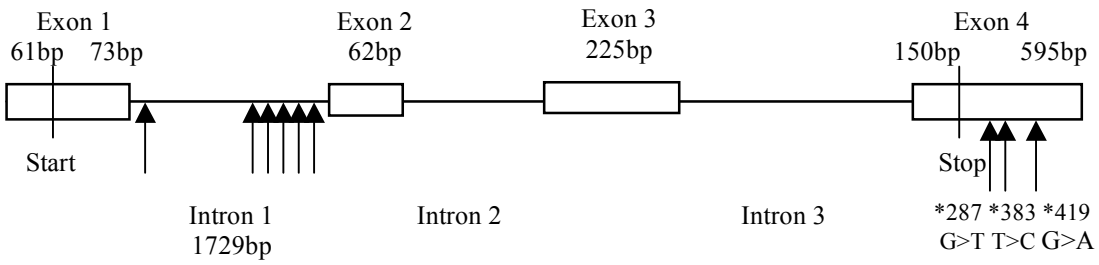

Figure 5.2: Genomic representation of the cattle *neurotensin* gene. Arrows indicate single nucleotide polymorphisms. * indicates nucleotide number in relation to the stop codon.

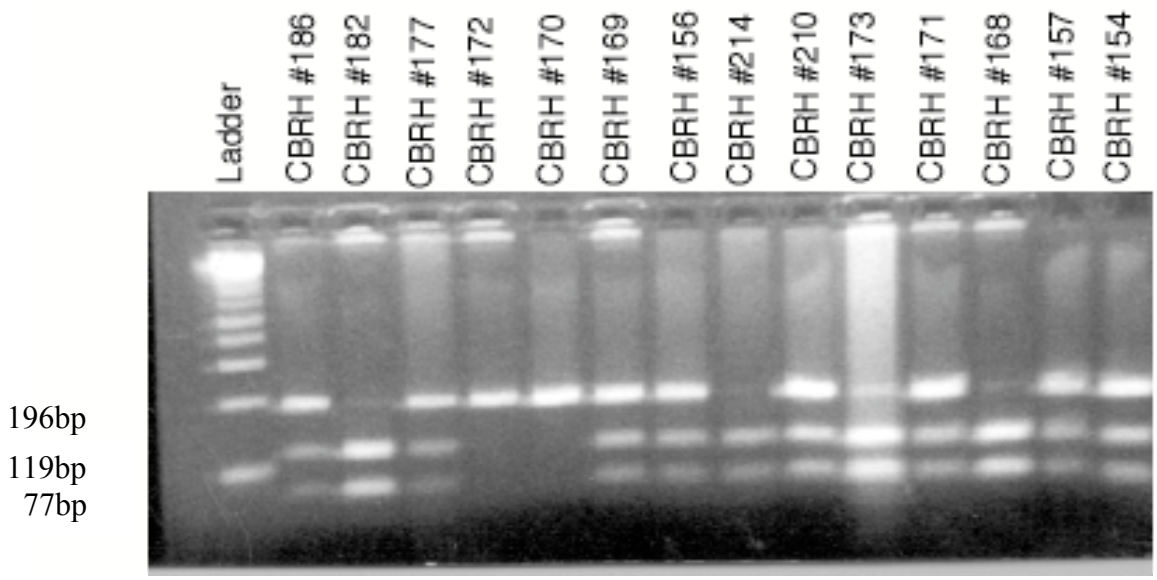

Figure 5.3: PCR-RFLP agarose gel electrophoreses of the *419G>A SNP in the 3'UTR of the *neurotensin* gene. Lane 3 = *GG* homozygote lane, 4 = *GA* heterozygote and lane 5 = *AA* homozygote.

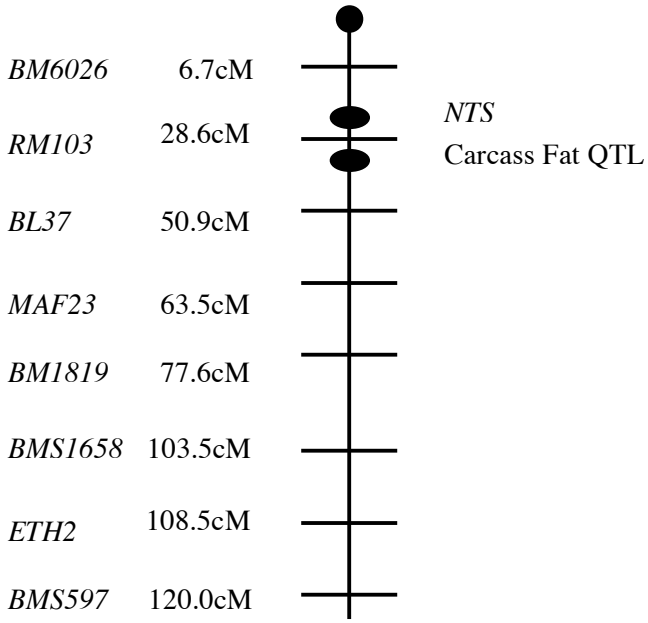

Figure 5.4: Linkage map of cattle *neurotensin* on chromosome 5 relative to microsatellite markers. *NTS* mapped between *BM6026* (13 cM) and *RM103* (4 cM). A QTL for carcass fat was previously identified by Stone *et al.* (2005) on cattle chromosome 5 in close proximity to *NTS*.

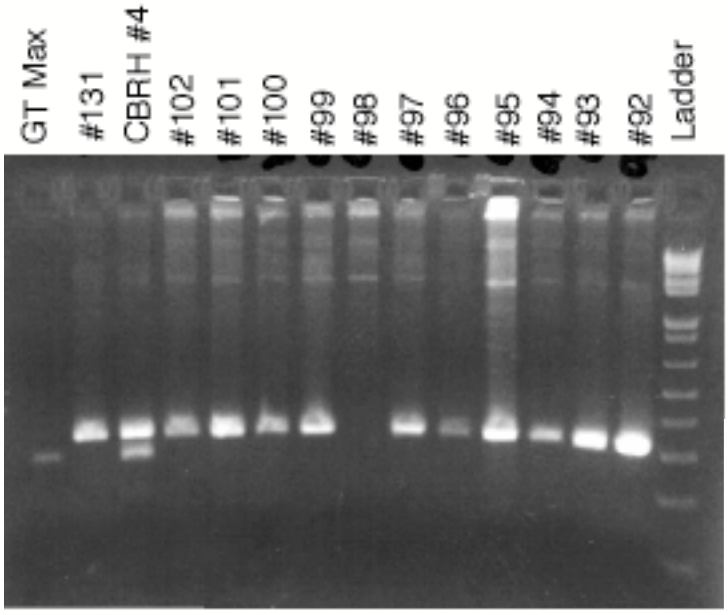

Figure 5.5: PCR-RFLP agarose gel electrophoreses of the *287G>T SNP in the 3'UTR of the *neurotensin* gene. Lane 1 = *TT* homozygote control, lane 2 = *GG* homozygote control and lane 3 = *GT* heterozygote control.

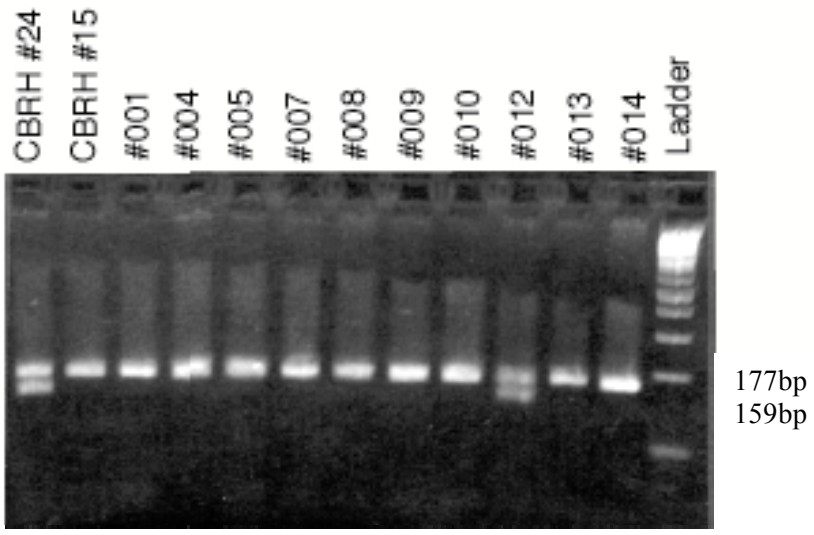

Figure 5.6: PCR-RFLP agarose gel electrophoreses of the *383T>C SNP in the 3'UTR of the *neurotensin* gene. Lane 1 = *CT* heterozygote control and lane 2 = *TT* homozygote control.

determine if these alleles were in linkage disequilibrium (LD). Chi-square analysis was used to determine that there was no LD between the *287G>T and *419G>A SNPs (P=0.0001) nor between the *383T>C and *419G>A SNPs (P=0.0001). In order to determine whether the *287G>T and *383T>C SNPs were in LD, the Fisher exact test was used (P=0.00033). The Fisher exact test is meant to be used if all samples have one or the other category, therefore, for multiple alleles you'd have to group the "not" together. Haplotypes were also determined for the sires and dams of the CBRH based on their offspring to further demonstrate independent segregation as shown in Figure 5.7.

5.5 Allele Frequencies

Allele frequencies of the *A* and *G* alleles at the *419G>A SNP were determined using the 145 Angus, Simmental, Charolais and Hereford bulls. Because the allele frequencies varied among the breeds (Table 5.1), chi-square analysis was used to determine if there was a significant interaction between breed type and allele frequency. Breed type separates breed based on their country of origin, being either of the British (Hereford and Angus) or Continental (Charolais and Simmental) types. Chi-square analysis using a continuity correction (used when at least one cell of the table has an expected frequency less than 5) revealed that there is not a significant (P=0.0589) interaction.

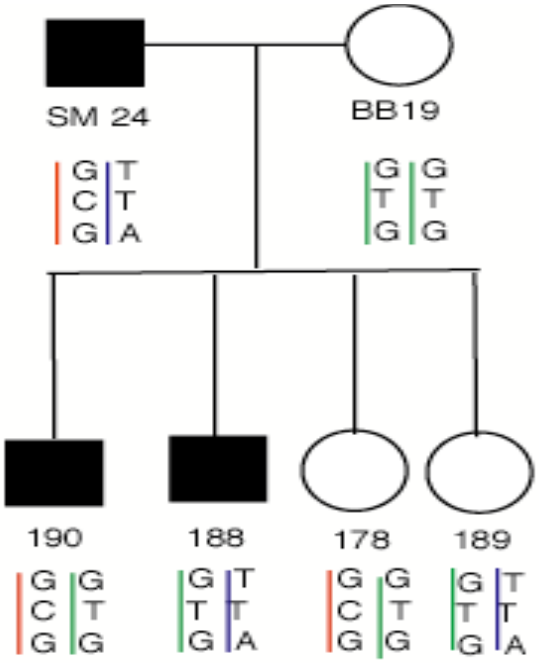

Figure 5.7: Haplotypes for the three SNPs in the 3'UTR of cattle *neurotensin* in one embryo transfer family.

Table 5.1: Allele frequency of SNP *419G>A in the 3'UTR of cattle *neurotensin*.

Breed	**Type**	**n**	***A* allele**	***G* allele**
Angus	British	55	0.23	0.77
Hereford	British	20	0.00	1.00
Simmental	Continental	18	0.11	0.88
Charolais	Continental	52	0.08	0.92
Total		**145**	**0.13**	**0.87**

The allele frequencies for the *287G>T and *383T>C SNPs were also identified in a random group of crossbred steers. Among the 95 steers genotyped at the *287G>T SNP, the *T* allele was shown to have a frequency of 0.02 and the *G* allele a frequency of 0.98. In the 28 steers genotyped at the *383T>C SNP the *C* allele had a frequency of 0.02 and the *T* allele of 0.98.

5.6 Association Studies

The *419G>A SNP was genotyped in the CBRH, the bull population and the steer population. In the CBRH, *NTS* genotype was analyzed for significant associations with growth and carcass traits (birth weight, carcass weight, rib eye area, grade fat, marbling, palatability, cutability, %lean and moisture levels). Significant associations between *NTS* genotype and rib eye area (REA), grade fat and moisture were found in the CBRH (Table 5.2). Rib eye area (cm^2) is defined as the amount of lean muscling being analyzed at the longissimus muscle at the 12^{th} rib, grade fat (mm) is the back fat depth over the 4^{th} quadrant of the longissimus dorsi and moisture (mg/g) is the amount of water present in the lean muscle. No associations were found for birth weight or carcass weight in the CBRH.

Cattle with an *AA* genotype had a significantly lower REA, higher grade fat and lower moisture level than *GA* or *GG* animals. These associations were also significant when using the *t* test. This test eliminated the *AA* genotypes, because of the limited

Table 5.2: Significant associations for SNP *419G>A in the CBRH using simple regression.

			***NTS* genotype**		
Trait	**P-value**	**r**	***AA***	***GA***	***GG***
			n=2	n=47	n=74
Mean REA[1] (cm^2)	0.0021	0.272	96.50 $\pm$ 0.71	103.00 $\pm$ 11.40	109.80 $\pm$ 13.30
Mean Grade fat (mm)	0.0242	0.202	12.25 $\pm$ 5.30	10.30 $\pm$ 3.20	8.80 $\pm$ 4.30
Mean Moisture (mg/g)	0.0249	0.201	721.16 $\pm$ 25.17	727.84 $\pm$ 16.20	734.06 $\pm$ 16.06

[1]REA = rib eye area

number of animals, and therefore only compared the *GA* versus *GG* genotypes (Table 5.3). No significant interactions were present for *NTS* genotype and breed type for any of the traits analyzed in the CBRH.

In the bull population, *NTS* genotype was analyzed for associations with end of trial weight, shipping weight, carcass weight, average fat, grade fat, yield grade, rib eye area, marbling, cutability, %lean, %fat, start of trial fat, end of trial fat and fat deviation. A significant association of *NTS* genotype with %fat (based on the dissection of the 7th bone-rib into bone, muscle and fat), end-of-trial (EoT) fat (ultrasound measurement of fat at 112 days on feed) and fat deviation (the rate at which fat is laid down in mm over the trial period) was found (Table 5.4). Animals with an *AA* genotype were identified to have higher %fat and EoT fat compared to *GA* or *GG* genotypes, while animals with a *GA* genotype had a lower fat deviation compared to *AA* or *GG* animals. No significant interaction effects were present between *NTS* genotype and breed type for start of trial (SoT) fat (ultrasound measurement of fat), EoT fat, %fat, fat deviation, REA or grade fat in the bull population.

In the steer population, carcass weight, lean meat yield, yield grade, quality grade, marbling, grade fat, rib eye are and hip height were analyzed for *NTS* genotype associations. A significant interaction effect was identified between *NTS* genotype and days on feed for REA, marbling (fat deposit within a muscle based on a 1-10 scale) and quality grade (classified as A, AA or AAA based on age and intramuscular fat) (Table 5.5). *NTS* genotype and type (British versus Continental) also showed a significant interaction effect for REA, grade fat, marbling and quality grade. Therefore, the steer

Table 5.3: Significant differences using the *t* test on cattle with *GG* versus *GA* genotypes for SNP *419G>A in the CBRH.

Trait	**P-value**
REA[1]	0.0047
Grade fat	0.0425
Moisture	0.0395

[1]REA=rib eye area

Table 5.4: Carcass and live trait associations using simple regression for cattle with various genotypes at the *419G>A SNP in the bull population.

			***NTS* Genotype**		
Trait	**P-value**	**r**	***AA***	***GA***	***GG***
			n=3	n=30	n=112
SoT Fat[1](mm)	0.1007	0.139	2.25±1.95	2.59±1.61	1.99±1.53
EoT Fat[2] (mm)	0.0362	0.178	5.17±4.65	4.93±2.63	3.89±2.38
Fat dev.[3]	0.0234	0.189	1.31±4.10	0.85±4.14	3.58±5.94
REA[4] (cm^2)	0.9394	0.006	95.67±18.85	96.47±12.05	96.52±13.35
Grade fat (mm)	0.3136	0.085	5.33±2.89	4.73±2.91	4.19±2.22
Fat (%)	0.0634	0.156	23.37±6.50	18.89±5.29	17.52±5.82

[1]SoT Fat = start of trial fat (ultrasound measurement)
[2]EoT Fat = end of trial fat (ultrasound measurement)
[3]Fat Dev. = fat deviation is the rate at which fat is put down
[4]REA = rib eye area

Table 5.5: Interaction effects between *NTS* genotype, days on feed and breed type on carcass traits in the steer population using proc MIXED model.

Treatment	**REA** **P-value**	**Grade Fat** **P-value**	**Marbling** **P-value**	**Quality Grade** **P-value**
***NTS**Type**[1]	<.0001	0.3687	0.0012	<.0001
***NTS**DOF**[2]	0.0178	<.0001	<.0001	<.0001
NTS**DOF**[2]Type**[1]	0.3731	0.6471	0.7223	0.7554

[1]Type = Breed type (British versus Continental)
[2]DOF = days on feed (119 versus 140)

population had to be analyzed separately for days on feed, as well as British versus Continental type for these traits.

The steer population was analyzed based on their means for REA, grade fat and carcass weight which were similar to those of the CBRH (Table 5.6). To determine if significant associations between genotype at the *419G>A SNP and marbling or quality grade existed in the steer population (Table 5.7), simple regression was used. Steers with an *AA* genotype had a higher quality grade followed by steers with a *GA* genotype and finally steers with a *GG* genotype. Steers with a *GA* genotype were shown to have lower marbling followed by steers with an *AA* genotype and steers with a *GG* genotype.

Using a *t* test, cattle of *GG* genotypes fed to 119 days and of the Continental type had significantly higher marbling and a significantly lower quality grade than cattle with *GA* genotypes (Table 5.8). Cattle with *AA* genotypes were not included in this analysis because of the limited number of such animals. In general, an increase in marbling tends to lead to an increase in quality grade, however in this study an increase in marbling led to a decrease in quality grade, therefore we can conclude that these varying results indicate that the *419G>A SNP associations with marbling and quality grade do not appear to be correct.

To determine if a dominant/recessive genotypic effect was present, an unpaired *t* test was used comparing one homozygote and the heterozygote against the opposite homozygote group. Quality grade and marbling would both fit a dominant/recessive inheritance model (Table 5.9). No significant associations were identified for REA or grade fat in the steer population.

Table 5.6: Comparison of the means and standard deviations for carcass weight, REA and grade fat in the CBRH, bull population and steer population.

Means	**CBRH**	**Bull population**	**Steer population**
REA[1] (cm^2)	107.04 ± 14.40	102.28 ± 13.90	100.76 ± 10.62
Grade fat (mm)	9.46 ± 3.70	4.02 ± 3.00	9.13 ± 4.05
Carcass weight (kg)	407.08 ± 16.70	348.61 ± 33.52	392.61 ± 20.62

[1]REA = rib eye area

Table 5.7: Significant associations using simple regression between carcass traits and genotypes at the *419G>A SNP in the steer population.

			***NTS* Genotype**		
Trait	**P-value**	**R**	***AA***	***GA***	***GG***
			n=4	n=89	n=399
QualityGrade (#A's)	0.0458	0.091	2.50±0.58	2.40±0.50	2.30±0.57
Marbling (1-10)[1]	0.0264	0.101	7.25±0.96	7.21±1.03	7.46±0.91
REA[2] (cm^2)	0.9253	0.004	92.00±6.06	101.46±10.04	100.69±10.72
Grade fat (mm)	0.6739	0.019	12±5.57	9±3.82	9±4.09

[1]1=practically devoid, 10=abundant
[2]REA = rib eye area

Table 5.8: Significant differences using the *t* test of cattle with *GG* versus *GA* genotypes for SNP *419G>A in the steer population.

Trait	**119 DOF**		**119 DOF &British**		**119 DOF &Continental**	
***NTS* Genotype**	***GA***	***GG***	***GA***	***GG***	***GA***	***GG***
n	49	188	26	94	23	94
Mean Quality Grade (# A's)	2.35 ±0.48	2.16 ±0.51	2.50 ±0.51	2.34 ±0.56	2.18 ±0.39	1.98 ±0.39
P-value	0.0185		0.1924		0.0294	
Mean Marbling (1-10)[1]	7.31 ±1.06	7.65 ±0.91	7.08 ±1.16	7.36 ±1.04	7.57 ±0.90	7.91 ±0.65
P-value	0.0286		0.2294		0.0348	

[1]1=practically devoid, 10=abundant

Table 5.9: Significant differences using the *t* test of cattle with *GG* versus *GA* or *AA* genotype at SNP *419G>A in the steer population suggest marbling or quality grade could be inherited as dominant/recessive traits.

	Steer population			
Trait	**Total**	**119 DOF**	**119 DOF &British**	**119 DOF &Continental**
Quality Grade P-value	0.0457	0.0084	0.1006	0.0324
Marbling P-value	0.0221	0.0183	0.1555	0.0411

Comparison of the MIXED model results in the SAS program across the three populations are represented in Table 5.10. Grade fat remains significant in the CBRH using this method of analysis, while REA does not. A trend towards significance for marbling (P=0.0518) in the Continental type animals of the steer population was also present. The bull population did not show any significant associations.

Table 5.10: Significant association comparison between the CBRH, bull and steer populations using the MIXED model procedure.

	CBRH		**Bull Population**		**Steer Population**	
Trait	British	Continental	British	Continental	British	Continental
REA[1] **(cm^2)**	94.61 ±4.94	112.02 ±12.59	88.16 ±8.05	105.23 ±11.64	99.47 ±10.71	102.18 ±10.37
P-value	0.8195	0.0749	0.3141	0.3729	0.1861	0.8032
Grade Fat (mm)	14.29 ±3.44	7.30 ±3.11	6.52 ±2.75	2.00 ±1.29	10.62 ±4.07	7.48 ±3.32
P-value	0.5716	0.0243	0.4981	0.5744	0.3404	0.5778
Marbling (1-10)[2]	5.16 ±0.58	4.47 ±0.75	1.27 ±0.53	0.50 ±0.53	7.12 ±1.00	7.74 ±0.73
P-value	0.8916	0.1212	0.1525	0.1075	0.5481	0.0518

[1]REA = rib eye area
[2]1=practically devoid, 10=abundant

6.0 DISCUSSION

6.1 Expression

NTS mRNA was successfully isolated from the brain and spinal cord repeating Carraway and Leeman (1973) who first isolated *NTS* from the hypothalamus of a calf. *NTS* was also found in the abomasum, rumen wall and a random stomach sample in this study. *NTS* was expressed in the fetal and early postnatal stomach of rats (Wang and Evers 1999). *NTS* was also isolated from the small intestine of a calf (Kitabgi *et al.* 1976) suggesting that *NTS* may exist in different areas of the gastrointestinal tract. Kitabgi *et al.* (1976) isolated a tridecapeptide with the same amino acid composition as that of *NTS* from extracts of bovine intestinal tissues. This material was as potent as *NTS* in inducing hypotension in anesthetized rats and therefore indicated that the intestinal tridecapeptide was identical to hypothalamic *NTS* and it was referred to as intestinal *NTS.* Although *NTS* was not isolated from the abomasum or large intestine of Philippine water buffalo (Baltazar *et al.* 1998), it was isolated from the small intestine. *NTS* was also successfully isolated from the small intestine in this study.

NTS mRNA was also found in the skin. Eedy *et al.* (1994) did not find *NTS* in human skin. Although *NTS* was not identified in liver in this study, it has been shown to be expressed in fetal rat liver, as well as three and seven days after birth (Wang and Evers 1999). Wang and Evers (1999) also showed that *NTS* was expressed at the earliest

fetal time in the small bowel. Although *NTS* expression was widely expressed in the gastrointestinal tract during fetal development in their study, expression was restricted to the small bowel in adults.

6.2 Allele Frequencies

Allele frequencies for the *419G>A SNP were calculated for four major beef breeds (Table 5.1). Although the cattle populations analyzed in this study were not all of purebred origin, they were considered to be representative of the beef cattle industry in western Canada. Because the allele frequencies drastically differed across the breeds, chi-square analysis was used to confirm that there was no significant difference between the allele frequencies in British versus Continental type cattle.

Cattle were analyzed separately for breed type because of the growth and carcass differences between the two types. The study by Wheeler *et al.* (2005) showed that British animals had a higher fat thickness, smaller REA and lower retail product yield than Continental type animals. These animals also had lower shear force values with a greater percentage of carcasses graded USDA choice compared to the Continental type. Therefore, the Continental cattle are generally leaner and heavier muscled, with less marbling. The two breed types did not differ significantly in live weight or hot carcass weight (Wheeler *et al*. 2005). There were also no differences in the sensory panel data for tenderness or beef flavor intensity.

The minor allele frequencies of the *287G>T and *383T>C SNPs were 0.02. These two SNPs were therefore not analyzed for genotype associations with production

traits, because if an association was found significant, the genotype frequency would be too low to produce an economic impact.

6.3 Mapping

NTS was linkage mapped to cattle chromosome 5 (Figure 5.3) using genotypes from eight microsatellite markers which were available from a previous study (Schmutz *et al.* 2001b). Stone *et al.* (2005) reported a QTL for carcass fat in cattle at the *hematopoietic protein 1* (*HEM1*) and *phosphodiesterase 1B* (*PDE1B*) genes on BTA5 (Figure 5.3).

NTS is located on human chromosome 12 (Marondel *et al.* 1996) (Figure 6.1). The *HEM1* (GenBank NM_005337) and *PDE1B* (GenBank NM_000924) genes were also shown to be located on human chromosome 12 in close proximity to the *NTS* gene (GenBank NM_006183). In dog, the *NTS* gene is on chromosome 15 (GenBank NM_006183). In mice, *NTS* is located on chromosome 10 (GenBank NM_024435) and in rats on chromosome 7.

A comparative map of *NTS*, in cow, human, rat, mouse and dog, along with different genes located in close proximity is represented in Figure 6.1. In all five mammalian species, *NT773* and *NTS* remain as a syntenic group.

The segment containing *KRTB*, *PDE1B*, *HEM1* and *LALBA* is inverted in humans compared to cattle. There also appears to be another inversion within this segment containing *LYZ* and *IFNG*. In the rat and mouse, the *LYZ* and *IFNG* genes are located on the same chromosome as the *NTS* gene and are in the same order as the cattle

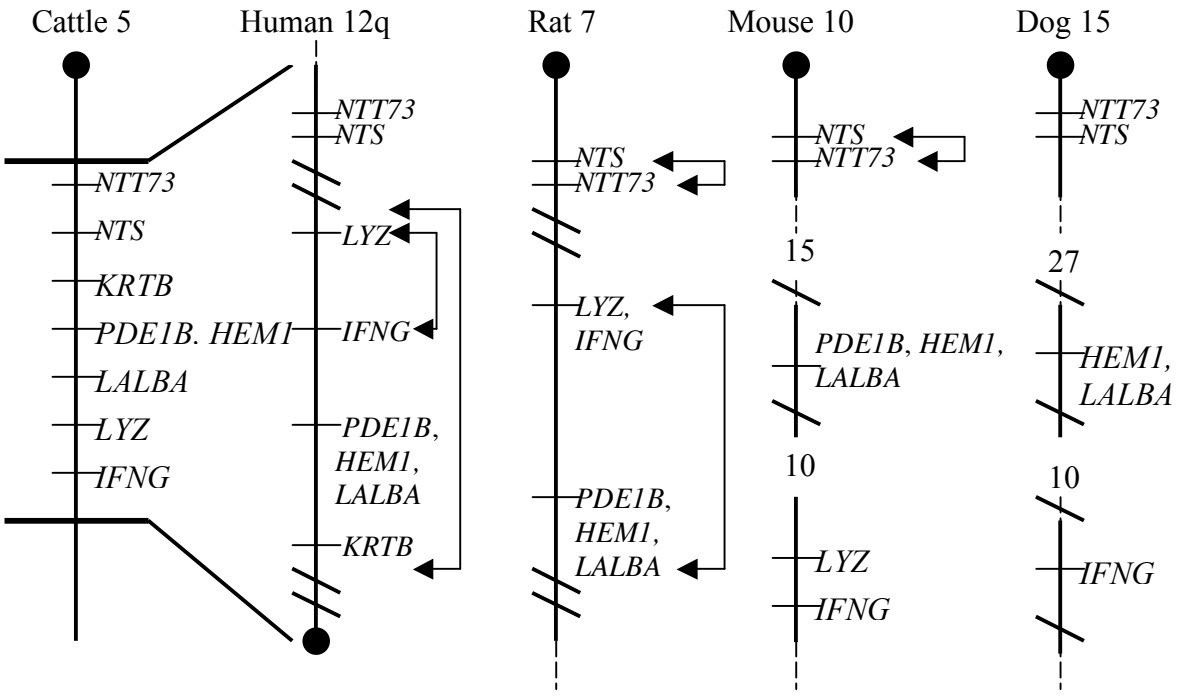

Figure 6.1: Comparative map of cow, human, rat, mouse and dog gene locations. Double slanted lines indicate chromosomal break point surrounding an inverted segment shown by arrows. Slanted lines indicate that the next segment is from another chromosome. Gene locations are adapted from: Stone *et al.* 2005, Solinas-Toldo *et al.* 1995 and NCBI map viewer.

chromosome. However, in rats these genes are inverted with *PDE1B*, *HEM1* and *LALBA* genes, while in mice the *PDE1B*, *HEM1* and *LALBA* genes are all located on a different chromosome than *NTS*. This is also the same for the dog in which the *HEM1*, *LALBA* and *IFNG* genes are all located on different chromosomes compared to *NTS*.

Therefore the conservation of chromosomal segments of this region of BTA5 is narrow. The locations of the genes distal to *NTS* do not appear to be conserved across species. Consequently, comparative gene mapping would have limited success with determining *NTS* chromosomal location across species.

Although the three SNPs occurred within 132 bp, they were not in LD. This suggests that these SNPs probably arose independently over a long period of time. The rare allele in the *419G>A SNP occurred at a frequency of 13%, whereas the rare allele of the other two SNPs occurred at only 2%. This suggests that the *419G>A SNP was either under some selection pressure or that it occurred longer ago.

6.4 Polymorphism Analysis

The *419G>A SNP was located in the 3'UTR of exon 4. Although it did not alter an amino acid, 3'UTR SNPs have been shown to affect function in other genes. The review by Grzybowska *et al.* (2001) states that: the 3'UTR plays a role in mRNA stability, translation and localization. Several human diseases are caused by mutations in the 3'UTR sequences such as myotonic dystrophy (Aslanidis *et al.* 1992) and Alzheimer's disease (Rajagopalan *et al.* 1998). Although the review by Grzybowska *et al*. (2001) identified that 3'UTR mutations have been shown to cause several human

diseases, these mutations differ in their function. For example, certain diseases have occurred as a result of altered stabilization via 3'UTR binding, translation suppression, antitermination mutations and inflated repeats leading to mutated transcripts lowering kinase synthesis (Grzybowska *et al*. 2001). A SNP in the 3'UTR of the *CAST* (*calpastatin*) gene has been shown to have a significant association with shear force in many beef breeds (Casas *et al.* 2006).

6.5 Association Studies

Genotype association studies revealed significant associations between the *419G>A SNP and REA, grade fat and moisture in the CBRH (Table 5.2). Significance for REA and grade fat may not have been observed in the bull population because these animals were fed to generate a lean breeding bull. Therefore, the diet used for the bull population was formulated to promote lean muscle growth (Bergen 1995) providing the bulls with a lower concentrate level, whereas the CBRH was fed a ration with a high concentrate level in order to put down fat (Fournier 2000).

Slaughter age also differed between the CBRH and the bull population. The CBRH herd was slaughtered at a fixed age, 19 months $\pm$ 2 weeks, whereas the slaughter age of the bull population was approximately 16 months. Based on the means for carcass weight, we can determine that the CBRH herd was slaughtered at heavier weights than the bull population.

The steer population showed means for grade fat and carcass weight that were similar to the CBRH rather than the bull population. The steer population also

represents a typical commercial feedlot group, which was the primary area of interest in finding an association of economic importance. A significant association between *NTS* genotype and quality grade was identified in the steer population. A significant association between genotype and marbling was also identified. Quality grade was measured as A, AA and AAA. Marbling was measured on a 1-10 scale with 10 representing abundant marbling. One would typically expect that higher marbling would result in higher quality grade. However, in the steer population, cattle of the various genotypes had inverse measured for quality grade and marbling score (Table 5.8).

Although cattle in all three populations had higher fat levels with the *AA* genotype (Table 5.2, 5.4, 5.7) than those with the *GG* genotype, no single measure of fat was consistently found to have significant association. Comparison of other significant production trait associations across the three populations could not verify a specific association. It was therefore concluded that the *419G>A SNP in the 3'UTR of the bovine *NTS* gene does not prove to be of economic importance to the beef cattle industry.

7.0 CONCLUSION

The cattle *NTS* gene was successfully amplified in brain, spinal cord, small intestine, rumen wall, abomasum and skin of random crossbred animals. Three SNPs in the 3'UTR and six in intron one were identified through sequence analysis. The *419G>A SNP in the 3'UTR was used to map *NTS* to BTA 5 between markers *BM6026* (13 cM, LOD=4.03) and *RM103* (4 cM, LOD=3.63) near a QTL for carcass fat (Stone *et al*. 2005).

The three SNPs in the 3'UTR were also analyzed in order to confirm that they were not in linkage disequilibrium with each other. The *287G>T and *383T>C SNPs were not analyzed for associations with production traits as a result of their low minor allele frequencies.

The *419G>A SNP was analyzed for growth and production trait associations across three different population of animals. No associations for growth traits were identified in any of the three populations used. Comparison of the significant production trait associations across the three populations could not verify a specific carcass trait association.

The original hypothesis stated that potential polymorphisms within the bovine *NTS* gene may be associated with growth traits in cattle, in particular with weaning weight and in turn yearling weight and hot carcass weight. This study was not able to confirm our hypothesis for the *419G>A SNP that was analyzed did not shown any

significant associations with growth or production traits across the three groups of animals studied. Therefore it was concluded that the *419G>A SNP in the 3'UTR of the bovine *NTS* gene does not prove to be of economic importance to the beef cattle industry.

8.0 FUTURE RESEARCH

In the central nervous system, *NTS* has been shown to produce potent hypothermia effects (Bissette *et al.* 1976), reduce locomotor activity and exhibit pain reducing effects (Clineschmidt *et al.* 1979). *NTS* has also been identified as a neuromodulator as shown by its similarity to neuroleptics (Nemeroff 1980), with decreased *NTS* signaling contributing to defects in sensorimotor gating in some animals with schizophrenia (Binder *et al.* 2001a).

In cattle, these effects of *NTS* on the CNS may influence animal behaviour. Hypothermia effects in beef cattle may lead to either an increase or decrease in feed consumption depending on the degree of hypothermia. A reduction in locomotor activity may also influence the amount of pasture being grazed or the amount of time spent at a feed bunk, while pain reducing effects may be beneficial at the time of castration and dehorning.

The study by Schmutz *et al.* (2001b) calculated the heritability of temperament at weaning and of habituation, across several full-sib families. From this, several QTL were detected for one and/or both of these traits. One of these QTL was found on chromosome 5, near the microsatellite *RM103*. This microsatellite is also located in close proximity to *NTS*.

Therefore, based on the identification of a QTL for behavior traits located near the *NTS* gene, along with its effects on the central nervous system and role as a neuroleptic, future research in the relationship between the *419G>A SNP located in the *NTS* gene and behavior in beef cattle may be plausible.

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Appendix A: Polymorphisms identified in intron 1 of the cattle *neurotensin* gene.

Polymorphisms are underlined; exon sequences are in bold.

TGTGCTCAGGTAAGCTCAGCTGCATTTCACAACTCCCTGAAATGATATCTTTTTCTGTTTTTGCAATGTG
TTGTTACTAATGCTAATGTATCTGGGGAGAAGAGTTTCTCCCTTATTCTGATTTGCTTTTTAGTGATAGA
AACTGGATATTTTCTTTCTCTGTATTTATGATTACAAGAACTGAAAATTTTCCCTTCCCTTTTTAAATTT
GCTAGGTAGTATAAGGGATTTCAATGAGACACTTCATGTTCCAGTTTGCAATGTAATCACTAGGAAAAAA
AAATGTAAAACTTAAATCAAGCTGATCTAATGAAAAGTACAGAAGTGGTGCCTGTCAAAATGCAAACTCA
TAATTCAAACTGATTGTATTTGAAATGYTTTATA......773bp......TAGYCTGTGATAAAATTGC
CAAGTACACATAATTCTAAATTGTTGCTTAATATAGAGAAACAAATCTTTCAAAGTGTGTTTTGTAAAAY
AGCATCTCAAATAAAATAAATCTCAGTGTCTTCCTTATTCCAAAATTATCAATAAGTAACTTCCTTTGGT
TTTATCTTTAAAAAATGTCTTGTACTTACCTTTTCATTATCTTGCTTTGAAAATATCAATATATATACAC
TCTATGTATATGYAGACTACTGCCAGTAAAAATTAACCAGCATATTTGATGACATCTTTGTAGTATAATC
AGGTTTTATTATCCTGAGATTCAGAGATTAAACAACTCCAGATCATCGAGAGCTCTCGGCMTAAAATCAT
RTGCCTATTTTTGTAAAAGTGCTTCTATTGTTCTATTCTGTTAGCCTAAAATAAAACTATAATTGTTTTT
TTAATATTATTATAAGGAGAATAAATACATAGCAAAGACAATAAACTTCTAAGACAAAGTTTTCTGAGAT
TAATCTTAAATATTCAAATTATAATTATATGATATGTGGTTATATAGTAAACATGTGGTTTTGATTTTAC
TCAG**ATC**

Appendix B: Polymorphisms identified in the 3'UTR of the cattle *neurotensin* gene.

Polymorphisms are underlined, stop codon is in bold.

TGAGAGGATAAATATTTTATTTACATGTGATTGTGATTTCTTTTAATGAAATATCAAATTATATTTGTGT
GGAAATGTGACAGAGCACAATTATTTTGTCTCTTCTACAGTTGTGGTTTATTAGATGTGATTTTTTTCTG
CATTACTATAAATTGGACTAAATGTTTTAAAATAAATCTAGATCTTGAGCATGAAATGTTGTGTATAATT
GGAGTAGATATTAATTAAGTCACATAGAATGTTTTGTCATTTTGCAAAGCACTTAATGGGTTGTTCAAGC
AGTTAAAATKTTTGCCATTCTAAACCAAATTAAAAGAGATTAGGACAGTTTTACCGCAAAGTCTAGCCTA
CCTGTCATTAACCCAGAACAAAGTAACACCTGTTTYATTATTTGAATGTTTCATTGAACTGAAACACATG
CRCTTTTCCAAGACTTACTCATTAATCTCAGAAATGGGGAAGGCAATAGAAGTAAAATAGGACCATCTTC
AAAGAATGATTAATGAATTATTGTTAATTATGCGTGTCTTA

Appendix C: Chi-square analysis for linkage disequilibrium

The five sires and 12 dams of the CBRH were genotyped for the *419G>A and *287G>T SNPs.

	***287G>T**			
***419G>A**	***G/G***	***G/T***	***T/T***	**Total**
A/A	0	0	0	0
A/G	2	3	0	5
G/G	12	0	0	12
Total	14	3	0	17

The frequency of the alleles was:

*287G>T $T = 0.09$ $G = 0.91$

*419G>A $A = 0.15$ $G = 0.85$

The observed probability of inheriting a *T* at the *287G>T SNP and an *A* at the *419G>A SNP was:

$$A + T = (0.09) \text{ X } (0.15) = 0.0135$$

The observed probability of inheriting a *G* at the *287G>T SNP and an *A* at the *419G>A SNP was:

$$A + G = (0.91) \text{ X } (0.15) = 0.136$$

The expected frequency of animals with a *T* at the *287G>T SNP and an *A* at the *419G>A SNP was:

(*A* + *T*) X n (number of animals) = 0.0135 X 17 = 0.2

The expected frequency of animals with a *G* at the *287G>T SNP and an *A* at the *419G>A SNP was:

(*A* + *G*) X n (number of animals) = 0.136 X 17 = 2.3

Alleles	Observed	Expected
A + *T*	3	0.2
A + *G*	2	2.3

In the Statview program, chi-square analysis was used to identify if the observed was significantly different from the expected. If the outcome is significant ($P<0.05$) then the alleles are not considered to be in linkage disequilibrium.

DF	Chi-square	Probability
1	444.059	.0001

Since the P value was equal to 0.0001, it was concluded that the *A* allele of the *419G>G SNP was not in linkage disequilibrium with the *T* to *G* allele of the *287G>T SNP.

Appendix D: Fisher exact test for linkage disequilibrium analysis

NOTE: This is meant to be used if all samples have one or the other category, therefore, for multiple alleles you'd have to group the "not" together.

This test was used to test if the T allele of the *287G>T SNP was in linkage disequilibrium with the alleles of the *383T>C SNP. There were a total of 18 animals (five sires and 13 dams of the CBRH) which were genotyped for the *287G>T and *383T>C SNPs. Of these animals the *T* allele of the *287G>T SNP and the *C* allele of the *383T>C SNP occurred in 4 animals. The *T* allele of the *287G>T SNP does not occur with the *T* allele of the *383T>C SNP. The *G* allele of the *287G>T SNP does not occur with the *C* allele of the *383T>C SNP and therefore it occurs with the *T* allele in 14 of the animals.

Construct a 2 X 2 contingency table:

	*287G>T	
*383T>C	*T* allele	Not *T* allele (*G* allele)
C allele	4 (A)	0 (B)
T allele	0 (C)	14 (D)

Formula:

$$P = \frac{(A + B)!\,(C + D)!\,(A + C)!\,(B + D)!}{N!\,A!\,B!\,C!\,D!} \qquad P = \frac{(4)!\,(14)!\,(14)!\,(4)!}{18!\,4!\,0!\,0!\,14!}$$

Where N = total animals (17 in this case)

From EXCEL:

14	0	0	4	18
87178291200	1	1	24	6.40237E+15

P = 0.000327

The Fisher exact test indicates that the probability of this happening by chance is 0.000327. Therefore we conclude that the *287G>T and *383T>C SNPs are not in linkage disequilibrium.

Appendix E: Cattle *NTS* manuscript (Animal Genetics 38, 428).

No association between the *neurotensin* (*NTS*) gene and production traits in beef cattle

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Source/description: Neurotensin (*NTS*) is a 510 bp gene which encodes a 13 amino acid peptide which is expressed in bovine hypothalamus[1] and skin (this study) (Accession No. DQ515198). Several studies have shown that intracerebroventricular injection of *NTS* decreased food intake in rats.[2-7] The purpose of this study was to determine if polymorphisms discovered within the *NTS* gene were correlated with growth and/or carcass traits in beef cattle.

SNP identification: The bovine *NTS* mRNA sequence (M18621) was used to design forward (5'- GAGAATAAACCCAGAAGACCC-3') and reverse (5'-GATCATTCATTAAGACACGC-3') primers. Using standard procedures, this primer set isolated a 592-bp fragment containing the majority of the 3'UTR and part of exon 4. Sequences of this amplicon were determined for four purebred beef cattle breeds (Charolais, Belgian Blue, Galloway and Simmental), as well as the five sires and 13 dams of the Canadian Beef Reference Herd.[8] A SNP was identified in the 3'UTR of

NTS (DQ515198.1:c.*419G>A, where *419 indicates 419 nucleotides 3' of the translation stop codon).

SNP genotyping: Primers that amplified a 196-bp fragment containing the c.*419G>A SNP were designed: forward (5'- GCCTACCTGTCATTAA CCC -3') and reverse (5'- CATTCTTTGAAGA TGGTCC -3'). The amplicon was digested with *Hhal* (Fermentas), producing fragments of 119 bp (c.*419G allele) and 196 bp (c.*419A allele).

Three groups of cattle were genotyped. The first group was the Canadian Beef Reference Herd, [8] which consisted of 17 full-sib families, five of which were purebred breeds and the remaining 12 were two-breed crosses. The second group consisted of 168 purebred yearling bulls of the Angus, Hereford, Simmental and Charolais breeds.[9] The final group consisted of 492 crossbred steers fed at the Lakeside Research facility in Brooks, Alberta. All animals were analysed for associations between the *NTS* genotype and carcass traits. The Canadian Beef Reference Herd was also analysed for associations between the *NTS* SNP and growth traits.

Allele frequencies for the c.*419G>A SNP were determined for the four purebred beef breeds. Frequencies of the c.*419A allele were 0.23 in Angus, 0.11 in Simmental, 0.08 in Charolais and 0.0 in Hereford. There was not a significant (P = 0.0589) interaction between breed type (British vs. Continental) and allele frequency, as tested with a chi-squared with a continuity correction. *NTS* was also mapped to bovine chromosome 5 using genotyping data from the Canadian Beef Reference Herd, with a

location 13 cM distal to *BM6026* (LOD = 4.03) and 4 cM proximal to *RM103* (LOD = 3.63), close to a reported quantitative trait locus for carcass fat.[10]

Statistical Analysis: The MIXED model procedure in SAS Version 9.1.3 was used to analyze a completely randomized design. Significant values were adjusted for multiple comparisons. An association was considered significant if the P-value was ≤ 0.05. All traits were analyzed seperately for British- and Continental- type animals.

Tests for association of the SNP with growth traits were not significant. Significant trends between carcass measurements and the c.*419G>A SNP genotypes were present in some populations (Table 1). However, associations with carcass traits were not found in all three groups of cattle and therefore, were not verified.

Acknowledgments: This study was funded by the Natural Science and Engineering Research Council of Canada through the Collaborative Research and Development program, and a contribution from Quantum Genetics, Inc.

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Table 1: Association of the *NTS* c.*419G>A SNP with traits in the Canadian Beef Reference Herd, bull and steer populations.

	Canadian Beef Reference Herd		Bull Population		Steer Population	
Trait	British	Continental	British	Continental	British	Continental
REA (cm^2)	94.61 ±4.94	112.02 ±12.59	88.16 ±8.05	105.23 ±11.64	99.47 ±10.71	102.18 ±10.37
P-value	0.8195	0.0749	0.3141	0.3729	0.1861	0.8032
Grade Fat (mm)	14.29 ±3.44	7.30 ±3.11	6.52 ±2.75	2.0 ±1.29	10.62 ±4.07	7.48 ±3.32
P-value	0.5716	0.0243	0.4981	0.5744	0.3404	0.5778
Marbling (1-10)[1]	5.16 ±0.58	4.47 ±0.75	1.27 ±0.53	0.50 ±0.53	7.12 ±1.00	7.74 ±0.73
P-value	0.8916	0.1212	0.1525	0.1075	0.5481	0.0518
Carcass weight (kg)	429.89 ±22.75	436.47 ±38.47	337.84 ±34.25	336.50 ±34.26	390.77 ±20.55	394.59 ±20.54
P-value	0.2759	0.9306	0.5369	0.2186	0.9235	0.5958

[1] 1=practically devoid, 10=abundant

Appendix F: Phenol/Chloroform Extraction of DNA from blood (adapted from Fitzsimmons *et al*. 1998)

1. Mix 500 µl of blood with 500 µl of lysis buffer, vortex.
2. Spin at 10000rpm for 5 min.
3. Remove supernatant, leaving pellet.
4. Add another 500µls of lysis buffer, vortex until pellet is resuspended.
5. Spin 10000rpm for 5 min.
6. Remove supernatant, leave pellet.
7. Repeat steps 5 and 6, until supernatant is clear or not red anymore.
8. Remove supernatant.
9. Resuspend pellet in 500µl of PCR extraction buffer.
10. Add 10 µl of 20 ngs/ml Proteinase K and incubate O/N at 65° C. If sample is really yellow, brown or red after incubation it should be phenol/chloroform extracted.

Phenol/chloroform Extraction

Mix sample with an equal volume of phenol/$CHCL_3$. Mix my inverting the tube for several minutes. Spin at 10000rpm for 5 min. Transfer top layer to a new eppendorf. Add an equal volume of $CHCL_3$ invert tube for several min. Spin at 10000rpm for 5 min. Transfer top layer to new eppendorf and carry on.

11. Precipitate – Add 1/10 the volume of 3M NaAcetate pH 5.5 ~ 50µl, mix by inversion, add 2X the volume of 95% ETOH~ 1000 µl, mix and store for several hours or O/N at -20° C.
12. Spin 13000rpm for 15 min. Remove supernatant. Wash 1X with 500µl of 70% ETOH (add ETOH then vortex). Spin at 13000rpm for 10 min. Remove supernatant.
13. Resuspend in 400 µl of 1XTE. Can be incubated for several hours or O/N at 55° C.
14. Store sample at 4° C until use. Use 1µl of this for your PCR template. For long term storage store at -80° C.

Lysis Buffer	250mls	500mls
Sucrose 0.32M	27.38gms	54.76gms
$MgCl_2$ 5mM	0.25gms	0.50gms
1% Triton X	2.5mls	5.0mls
Tris 10mM pH 7.5	1.25mls	2,5mls of 2M Tris

PCR Extraction Buffer	250mls	500mls
KCl 50mM	0.93gm or 12.5mls	1.86gms or 25mls of 1M KCl
Tris-HCl 10mM pH 8.3	1.25mls	2.5mls of 2M Tris
$MgCl_2$ 2.5mM	0.12gms	0.25gms
Gelatin 0.1mg/ml	0.25gms	0.50gms
Tween 20 0.45%	1.125mls	2.25mls
Nonident P40 0.45%	1.125mls	2.25mls

Appendix G: DNA extraction from fresh tissue (adapted from Sambrook *et al.* 1989)

1. Drop freshly excised tissue into liquid nitrogen in a stainless steel blender.
2. Blend until tissue is ground to a powder
3. Allow liquid nitrogen to evaporate, add powdered tissue to 10 volumes of extraction buffer in a beaker
4. Shake the beaker to submerge the powder
5. Transfer solution to a 50 ml centrifuge tube, incubate for 1 hour at 37° C
6. Add proteinase K to a final concentration of 100 ng/ml. Gently mix in the enzyme
7. Place in a water bath for 3 hours at 50° C. Mix periodically.
8. Cool solution to room temperature. Ass equal volume of phenol equilibrated with 0.5M Tris Cl, pH 8.0. Mix the two phases by inverting for 10 min.
9. Transfer aqueous phase to a clean centrifuge tube and repeat the extraction with phenol twice.
10. Dialyze the aqueous phases at 4° C four times against 4 liters of a solution of 50mM Tris Cl and 10mM EDTA. Allow room for the volume of the sample to increase 1.5 to 2.0 fold.
11. Measure the absorbance of the DNA at 260nm and 280nm.
12. Calculate concentration of DNA

Appendix H: High Salt DNA extraction from white blood cells (adapted from Montgomery and Sise 1990)

1. While blood cells collected following the lysis of red blood cells in 2.5 volumes of cold sterile lysing solution.
2. Hold on ice for approximately 2-10 min until red blood lysis is observed (change in color).
3. Spin at a relative centrifugal force (RCF) of 2000 for 10 min at 4° C.
4. Resuspend pelleted white blood cells with a Pasteur pipette and wash twice in 10ml of Tris buffered saline.
5. Pellet cells between washes at an approximate RCF of 1000 for 3 min.
6. Resuspend cells by vigorous vortexing in 9ml of TE.
7. Add 50 µl of Proteinase K and 0.5ml 0.5M EDTA, pH 8.0, and mix.
8. Add sodium dodecylsulphate while gently swirling tubes.
9. Incubate at 50° C in a water bath, mixing occasionally, for 3 hours.
10. Add 4.3ml of a saturated NaCl solution and shake vigorously for 30 sec.
11. Spin at RCF 2000 for 10 min.
12. Transfer supernatant DNA to a clean glass tube and add two volumes of 95% ethanol.
13. Spool out DNA and resuspend in TE and measure the concentration of the DNA in a scanning spectrometer.

Appendix I: RNA extraction method (TriZol Method, Gibco, Gaithersburg, MD)

1. Grind tissue sample in 1ml of reagent per 50-100mg of tissues. The sample should not exceed 10% of the volume of TRIzol reagent used for grinding.
2. Incubate samples for 5 min to permit the complete dissociation of nucleoprotein complexes.
3. Add 0.2 ml of chloroform per 1 ml of TRIzol reagent. Shake tubes by hand for 15 sec and incubate for 2 or 3 min.
4. Centrifuge the samples at no more than 12,000 x g for 15 min at 4° C.
5. Transfer the aqueous phase (top layer containing the RNA) to a fresh tube. Add 0.5 ml of isopropanol per 1 ml of TRIzol reagent.
6. Incubate samples for 10 min at -20° C and centrifuge at no more than 12,000 x g for 10 min.
7. Remove the supernatant. Wash pelleted RNA once with 75% ethanol per 1 ml of TRIzol reagent.
8. Mix sample and centrifuge at no more than 7,500 x g for 5 min at 4° C.
9. Briefly dry the RNA pellet (do not dry completely as this will decrease its solubility).
10. Dissolve RNA in DEPC-treated water and incubate for 10 min at 55° C to 60° C. Store RNA as 8 µl aliquot, at -80° C.

Appendix J: cDNA synthesis (Superscript^{TM} Gibco, Gaithersburg, MD)

DNase I Digestion of RNA Preparation

1. Add the following to a 0.5 ml tube on ice:

Total RNA	1-2 µg
10X reaction buffer	1 µl
Amplification grade DNase I	1 µl
DEPC-treated water	to 10 µl

2. Incubate at room temperature for 15 min.
3. Add 1 µl of 25 mM EDTA.
4. Incubate for 15 min at 65° C to heat inactivate DNase I, and then place on ice for 1 min.
5. Collect the reaction by brief centrifuge.

First strand cDNA synthesis using Oligo (dT)

1. Mix and briefly centrifuge each sample before use.
2. Prepare RNA/primer mixtures in sterile 0.5 ml tubes as follows:

1 to 5 µg total RNA	n µl
Oligo $(dT)_{12-18}$ (0.5 µg/µl)	1 µl

3. Incubate each sample at 70° C for 10 min and incubate on ice for at least 1 min.
4. Prepare the following reaction mixture:

10X PCR buffer	2 µl
25 mM $MgCl_2$	2 µl
10 mM dNTP mix	1 µl
0.1 M DTT	2 µl

5. Add 7 µl of reaction mixture to each RNA/primer mixture, mix gently and collect by brief centrifugation.
6. Incubate at 42° C for 5 min.
7. Add 1 µl of SUPERSCRIPT II RT to each tube, mix, and incubate for 42° C for 50 min.
8. Terminate the reactions at 70° C for 15 min. Chill on ice.
9. Collect the reactions by brief centrifugation and add 1 µl of RNase H to each tube and incubate for 20 min at 37° C.
10. Store cDNA as 5 µl aliquots at -70° C.