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QUANTIFICATION OF HSP70 MRNA AS AN INDICATOR OF STRESS IN CATTLE

by

Jan Michelle Kennie 

A thesis submitted to the faculty of Graduate Studies and research in partial fulfillment of the requirements for the degree of Master of Science

in

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ABSTRACT

Cattle are subjected to a variety of stressors during the production cycle including extreme temperatures, handling, and transport which can have a negative impact on their health and production efficiency. The objective was to measure the relative expression of Hsp70 mRNA in bovine lymphocytes and fibroblasts to determine if the expression of Hsp70 mRNA is a useful indicator of the level of stress to which the cells were exposed. Hsp70 expression is induced in response to stress and helps the cells to survive the insult by protecting other proteins from damage. Bovine lymphocytes and fibroblasts were heat-shocked *in vitro* and the relative expression of Hsp70 mRNA was determined using RT-MIMIC PCR. Also, lymphocyte samples were taken from heifers exposed to a variety of stressors and relative Hsp70 mRNA expression was measured. Hsp70 mRNA was detected in lymphocytes from all animals examined except for one animal. Relative Hsp70 mRNA expression was found to vary between individual animals exposed to the same treatments. Further study is needed for application of the level of Hsp70 mRNA expression to be used as an indicator of stress in cattle.

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Quantification of Hsp70 mRNA as an indicator of stress in cattle" submitted by Jan Michelle Kennie in partial fulfillment of the requirements for the degree of Master of Science in Animal Science.

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Date May 5/2000

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TABLE OF CONTENTS

Chapter **Page**

1. Introduction

1.1. Stress in Cattle Production..... 1

1.1.1. Adrenal Responses to Stress..... 1

1.1.2. Quantification of the Impact of Stress on Cattle..... 4

1.1.3. Heat Shock Proteins and the Stress Response..... 7

1.1.3.1. Induction of the Stress Response..... 8

1.1.3.2. Transcriptional Regulation of the Stress Response..... 9

1.1.3.2.1. Heat Shock Transcription Factor 1..... 11

1.1.3.2.2. Heat Shock Transcription Factor 2..... 13

1.1.3.2.3. Heat Shock Transcription Factor 3..... 13

1.1.3.2.4. Heat Shock Transcription Factor 4..... 14

1.1.3.3. Translational Regulation of the Stress Response..... 14

1.1.3.3.1. Initiation of Translation..... 14

1.1.3.3.1. Phosphorylation of eIF2 During Heat Shock..... 15

1.1.3.3.3. Effect of Heat Shock on eIF4F Activity..... 16

1.1.3.3.4. Preferential Translation of Hsp mRNA..... 17

1.1.3.4. Heat Shock Proteins as Molecular Chaperones..... 18

1.1.3.5. Classification of Heat Shock Proteins..... 18

1.1.3.5.1. Large Heat Shock Proteins..... 20

1.1.3.5.2. Hsp90 Family..... 20

1.1.3.5.2.1. Hsp90 and Glucocorticoid receptors..... 20

1.1.3.5.2.2. Hsp90 and heme-regulated eIF-2α kinase..... 22

1.1.3.5.3. Hsp70 Family..... 23

1.1.3.5.4. Hsp60 Family..... 24

1.1.3.5.5. Hsp40 Family..... 25

1.1.3.5.6. Small Heat Shock Proteins..... 25

1.1.3.6. Heat Shock mRNA and Protein Expression in Farm Animals..... 26

1.2. Reverse Transcription-Polymerase Chain Reaction..... 30
1.2.1. Competitive PCR..... 31
1.3. Conclusion and Objectives..... 34

2. Materials and Methods

2.1. Isolation and heat shock of Lymphocytes..... 36
2.1.2. Isolation of Total RNA From Bovine Lymphocytes..... 37
2.2. Quantification of Bovine Stress Protein mRNA - Method Development..... 38
2.2.1. RT-PCR Using Human Hsp Primers..... 38
2.2.2. RT-PCR Using Bovine Hsp Primers..... 41
2.2.3. Quantification of Relative Differences in Hsp mRNA..... 43
2.3. *In Vitro* Thermal Shock of Bovine Lymphocytes..... 51
2.4. *In Vivo* Controlled Environment Experiments..... 52
2.4.1. Experiment One - Temperature Exposure..... 52
2.4.2. Experiment Two - Transport Stress..... 53
2.5. *In Vitro* Heat Shock of Bovine Fibroblast Cell-line..... 54
2.6. Statistical Analysis..... 54

3. Results

3.1. Quantification of Bovine Stress Protein mRNA – Method Development..... 55
3.1.1. RT-PCR Using Human Hsp Primers..... 55
3.1.2. RT-PCR Using Bovine Hsp Primers..... 55
3.1.3. Quantification of Relative Differences in Hsp mRNA using PCR MIMICS..... 59
3.2. *In Vitro* Thermal Shock of Bovine Lymphocytes..... 67
3.2.1. Determination of Hsp70 MIMIC Concentration..... 67
3.2.2. Relative Expression of Hsp70 mRNA in Bovine Lymphocytes Exposed to *In Vitro* Thermal Shock..... 71
3.3. *In Vivo* Controlled Environment Experiment..... 84
3.3.1. Experiment One – Movement/Temperature Exposure..... 84
3.3.2. Experiment Two – Transport Stress..... 87

3.4. *In Vitro* Heat Shock of Bovine Fibroblast Cell-line..... 92

4. Discussion

4.1. RT-PCR Using Human Hsp Primers..... 96

4.2. RT-PCR Using Bovine Hsp Primers..... 96

4.3. Quantification of Relative Differences in Hsp mRNA Using PCR MIMICs.... 98

4.4. *In Vitro* Thermal Shock of Bovine Lymphocytes..... 99

4.5. *In Vivo* Controlled Environment Experiment..... 101

4.5.1. Experiment One – Movement/Temperature Exposure..... 101

4.5.2. Experiment Two - Transport Stress..... 102

4.6. *In Vitro* Heat Shock of Bovine Fibroblast Cell-line..... 103

5. Conclusions..... 105

6. Future Considerations..... 104

7. References..... 106

8. Appendices..... 119

Appendix A. Lymphocyte Isolation Buffers..... 119

Appendix B. Gel Electrophoresis Running Buffer..... 120

Appendix C. RNA Quality..... 121

Appendix C. Determination of DNA Concentration in PCR Product Bands and of Hsp70:MIMIC Ratios..... 122

LIST OF TABLES

		PAGE
Table 1-1	Major heat shock proteins in mammals	19
Table 2-1	Heat shock protein primer pairs used for analysis by RT-PCR	40
Table 2-2	PCR primer pairs used for MIMIC construction and analysis for heat shock protein mRNA	46
Table 3-1	Relative expression of Hsp70 mRNA in bovine lymphocytes from animal #6 incubated at 38°C, 42°C, or 34°C as determined by RT-MIMIC PCR	73
Table 3-2	Relative expression of Hsp70 mRNA in bovine lymphocytes from animal #44 incubated at 38°C, 42°C, or 34°C as determined by RT-MIMIC PCR	75
Table 3-3	Relative expression of Hsp70 mRNA in bovine lymphocytes from animal #41 incubated at 38°C, 42°C, or 34°C as determined by RT-MIMIC PCR	77
Table 3-4	Relative expression of Hsp70 mRNA in bovine lymphocytes from animal #28 incubated at 38°C, 42°C, or 34°C as determined by RT-MIMIC PCR	79
Table 3-5	Relative expression of Hsp70 mRNA in bovine lymphocytes from animal #26 incubated at 38°C, 42°C, or 34°C as determined by RT-MIMIC PCR	81
Table 3-6	Relative expression of Hsp70 mRNA in bovine lymphocytes from blood samples taken from heifers before movement and after movement to a new pen in either a thermoneutral room (warm) or a below thermoneutral room (cold)	86

Table 3-7 Relative expression of Hsp70 mRNA in bovine lymphocytes from blood samples taken from heifers before transport and after transport for one hour..... 90

Table 3-8 Core body temperature, neutrophil to lymphocyte ratio, plasma cortisol, plasma T_3 and plasma T_4 concentrations before and after transport..... 91

Table 3-9 Relative expression of Hsp70 mRNA in bovine fibroblasts measured using RT-MIMIC PCR..... 95

LIST OF FIGURES

PAGE

Figure 1-1 The integrated stress responses of the hypothalamic-pituitary -adrenal axis (HPA) and the sympatho-adrenal medullary axis..... 3

Figure 1-2 Inducers of heat shock gene transcription and protein synthesis..... 10

Figure 2-1 Construction of PCR MIMICs..... 45

Figure 3-1 RT-PCR analysis of control (38°C) and *in vitro* heat-shocked (42°C) bovine lymphocyte RNA for Hsc70 mRNA..... 57

Figure 3-2 RT-PCR analysis of control (38°C) and *in vitro* heat-shocked (42°C) bovine lymphocyte RNA for Hsc70, Hsp70-1 and Hsp70-2 mRNA..... 57

Figure 3-3 Expression of Hsc70 mRNA in lymphocyte samples taken from animal 323 before and after movement to a new pen, and from animal 837 before and after transport for one hour..... 58

Figure 3-4 Expression of Hsp70-2 mRNA in lymphocyte samples taken from animal 323 before and after movement to a new pen, and from animal 837 before and after transport for one hour..... 58

Figure 3-5 First and second rounds of PCR in the construction of the Hsp70 and Hsc70 PCR MIMICs..... 60

Figure 3-6 Gel electrophoresis of purified Hsc70 and Hsp70 PCR MIMICS..... 60

Figure 3-7 Estimation of Hsc70 and Hsp70 PCR MIMIC yield..... 61

Figure 3-8 RT-PCR analysis for Hsp70 mRNA of control lymphocyte RNA for 15, 20, 25, 30, 35, or 40 PCR cycles..... 61

Figure 3-9 RT-PCR analysis for Hsp70 mRNA starting with 100, 200, or 400 ng of total RNA..... 62

Figure 3-10 Results from an optimized RT-PCR reaction using new primers designed to amplify a 870 base pair fragment corresponding to Hsp70 mRNA..... 62

Figure 3-11 Results from an optimized RT-PCR reaction using new primers designed to amplify a 934 base pair fragment corresponding to Hsc70 mRNA..... 63

Figure 3-12 Ten-fold dilution series of 100 attomole/µl Hsp70 MIMIC..... 65

Figure 3-13 Two-fold dilution series of 10^{-1} attomole/µl Hsp70 MIMIC..... 65

Figure 3-14 RT-PCR using PCR MIMICs to determine the relative expression of Hsp70 mRNA in RNA from bovine lymphocytes incubated at 38°C or 42°C for one hour..... 66

Figure 3-15 RT-PCR analysis for Hsp70 mRNA of control RNA from animals 44, 41, 6, and 26 without Hsp70 MIMIC..... 69

Figure 3-16 Ten-fold serial dilutions of Hsp70 MIMIC with control RNA from animal 26 and 6..... 69

Figure 3-17 Two-fold serial dilutions of Hsp70 MIMIC with control RNA from animal 6 and 26..... 70

Figure 3-18 RT-MIMIC PCR analysis for relative expression of Hsp70 mRNA in lymphocyte samples from animal 6 incubated at 38°C, 42°C, and 34°C..... 72

Figure 3-19 RT-MIMIC PCR analysis for relative expression of Hsp70 mRNA in lymphocyte samples from animal 44 incubated at 38°C, 42°C, and 34°C..... 74

Figure 3-20 RT-MIMIC PCR analysis for relative expression of Hsp70 mRNA in lymphocyte samples from animal 41 incubated at 38°C, 42°C, and 34°C..... 76

Figure 3-21 RT-MIMIC PCR analysis for relative expression of Hsp70 mRNA in lymphocyte samples from animal 28 incubated at 38°C, 42°C, and 34°C..... 78

Figure 3-22 RT-MIMIC PCR analysis for relative expression of Hsp70 mRNA in lymphocyte samples from animal 26 incubated at 38°C, 42°C, and 34°C..... 80

Figure 3-23 RT-MIMIC PCR analysis for relative expression of Hsp70 mRNA in lymphocyte samples from animal 101 incubated at 38°C, 42°C, and 34°C. 82

Figure 3-24a Diagram of the thermocycler block showing the placement of tubes 1-14 during the RT-PCR reaction..... 83

Figure 3-24b Gel electrophoresis showing the products of RT-PCR for tubes 1-14.... 83

Figure 3-25 Gel electrophoresis of RT-PCR reactions from tubes placed in the top two rows of the thermocycler..... 83

Figure 3-26 RT-MIMIC PCR analysis for relative expression of Hsp70 mRNA in lymphocyte samples from animals 314, 722, 212, 349, 69, 72, 25, and 14 before and after movement to a new pen..... 85

Figure 3-27 RT-MIMIC PCR analysis for relative expression of Hsp70 mRNA in lymphocyte samples from animals 12, 116, and 11 before transport and after transport..... 88

Figure 3-28 RT-MIMIC PCR analysis for relative expression of Hsp70 mRNA in lymphocyte samples from animals 6, 18, and 27 before transport and after transport..... 89

Figure 3-29 Hsp70 mRNA expression in total RNA extracted from bovine fibroblasts incubated at 38°C (control), 42°C, or 45°C for one and two hours..... 93

Figure 3-30 Hsp70 mRNA expression in total RNA extracted from bovine fibroblasts incubated at 38°C (control), and 42°C, or 45°C for one and two hours..... 94

LIST OF ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
CCM	complete culture media
cDNA	complementary deoxyribonucleic acid
CP	crude protein
CRH	corticotrophin releasing hormone
DEPC	diethylpyrocarbonate
DM	dry matter
DNA	deoxyribonucleic acid
DTT	dithiothreitol
eIF	eukaryotic initiation factor
ER	endoplasmic reticulum
FCS	fetal calf serum
GDP	guanine triphosphate
GR	glucocorticoid receptor
Grp	glucose regulated protein
GTP	guanine triphosphate
HBD	hormone binding domain
HPA	hypothalamic-pituitary-adrenal axis
HRI	heme-regulated inhibitor
HSBP	heat shock binding protein
Hsc	heat shock cognate protein
HSE	heat shock element
HSF	heat shock transcription factor
Hsp	heat shock protein
mRNA	messenger ribonucleic acid
NE	net energy

PBS	phosphate-buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT	reverse transcription
RT-PCR	reverse transcription–polymerase chain reaction
T_3	triiodothyronine
T_4	thyroxine
UTR	untranslated region

1. INTRODUCTION

1. 1. STRESS IN CATTLE PRODUCTION

Cattle encounter many different types of stressors throughout the production cycle. These include weaning, vaccination, handling, transport, infection, branding, and thermal extremes. The reaction of the animal to these stressors is believed to be a function of the interaction of genetic factors, age, previous experiences, the perception of the stressor to the animal, and the physiologic state of the animal at the time (Zavy *et. al.*, 1992; Grandin, 1997). The inability to adapt to stress can result in a negative impact on the health and production efficiency of the animal. Producers, veterinarians, and researchers have long been able to identify factors that impose stress on the animal, however, the major challenge has been to identify and measure which stressor or combination of stressors compromise the animals' health to the point where intervention and treatment are required. Assessment of stress caused by production practices and environmental factors is of interest not only for production efficiency, but also for animal welfare and public food safety issues. Knowing the level of stress and the combination of stressors that are most likely to have ill effects on the animal will enable producers and veterinarians to determine if, and/or when antibiotics should be administered and whether herd management should be altered.

1.1.1. ADRENAL RESPONSES TO STRESS

The adrenal hormones (cortisol, epinephrine, norepinephrine) are secreted by the adrenal gland in response to a stressor (i.e. infection, trauma, hypoglycemia, anxiety). Stressors are considered to be any event or condition that have the potential to activate the hypothalamic-pituitary-adrenal axis (HPA) and the sympatho-adrenal medullary axis (Minton, 1994). Figure 1-1 illustrates a typical adrenal response to stress by the HPA and its interaction with the sympathetic nervous system. The stressor, which may be physical and/or psychological, activates corticotrophin releasing hormone (CRH) neurons in the

hypothalamus which stimulate the release of CRH from the hypothalamus. CRH acts on the anterior pituitary and causes the release of adrenocorticotrophic hormone (ACTH). ACTH exerts its effect on the adrenal cortex where it stimulates secretion of cortisol into the bloodstream. Generally, increased cortisol concentrations lead to increased energy mobilization and redistribution, repressed inflammatory and immune responses, and inhibition of growth and reproductive function. The stressor also activates adrenergic neurons in the hypothalamus which stimulate the release of norepinephrine and epinephrine. Epinephrine and small amounts of norepinephrine are secreted by the adrenal medulla. Norepinephrine is primarily a neurotransmitter. Epinephrine release rapidly activates glycogenolysis which, along with the actions of cortisol, leads to an increase in glucose production. The magnitude and duration of adrenocortical responses depend on the nature, intensity, and duration of the stimulus. The response of individual animals to an identical stimulus may be very different due to the animal's perception of the stimulus and the novelty of the event.

Cortisol regulates its own secretion in a classic example of feedback regulation. When plasma cortisol level has reached a threshold, cortisol secretion is inhibited in three ways. First, cortisol feedback on the anterior pituitary blocks the stimulatory action of CRH on corticotroph cells, inhibiting the release of ACTH. This occurs within minutes of reaching the cortisol threshold. Second, within hours, increased cortisol concentration leads to the inhibition of ACTH synthesis by blocking gene transcription. Third, cortisol blocks the release and synthesis of CRH. It has been suggested that ACTH can feed back on the hypothalamus, blocking the release of CRH. However, prolonged stress can cause hyperplasia of the adrenal cortex because of continuous ACTH secretion.

The activity of the HPA is normally regulated within a narrow range with marked secretions occurring due to a pattern established by the circadian rhythm. In humans plasma ACTH and cortisol levels peak about two hours before awaking and a morning peak in cortisol level occurs approximately two hours after awaking (Genuth, 1990). Cortisol levels are lowest just before sleep. The circadian rhythm can be interrupted and overridden by stress and negative feedback.

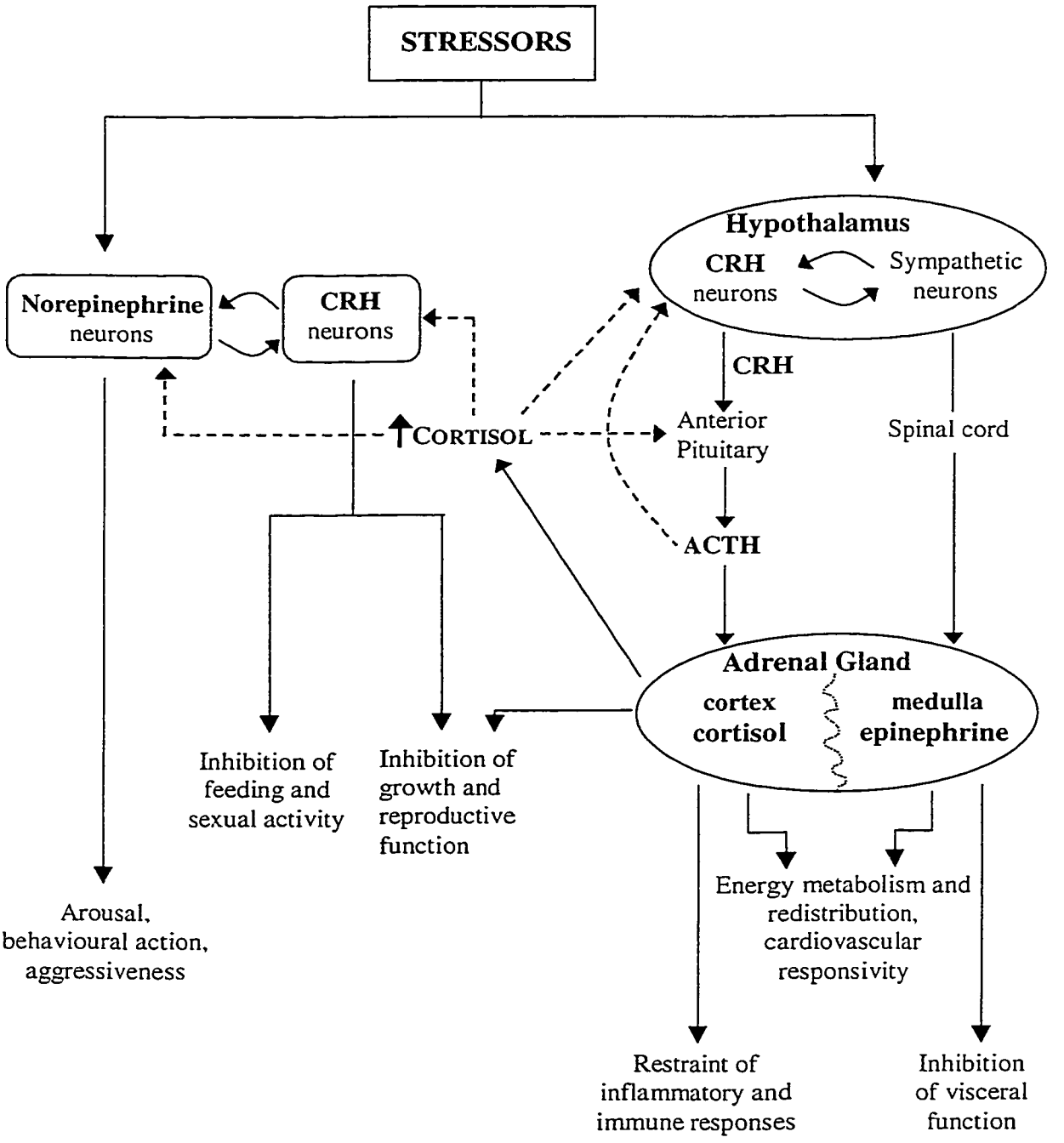

Figure 1-1. The integrated stress responses of the hypothalamic-pituitary-adrenal axis (HPA) and the sympatho-adrenal medullary axis. Solid lines represent activation/stimulation and dotted lines represent negative feedback (Adapted from Genuth, 1990).

1.1.2. Quantification of the Impact of Stress on Cattle

The plasma levels of the adrenal hormones are commonly used by researchers as indices of stress. However, some researchers caution that cortisol, epinephrine, and norepinephrine are highly variable and absolute comparisons between studies should not be made (Zavy *et. al.*, 1992; Grandin, 1997). Also, plasma concentrations of adrenal hormones can reflect acute stress but do not reflect the degree of stress (Cooper *et. al.*, 1995). Cortisol has been the most studied of these hormones since measuring plasma epinephrine and norepinephrine is relatively more difficult and highly variable compared to measuring peripheral cortisol concentration. The plasma half-life of epinephrine and norepinephrine is approximately two minutes while the plasma half-life of cortisol is approximately 70 minutes. Rulofson and co-workers (1988) transported five Red Angus bulls 320 km and found that plasma norepinephrine and epinephrine concentrations increased 2-fold over resting levels. Much greater increases in plasma concentration were expected but, because of the short half-lives of norepinephrine and epinephrine, they may have been metabolized in the ten-minute interval between offloading and venipuncture.

There are many studies reporting an increase in cortisol concentration in response to one or a combination of treatments including weaning, handling, transport, restraint, and dehorning. Warriss *et. al.* (1995) reported that cortisol concentration in steers transported for 5, 10, or 15 hours were all significantly higher over control levels. An effect of length of transport was seen. Cattle transported for 5 hours had significantly greater cortisol concentrations than cattle transported for 10 or 15 hours. The authors suggested that the decrease in cortisol levels during the longer journeys was the result of the animals becoming adapted to being transported. Cooper *et. al.* (1995) measured plasma cortisol concentration in steers subjected to restraint and dehorning and in control steers subjected to headgate restraint only. Plasma cortisol concentrations were significantly greater in dehorned steers than in control steers from 10 to 60 minutes after dehorning or headgate restraint.

Breed of the animal affects cortisol concentrations in animals exposed to handling, weaning, mixing, and transport. Zavy *et. al.* (1992) subjected castrated beef calves to initial handling, weaning, remixing, and transport and measured cortisol levels

after each stressor. Half of the calves had a *Bos indicus* genetic component while the other half were of *Bos taurus* breeding. *Bos indicus* calves had higher cortisol levels than *Bos taurus* calves throughout the study.

Grandin (1997) compared reported cortisol levels in cattle during handling from different studies. Cortisol levels were found to be highly variable; baseline cortisol levels ranged from 0.5 to 9 ng/ml, while levels during handling ranged from 13 to 93 ng/ml. Although it seems that cortisol level can distinguish between a very low and a very high level of stress, Grandin cautioned that an animal's response to a particular stress is individual and many different factors (i.e. genetics, age, previous experiences) interact making it difficult to use the measured cortisol concentration as an accurate index of stress.

Stress is thought to be an immunosuppressant, which can increase the susceptibility of an animal to disease (Wong *et. al.*, 1992). The immunosupression is believed to be due to increases in cortisol concentrations during stress (Stull *et. al.*, 1999). This effect of stress can be seen in changes in neutrophil-to-lymphocyte ratio (N:L). Friend *et. al.* (1985) compared physiological characteristics of Holstein bull calves in four types of housing; stall, pen, hutch, and yard. Basal cortisol concentration was not significantly different between housing types, although levels tended to be highest in stall and hutch housing types. N:L was significantly higher in calves housed in stall and hutch housing types. It was concluded that individually confining calves in stalls could be detrimental to the animal's immune function and therefore more stressful to the animal. In another study on dairy bull calf housing, Stull and McDonough (1994) compared physiological responses of group-penned calves with calves housed in stalls for 16 weeks of production. Cortisol concentration was found to be significantly higher in stall-housed calves than grouped-penned calves from week 8 to week 16. Similarly, N:L in stall-housed calves versus group-penned calves were higher during week 8 to week 16.

Wong *et. al.* (1992) intensely exercised four Thoroughbred horses on a treadmill and measured serum cortisol concentration and N:L at 24 hours before exercise and 30 minutes, 6, 24, 48, 72, and 120 hours after exercise. Mean serum cortisol concentration increased significantly at 30 minutes, decreased significantly at 6 hours, and returned to pre-exercise levels by 24 hours. Negative feed back inhibition or a diurnal effect (the

samples were collected in the afternoon) may have caused the decrease in cortisol concentration at 6 hours. Conversely, mean N:L was significantly higher at 6 hours after exercise and returned to pre-exercise levels by 24 hours. These results suggested that the horses had undergone stress from the strenuous exercise.

Neutrophil-to-lymphocyte ratio is not an accurate indicator of health status or intensity of a stressor on its own. N:L was shown to detect disease in calves by Adams *et. al.* (1992) who measured N:L in 35 beef calves at birth, at 24, and 48 hours, and in 22 of these calves at 3 weeks. At 3 weeks of age, five of these calves had mild clinical signs of disease such as nasal discharge and fever. Comparison of N:L between the ill calves and the healthy calves revealed that the ill calves had significantly higher N:L values at birth, at 24 and 48 hours and at 3 weeks of age. Although N:L indicated a disease state, there was no indication of severity of the disease until mild clinical signs appeared at 3 weeks of age. These calves did not need medical treatment and grew normally.

Kleinbeck and McGlone (1999) compared N:L in 4-week old pigs reared indoors versus outdoors. Pigs reared indoors had significantly lower N:L. This suggested that the outdoor-reared pigs were showing signs of stress. However, the outdoor-reared pigs showed similar weight gain and pig survival as the indoor-reared pigs, making it unlikely that that outdoor-reared pigs suffered from more than mild stress. Stull *et. al.* (1999) measured serum cortisol concentration and N:L in a total of 119 pigs from five commercial swine farms in California. Blood samples were taken on day 28, 56, 84, 112, 140, and 168. Age of the animal and facility significantly effected cortisol and N:L ratio. No correlation was found between cortisol and N:L. It was concluded that these physiological parameters were not good indices of stress and health status when making comparisons between pigs of different ages.

Although N:L ratios do not appear to be generally useful indicators of stress in all species, they do appear to have some value in the monitoring of stress in ruminants when used in conjunction with other indicators.

1.1.3. HEAT SHOCK PROTEINS AND THE STRESS RESPONSE

In 1962, Ritossa described puffs on the salivary gland chromosomes of a fruit fly, *Drosophila busckii* (Ritossa, 1962). The puffs were shown to be associated with newly synthesized RNA. These puffs appeared quickly after a sub-lethal heat shock and once the heat shock was removed and the temperature of the cells returned to normal, the puffs disappeared. Ritossa (1962) also established that the chromosomal puffs could be induced by dinitrophenol and sodium salicylate. This study was the first to report a response to heat shock at the molecular level and this response was referred to as the heat shock response. The chromosomal puffs were later identified to be sites of active transcription of genes encoding a group of proteins now referred to as heat shock proteins (Hsps) (Tissieres *et. al.*, 1974).

The heat shock response has been observed in cells from many organisms, from bacteria to man, and is recognized as a universally conserved cellular defense program. In fact, the response is considered to be the most highly conserved genetic system described to date. Acute exposure to heat has been shown consistently to induce a heat shock response *in vitro* and *in vivo*. Other types of environmental insults such as amino acid analogs, oxidants, heavy metals, physical trauma, infection, (reviewed in: Lindquist, 1986; Subjeck and Shyy, 1986; Lindquist and Craig, 1988; Macario, 1995) and even psychological stress (Isosaki and Nakashima, 1998) have been shown to elicit a heat shock response. Because so many different types of stress have been shown to elicit a heat shock response, the response is more generally referred to as the stress response and the proteins induced, stress proteins.

Hsps and the stress response have been studied intensively for the last twenty years because of the universality of the response, the conservation of structure and function of the Hsp genes and proteins, and the continuous discovery of novel functions of Hsps in normal and stressed organisms. The high conservation of the stress response in all living cells suggests that heat shock genes and proteins play a crucial role in the prevention, protection, and recovery of cells from stress. Also, many of the stress proteins themselves or their close relatives are present in organisms under normal conditions and these stress proteins play important roles in a variety of normal cellular processes. The

various functions, features, and regulation of expression of heat shock genes and proteins in normal and stressed cells will be discussed with emphasis on the mammalian stress proteins, particularly Hsp70 and Hsp90.

1.1.3.1. *INDUCTION OF THE STRESS RESPONSE*

In the presence of a stressor, the cell responds by shutting down the expression of most proteins, with the exception of the stress proteins whose expression increases dramatically. Generally, once the stressor is removed the normal pattern of protein synthesis resumes. The magnitude of transcription and preferential translation of Hsps and the global repression in protein synthesis of non-Hsp proteins varies substantially among cell types, species, and in relation to the degree of heat shock (Duncan, 1996). These variables also influence the rate of recovery from the stress. These variations must be taken into account, however, this discussion will focus on the response to a relatively severe heat shock.

The means by which the cell is able to recognize stress and respond to it by increasing the expression of Hsps is incompletely understood. Hightower (1980) suggested that the accumulation of abnormally folded proteins in the cell initiated a stress response. This idea has been supported by several studies that have demonstrated that the presence of abnormally folded or partially denatured proteins in bacterial and animal cells activates the stress response. Goff and Goldberg (1987) fed amino acid analogues to bacteria which led to the production of abnormally folded proteins and the subsequent induction of a stress response. Kelley and Schlesinger (1978) demonstrated that adding amino acid analogues to animal cells was enough to induce a stress response. Finally, Anathan *et. al.* (1986) injected denatured proteins into frog oocytes which immediately resulted in a stress response. In fact, most of the factors known to induce the stress response are known to be protein denaturants. The stress response can be induced by viral infection, inflammation, and fever (Figure 1-2) (Macario, 1995; Jindal, 1996; Leppa and Sistonen, 1997), all of which can result in altered protein synthesis or the presence of abnormal proteins in the cell. Non-stressor inducers of the stress response include growth

factors, development, differentiation, and stage of the cell cycle (Figure 1-2) (Milarski and Morimoto, 1986; Williams *et. al.*, 1989).

1.1.3.2. *TRANSCRIPTIONAL REGULATION OF THE STRESS RESPONSE*

Transcription of Hsp genes is induced by heat shock while transcription of other (i.e. non-Hsp) genes is suppressed in response to heat shock. Transcriptional control of Hsp gene expression is achieved by a conserved regulator called the heat shock transcription factor (HSF) which when induced by heat shock or other physiological stress binds to a highly conserved promoter element upstream of heat shock genes referred to as the heat shock element (HSE) (Voellmy, 1994; Morimoto *et. al.*, 1996; Satyal and Morimoto, 1998). Heat shock gene transcription is activated when the HSF binds to the HSE. Unlike yeast and *Drosophila* where there is only one gene encoding HSF, multiple HSF genes have been cloned in vertebrates. There are two HSF genes in the mouse (HSF1, HSF2), three in the chicken (HSF1, HSF2, HSF3) and three in the human (HSF1, HSF2, HSF4) genomes respectively (Rabindran *et. al.*, 1991; Sarge *et. al.*, 1991; Schuetz *et. al.*, 1991; Nakai and Morimoto, 1993; Nakai *et. al.*, 1997).

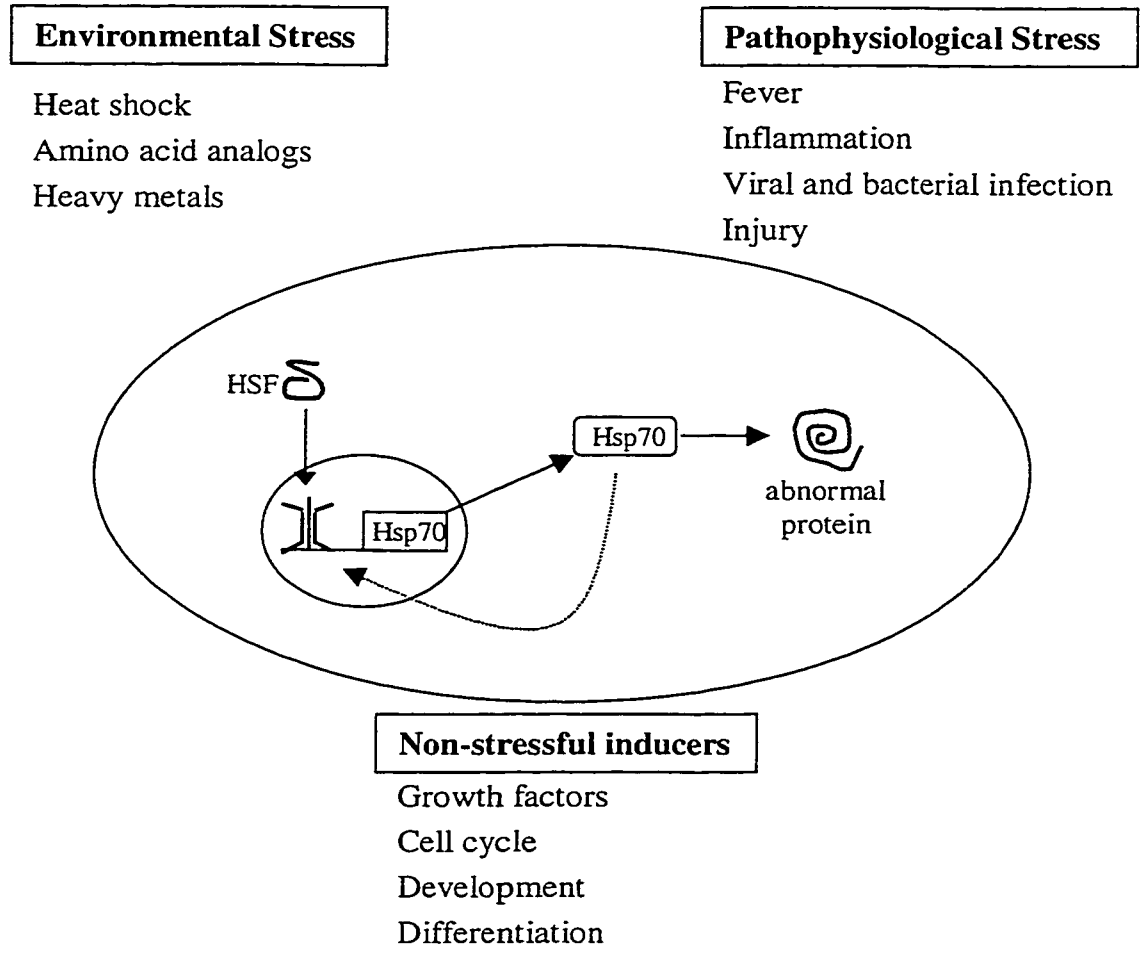

Figure 1-2. Inducers of heat shock gene transcription and protein synthesis. The activation of the heat shock transcription factor (HSF) occurs in response to environmental, pathophysiological, and /or non-stressful conditions. In this example, Hsp70 expression is elevated in response to the presence of abnormal protein. The dotted line indicates possible negative feedback regulation by Hsp70. (Adapted from Morimoto *et. al.*, 1996)

1.1.3.2.1. *Heat Shock Transcription Factor 1*

HSF1 is rapidly activated in response to heat shock and other stress signals (Baler *et. al.*, 1993; Sarge *et. al.*, 1993). HSF1 is highly conserved, and there is 85-95% conservation in sequence between the human, mouse, and chicken HSF1 (Morimoto *et. al.*, 1996). HSF1 exists mostly in the cytoplasm in monomeric form in non-stressed cells, although some HSF1 can be found in the nuclear compartments (Baler *et. al.*, 1993; Sarge *et. al.*, 1993). Upon activation HSF1 immediately undergoes conformational changes, trimerization, and translocation to the nucleus where the HSF1 trimer binds to the HSE, thereby activating transcription of Hsp genes (Baler *et. al.*, 1993; Sarge *et. al.*, 1993). Trimerization of HSF1 is essential for high-affinity binding to the HSE. Exactly how HSF1 undergoes conformational changes and trimerization as a result of stress is not completely understood. It is known that the human and mouse HSF1 are both constitutively and inducibly phosphorylated at serine residues (Sarge *et. al.*, 1993). Voellmy (1994) suggested that phosphorylation may be an event in the final activation step that enables transcription-activating function. However, it is possible that inducible phosphorylation modulates the trimerization of HSF1 or stabilizes the trimeric conformation. Also, the substantial inducible phosphorylation of HSF1 may mask dephosphorylation events at the constitutively phosphorylated residues.

Transcriptional activity attenuates upon recovery of the cell to control conditions or after a period (2-3 hours) of continuous exposure to heat shock (or other stress conditions). Attenuation of HSF1 is characterized by decreased transcriptional activity, conversion back to the monomeric state, loss of DNA binding, dephosphorylation and relocalization to the cytoplasm (Abravaya *et. al.*, 1991, Abravaya *et. al.*, 1992; Sarge *et. al.*, 1993) Attenuation and release of HSF1 from the HSE is correlated with increased levels of Hsp70 (Abravaya *et. al.*, 1991) and HSF1 activity attenuates more quickly in mammalian cells overexpressing Hsp70 (Shi *et. al.*, 1998). This suggests a feedback mechanism whereby Hsp70 negatively regulates HSF activation. Studies have shown that Hsp70 forms a stable complex with HSF1 and that this complex is able to bind DNA (Abravaya *et. al.*, 1992; Baler *et. al.*, 1992). Hsp70 and HSF1 are most readily found associated during the attenuation phase suggesting that Hsp70 may play a role in

disassembly of the HSF1 trimer. However, there is no direct evidence as yet to the exact role of Hsp70 in regulating HSF1 activity. Recently, Satyal *et. al.* (1998) carried out studies to identify proteins that interact with the trimerization domain of HSF1. They cloned a protein, heat shock binding protein1 (HSBP1), which is found in the nucleus and interacts *in vivo* with HSF1 trimers and Hsp70 during attenuation. Overexpression of HSBP1 in mammalian cells led to the repression of HSF1 tranasactivation as HSBP1 negatively affected HSF1 DNA binding activity (Satyal *et. al.*, 1998). This indicated that Hsp70 does not work alone in deactivating HSF1 activity.

Morimoto *et. al.* (1996) proposed a possible model for the activation and attenuation of mammalian HSF1 activity: (i) heat shock causes an increase in the level of misfolded or unfolded proteins in the cell. Hsp70 and other chaperones are required to stabilize these proteins until correct folding can be directed. Hsp70 and other chaperones become sequestered by the abnormal proteins which releases HSF1 from its negatively regulated state and the HSF1 monomer moves to the nucleus. (ii) In the nucleus HSF1 undergoes conformational changes, trimerizes, and binds to the HSE in the promoters of heat shock genes. Although HSF1 is bound to DNA, transcription is not yet activated. (iii) HSF1 undergoes stress-induced phosphorylation which correlates with an increase in Hsp70 and other heat shock gene transcription. Heat shock protein synthesis is thereby elevated. (iv) As protein damage is repaired, Hsp70 is released from its association with the repaired proteins and associates with the HSF1 trimers. Hsp70 (and HSBP1?) facilitate the release of HSF1 from the HSE. (v) Hsp70 and HSF1 remain in a complex as HSF1 trimers are converted back to the monomeric state and Hsp70 maintains the HSF1 monomers in a latent state. Even with the discovery of HSBP1, this outline may still serve as a simplified model for the activation and attenuation of mammalian HSF1 activity.

1.1.3.2.2. *Heat Shock Transcription Factor 2*

HSF2 is activated in response to non-stressful conditions such as development and differentiation and does not appear to be affected by heat shock and other physiological stress. Unlike HSF1 which is activated within minutes of heat shock, HSF2 activation occurs over a much longer period of time, requiring 16-24 hours, and remains activated for 72 hours (Sistonen *et. al.*, 1994). HSF2 exists in its latent form as a dimer and was shown to be altered to the transcriptionally active trimer hemin treatment in human erythroleukemia K562 cells (Sistonen *et. al.*, 1992). It appears that HSF1 and HSF2 are regulated independently. Sistonen *et. al.* (1994) induced HSF2 DNA-binding activity by addition of hemin to human erythroleukemia K562 cells and by subsequent heat shock were able to induce HSF1 activity. Other studies in mouse tissues indicate that the activation of HSF2 is developmentally regulated and examples involve embryo preimplantation and testis development (Sarge *et. al.*, 1994, Rallu *et. al.*, 1997).

1.1.3.2.3. *Heat Shock Transcription Factor 3*

HSF3 has been observed only in chicken cells. HSF3 responds to heat shock and is co-expressed with HSF1, however the activation of HSF3 is delayed in comparison to HSF1 and the two transcription factors are independently regulated (Nakai and Morimoto, 1993; Nakai *et. al.*, 1995). Tanabe *et. al.* (1998) developed cells deficient in HSF3 and found that although HSF1 DNA binding activity was activated, the heat shock response was significantly impaired. Re-introduction of HSF3 into these cells restored the normal heat shock response indicating the importance of HSF3 in the transcription of heat shock genes. There has been no explanation for why avian cells have developed two HSFs that appear to respond to the same stimulus.

1.1.3.2.4. *Heat Shock Transcription Factor 4*

HSF4 has only been characterized in human cells and not much is known about this transcription factor. It is very different from the other vertebrate HSFs as its expression is tissue-specific (preferentially expressed in the human heart, brain, skeletal muscle, and pancreas) and it has no activator function (Nakai *et. al.*, 1997). It may in fact act as a repressor reducing the basal level expression of stress-inducible genes (Nakai *et. al.*, 1997).

1.1.3.3. *TRANSLATIONAL REGULATION OF THE STRESS RESPONSE*

When the physiological temperature of an organism becomes elevated above normal, the synthesis of most proteins is drastically inhibited and the synthesis of Hsps is markedly enhanced. In mammalian cells, non-Hsp mRNAs are not degraded and there is not any substantial modification of these mRNAs when heat shock occurs (Storti *et. al.*, 1980; De Benedetti and Baglionli, 1986). Inhibition of translation in mammalian cells is primarily achieved via regulation of initiation (Panniers, 1994). Regulation of initiation can occur at many different stages during the initiation process and is mainly achieved by the phosphorylation/dephosphorylation of several eukaryotic initiation factors (eIFs). Only the eIFs that have been implicated in the inhibition of initiation of translation during heat shock will be discussed.

1.1.3.3.1. *Initiation of Translation*

Generally, under normal physiological conditions, translation is initiated when eIF2 forms a complex with guanine triphosphate (GTP) and initiator Met-tRNA. eIF2 mediates binding of Met-tRNA to the 40S ribosomal subunit, resulting in the 43S preinitiation complex. With the help of many initiation factors including the eIF4F cap-binding complex, the preinitiation complex binds to the 7-methyl guanosine cap (5'-m^7GpppG) at the 5' end of the mRNA, scans for the correct AUG (start) codon, and

complexes with a 60S ribosomal subunit to form an 80S ribosome capable of polypeptide chain elongation. The initiation factors are then released and are available to participate in further rounds of initiation.

Once the preinitiation complex binds RNA, hydrolysis of GTP occurs releasing eIF2 bound to guanine diphosphate (GDP). The eIF2•GDP complex is inactive and GDP must be exchanged for GTP in order to promote another round of initiation. GDP dissociates very slowly from eIF2, too slowly to keep up with the rate of initiation. Another initiation factor, the guanine nucleotide exchange factor (eIF2B), increases the rate of GDP release by catalyzing the exchange of GDP for GTP. Phosphorylation of eIF2 at serine 51 prevents the release of GDP from eIF2 by eIF2B. Instead a stable eIF2•GDP•eIF2B complex forms and GTP exchange cannot occur. There is a limited amount of eIF2B present therefore only a portion of eIF2 needs to be phosphorylated in order to deplete available eIF2B. This leads to the almost complete shutdown of protein synthesis due to the lack of available eIF2B to exchange GDP for GTP with unphosphorylated eIF2.

1.1.3.3.2. *Phosphorylation of eIF2 During Heat Shock*

Heat shock has been shown to induce the phosphorylation of eIF2 in mammalian cells (Duncan and Hershey, 1984; Scorsone *et. al.*, 1987) and appears to be the main site of inhibition of protein synthesis during severe heat shock. However, as shown recently by Scheper *et. al.* (1997), substantial phosphorylation of eIF2 does not occur under conditions of mild heat shock. Cells heat-shocked above 41.5°C were found to have greatly reduced protein synthesis, reduced activity of eIF2B and a gradual increase in eIF2 phosphorylation. In cells heat-shocked at temperatures ranging from 40.5 to 41.5°C a comparable decrease in protein synthesis and eIF2B activity was observed without any changes in eIF-2 phosphorylation. eIF2B activity recovered very quickly in cells exposed to the mild heat shock temperature (40.5 or 41°C) while eIF2B activity was only partially recovered after four hours in more severely heat-shocked cells. These results are in agreement with other studies that have found that inhibition of eIF2B under mild heat

shock conditions is not caused by phosphorylation of eIF-2 (Mariano and Siekierka, 1986; Duncan and Hershey, 1989). The mechanism by which eIF2B is inhibited under mild heat shock conditions is still unknown and this mechanism probably inactivates eIF2B during more severe heat shock along with the phosphorylation of eIF2.

1.1.3.3.3. *Effect of Heat Shock on eIF4F Activity*

eIF4F is a large complex consisted of several initiation factors including eIF4E, eIF4G, and eIF4A. This complex binds to the 7-methylguanosine triphosphate (cap) structure found at the 5' end of most mRNAs. eIF4A is a RNA helicase and is important for the translation of mRNAs with substantial secondary structure in their 5'-untranslated regions. eIF4E is necessary for efficient cap binding and its activity is regulated through phosphorylation/dephosphorylation and through inhibitory binding proteins (4E-BPs). Heat shock leads to dephosphorylation of eIF4E resulting in a decrease in eIF4F activity (Duncan and Hershey, 1989). However, dephosphorylation of eIF4E does not appear to occur under mild heat shock conditions, only under more severe heat shock conditions (Duncan and Hershey, 1989; Scheper *et. al.*, 1997). The 4E-BPs bind to eIF4E blocking its availability for formation of the eIF4F complex which leads to the inhibition of protein synthesis. Phosphorylation of 4E-BP causes the dissociation of the eIF4E•4E-BP complex making eIF4E available for the formation of eIF4F, promoting translation. Scheper *et. al.* (1997) were surprised to find that heat shock led to an increase in 4E-BP phosphorylation even though protein synthesis was greatly decreased which indicated that 4E-BP activity did not necessarily regulate protein synthesis after heat shock. It seems that, much like eIF2B activity, other factors and/or events are involved in inhibiting translation during heat shock that have yet to be determined.

1.1.3.3.4. *Preferential Translation of Heat Shock Protein mRNA*

Heat shock can cause an almost entire shutdown of protein synthesis except for the heat shock proteins. Heat shock protein mRNAs appear to be resistant to inhibition of translation through a reduction in eIF4F and eIF2B activity. Joshi-Barve and co-workers (1992) developed a mammalian system that was deficient in eIF4E and found that protein synthesis continued at approximately 8% of control rate. Further analysis revealed that this protein synthesis was due to a small number of proteins, most of which were identified as Hsps. This study indicates that Hsp mRNAs have little or no requirement for cap-recognition machinery. This study and others have led some researchers to suggest that initiation of translation of Hsp mRNAs occurs through internal initiation where ribosomes bind at specific sequences in the 5'-untranslated region (5'-UTR), called internal ribosome entry sites (IRES), instead of entering at the 5' end. There has not been any conclusive evidence for this theory.

The 5'-UTR of *Drosophila* Hsp mRNAs have been studied in order to determine how preferential translation of Hsp mRNA is achieved during heat shock. The 5'-UTR of Hsp mRNAs are essential for their preferential translation during heat shock as deletion of most or all of this region prevents translation of heat shock mRNAs (Klemenz *et. al.*, 1985; McGarry and Lindquist, 1985). The sequence composition of 46-50% adenine makes secondary structure formation in the 5'-UTR of Hsp mRNAs very unlikely and inserting stable stem-loop structures into the cap-proximal region of the UTR inhibits translation of heat shock mRNAs during heat shock (Hess and Duncan, 1996). It appears that a lack of secondary structure in the 5'-UTR of Hsp70 is necessary, but probably not sufficient, for preferential translation of Hsp70 mRNA during heat shock (Hess and Duncan, 1996). The fact that stem-loop structures and a 5' extension of the Hsp70 5'-UTR decrease or inhibit translation is evidence against a cap-independent internal initiation mechanism. Researchers have attempted to identify important sequence elements in the 5'-UTR of Hsp mRNAs, but none have determined to date. However, it is believed that these sequences do exist. More studies are needed to determine how Hsp mRNAs are preferentially translated during heat shock.

1.1.3.4. *HEAT SHOCK PROTEINS AS MOLECULAR CHAPERONES*

Some stress proteins have been recognized as molecular chaperones (Table 1) (for review see: Ellis, 1987; Ellis and van der Vies, 1991; Gething and Sambrook, 1992). Molecular chaperones help other polypeptides to fold correctly while they are being synthesized in the ribosome, to direct proper re-folding after partial denaturation, and to transport other polypeptides to their final destination in the cell which may include allowing them to pass through biological membranes. The molecular chaperones have the ability to bind reversibly to polypeptides to facilitate or prevent their interaction with other polypeptides. They also act in the presentation of proteins to proteases for degradation. It is important to note that all molecular chaperones are not stress proteins and not all stress proteins are molecular chaperones. Cell proteins become vulnerable to denaturation and degradation during times of stress and the protective role of Hsps as molecular chaperones is thought to be one of their most important functions.

1.1.3.5. *CLASSIFICATION OF HEAT SHOCK PROTEINS*

The major heat shock proteins have been classified into six protein families identified by their molecular sizes: (i) large Hsps (proteins exceeding 100 kDa); (ii) Hsp90 family (proteins from 83 to 90 kDa); (iii) Hsp70 family (proteins from 66-78 kDa); (iv) Hsp60 family; (v) Hsp40 family; (vi) small Hsps (proteins from 15-30 kDa) (Leppa and Sistonen, 1997). The limits between families are not well defined and researchers in different disciplines may group families differently, however the families defined above serve as a guide to organize known and new information on Hsps. The following discussion will outline the major Hsps in each family with emphasis on the mammalian Hsps and their primary function(s) in normal and stressed cells (Table 1-1).

Table 1-1. Major heat shock proteins in mammals.

PROTEIN	LOCATION	FUNCTIONS/FEATURES
LARGE Hsps		
Hsp110	Cytosol, nucleus, Nucleolus	Molecular chaperone; thermotolerance.
Hsp90 FAMILY		
Hsp90	Cytosol	Stabilize inactive hormone receptors; interact with certain protein kinases; molecular chaperone.
Hsp70 FAMILY		
Hsp70 (inducible)	Cytosol, nucleus	Molecular chaperone; thermotolerance.
Hsc70 (constitutive)	Cytosol, nucleus	
Grp78	ER	Molecular chaperone; constitutive but slightly heat-inducible.
Grp75	Mitochondria	
Hsp60 FAMILY		
Hsp60	Mitochondria	Molecular chaperone; possible role in autoimmune diseases.
Hsp40 FAMILY		
Hsp40	Cytosol	Thermotolerance; molecular chaperone; associated with Hsp70 molecular chaperone activity.
SMALL Hsps		
Hsp28	Cytosol, nucleus	Molecular chaperone; thermotolerance

1.1.3.5.1. *Large Heat Shock Proteins*

Hsp110 is the most characterized protein in this family. In mammalian cells this protein is constitutively expressed and is present in the cytoplasm, nucleus, and nucleolus at low levels (Subjeck *et. al.*, 1983; Subjeck and Shyy, 1986). Hsp110 expression increases following heat shock, with much of the protein now being highly concentrated within the nucleolus near the region where ribosomal RNA genes are being transcribed (Subjeck *et. al.*, 1983). The functions of Hsp110 are not entirely understood, however Hsp104, the yeast homologue of mammalian Hsp110, has been shown to play a role in cell survival and acquired thermotolerance (Sanchez and Lindquist, 1990; Parsell *et. al.*, 1991). Recently, Oh *et. al.* (1997) observed that the overexpression of Hsp110 *in vivo* conferred substantial heat resistance on Rat-1 and HeLa cells. They also demonstrated that Hsp110 is highly efficient in selectively recognizing denatured proteins and holding them until refolding is favored.

1.1.3.5.2. Hsp90 Family

Hsp90 is one of the most abundant proteins in non-stressed mammalian cells (1-2% of cytosolic proteins) and its presence increases further following stress. Hsp90 has been researched extensively due to its association with a number of transcription factors and protein kinases (reviewed in Pratt and Toft, 1997; Pratt 1998). The role of Hsp90 in association with one of these transcription factors, the glucocorticoid receptor (GR), is of particular interest to this research due to the role of the GR in the hormonal response to stress.

1.1.3.5.2.1. Hsp90 and Glucocorticoid Receptors

Several laboratories were able to purify molybdate-stabilized progesterone and glucocorticoid receptors and the major protein purified was 90-kDa (for review see Pratt,

1992; Pratt, 1993). This 90-kDa protein was later identified to be Hsp90 (Catelli *et. al.*, 1985; Sanchez *et. al.*, 1985). Research indicates that the glucocorticoid receptor•Hsp90 complex consists of one molecule of steroid-binding protein associated with two molecules of Hsp90 (Denis *et. al.*, 1987; Mendel and Orti, 1988; Lefebvre *et. al.*, 1989).

Steroid receptor•Hsp90 heterocomplex assembly is required for hormonal regulation of receptor activity, but it is not clear how this regulation occurs. It is known that Hsp90 binds to the hormone-binding domain (HBD) of the GR (Pratt *et. al.*,1988; Denis *et. al.*, 1988). Hsp90 must be bound in order for the HBD to be in a high affinity steroid-binding conformation as it was found that in the absence of Hsp90 there was no binding of steroid by intact GR (Bresnick *et. al.*, 1989). Once steroid binds to the receptor•Hsp90 heterocomplex, Hsp90 dissociates from the GR (Mendel *et. al.*, 1986; Sanchez *et. al.*, 1987) and this transformation somehow allows the receptor to dimerize, bind to DNA and begin transcriptional activating activity.

Two other hsps have been identified as members of the GR complex, Hsp70 and Hsp56 (Wrange *et. al.*, 1984; Sanchez, 1990; Sanchez *et. al.*, 1990). Hsp70 has been shown to interact with the HBD (Scherrer *et. al.*, 1993) and is required for assembly of the receptor•Hsp90 heterocomplex (Hutchison *et. al.*, 1994a). Although Hsp70 remains associated with the receptor after steroid binding (Srinivasan *et. al.*, 1994), it is not required for nuclear import of the GR as shown by Yang and DeFranco (1994) who demonstrated that an antiserum against Hsp70 blocked nuclear import of SV40 large T antigen but not import of GR. Hsp56, now commonly referred to as FKBP52, was determined to be an immunophilin of the FK506 binding class (Yem *et. al.*, 1992; Tai *et. al.*, 1992). Immunophilins are proteins that bind immunosuppressive agents such as rapamycin and FK506 and they have peptidylprolyl isomerase activity suggesting a possible role in protein folding. The function of Hsp56 in the receptor heterocomplex has not been determined.

Hsp90, Hsp70, Hsp56, as well as other proteins have been shown to exist in heterocomplexes in the cytosol independent of steroid receptors (Sanchez *et. al.*, 1990; Perdew and Whitelaw, 1991). Hutchison *et. al.* (1994b) determined that all the factors required for GR•Hsp90 heterocomplex assembly were prebound to Hsp90. Using a monoclonal antibody, Hsp90 was immunoabsorbed from reticulocyte lysate. The washed

immunopellet was shown to contain Hsp90, Hsp70, and all other factors required for heterocomplex assembly and activation of steroid binding activity. It was concluded that the components required for assembly of the GR into a functional heterocomplex are preassociated with Hsp90 in a structure they have called a foldosome (Hutchison *et. al.*, 1994b).

An obvious question that emerges from these studies is the relationship between increased expression of Hsp90 mRNA and protein following stress and its association with steroid receptors. Although Hsp90 has been shown to increase following stress (Guerriero and Raynes, 1990; Wang and Edens, 1993), the effect of this increase on steroid receptor association with Hsp90 has not been studied. Most research on Hsp90 has focused on its function in the receptor•Hsp90 heterocomplex.

1.1.3.5.2.2. Hsp90 and heme-regulated eIF-2α kinase

Hsp90 interacts with the heme-regulated eIF-2α kinase called heme-regulated inhibitor (HRI) which is expressed in an erythroid-specific manner. Deficiencies in heme result in inhibition of protein synthesis in rabbit reticulocyte lysate. The heme deficiency activates HRI which phosphorylates the initiation factor eIF-2 on the α-subunit resulting in the inhibition of protein synthesis (Pratt and Toft, 1997). A 90-kDa protein, later identified to be Hsp90, was found to co-purify with HRI and Hsp90 was shown to have a stimulatory effect on HRI (Rose *et. al.*, 1987; Rose *et. al.*, 1989). It was suggested that Hsp90 may regulate HRI in a similar manner as the GR; HRI is inactive when complexed with Hsp90 and this complex dissociates when activated by heme deficiency. Furthermore, the HRI complex has been shown to contain Hsp70 and FKBP52 (Hsp56) (Matts *et. al.*, 1992) which suggests that the heterocomplex of Hsp90, Hsp70, and Hsp56 found in the cytosol is an important complex required to regulate protein complexes other than the steroid receptors.

1.1.3.5.3. *Hsp70 Family*

The Hsp70 family is one of the most studied due to the fact that one of its members, Hsp70, is highly heat-inducible and all organisms examined to date produce proteins from the Hsp70 family in response to elevated temperatures. For these reasons Hsp70 will be the focus of the research described in this thesis. Two important members of the mammalian Hsp70 family are the cytosolic proteins, Hsc70 and Hsp70. Hsc70, also referred to as Hsp73 or the Hsp70 cognate, is expressed constitutively in all cells and is slightly heat-inducible (Lindquist and Craig, 1988). Hsp70, also known as Hsp72, is not usually expressed in the non-stressed cell, but is highly inducible by heat shock and other cell stressors (Lindquist and Craig, 1988). Hsc70 and Hsp70 are very similar in biochemical properties and have a high sequence homology (~95%). Other Hsp70 family members include glucose-regulated protein (Grp)-78 which is found in the endoplasmic reticulum and the mitochondrial Grp75. Grp78 and Grp75 are constitutively expressed, slightly heat-inducible, and act as molecular chaperones in their respective cellular compartment (Macario, 1995).

The role of Hsp70 proteins in cellular trafficking of proteins was uncovered when researchers characterized a clathrin-uncoating ATPase that disassembled the clathrin cages of coated vesicles in an ATP-dependant reaction (Schlossman *et. al.*, 1984). The clathrin-uncoating ATPase was later identified as Hsc70 (Chappell *et. al.*, 1986). The Hsp70 proteins have since been found to be the main components of the chaperone system in almost all cellular compartments. Increased levels of Hsp70 during stress enable the cell to survive by binding to partially denatured proteins and directing their refolding or degradation once the stress has been removed. The constitutively expressed Hsp70 proteins (i.e. Hsc70, Grp78, Grp75) are essential for protein synthesis, translocation across membranes, folding, degradation, and assembly and disassembly of macromolecular complexes under normal physiological conditions (Nelson *et. al.*, 1992; Shi and Thomas, 1992; reviewed in Mayer and Bukau, 1998).

Hsp70 has been shown to be one of the main factors required for thermotolerance in mammalian cells. Cells exposed to elevated, but non-lethal, temperatures become transiently resistant to further exposure to heat shock temperatures, referred to as

acquired thermotolerance. The need for initial exposure to a heat stress to confer thermotolerance has been shown to be overcome in *Drosophila* by the overexpression of Hsp70 (Feder *et. al.*, 1992). Recently, Nollen *et. al.* (1999) investigated the chaperone function and involvement in heat resistance of Hsp70 in O23 hamster fibroblasts. OT cells, which are O23 cells that expressed the tetracycline-responsive tTA protein, were stably transfected with a plasmid encoding Hsp70 under the control of a tTA-regulated promoter. These OT70 cells were further transfected with a plasmid containing either a cytoplasmic or nuclear firefly luciferase gene regulated by a stress-inducible Hsp70 promoter. Some of these cells were exposed to an initial heat shock for the induction of thermotolerance. Others were grown in medium without tetracycline which induced Hsp70 expression alone at the same level as in the thermotolerant cells. Both groups of cells were then exposed to heat shock at 45°C. Similar levels of Hsp70 were measured in OT70 and thermotolerant cells, however, in the thermotolerant cells levels of Hsp25 and Hsp40 also increased. The level of recovery of cytoplasmic luciferase activity following heat shock was found to be the same in both cell types. However, Hsp70 expression alone was not sufficient to reach the level of recovery of nuclear luciferase activity following heat shock in thermotolerant cells. This suggested that for cytoplasmic chaperone activity, Hsp70 expression is sufficient, while nuclear chaperoning activity requires other factors, possibly including Hsp40, in addition to Hsp70. In addition, the ability of the OT70 and thermotolerant cells to survive heat treatments was compared. Cell survival was found to increase with increasing expression levels of Hsp70 alone, however, the OT70 cells were found to be more sensitive to thermal killing than the thermotolerant cells. This also suggested that other factors are required to fully protect the cell.

1.1.3.5.4. *Hsp60 Family*

Members of the Hsp60 family are called chaperonins. Hsp60 protein expression increases in response to heat shock and other stressors. The mammalian Hsp60 proteins are ring-shaped molecular chaperones that are synthesized in the cytoplasm and are then translocated to the mitochondria where they use the energy of ATP hydrolysis for protein

folding (for review see Gething and Sambrook, 1992; Welsh, 1992). Hsp60 has also been shown to cause antibody and T-cell immune responses during infections by bacteria and parasites and has been implicated in the development of certain autoimmune diseases due to T-cell cross reactivity to Hsp60 (reviewed in: Young, 1990; Jindal, 1996).

1.1.3.5.5. *Hsp40 Family*

The bacterial version of Hsp40, called DnaJ, has been extensively studied and has been found to act as a molecular chaperone in important cellular activities (Macario, 1995). DnaJ also associates with, and modulates the chaperoning activity of, DnaK (bacterial homologue of Hsp70) (reviewed in Ang *et. al.*, 1991). Recently, the yeast Hsp40 co-chaperones, Sis1 and Ydj1, have been shown to regulate Hsp70 protein folding activity (Lu and Cyr, 1998). In humans, Hsp40 is located in the cytosol and is believed to work in association with Hsp70 as a molecular chaperone (Macario, 1995). Hsp40 has been found to colocalize with Hsp70 into the nucleus after heat shock in HeLa cells (Hattori *et. al.*, 1993).

1.1.3.5.6. *Small Heat Shock Proteins*

Only a few members of this family have been found in mammalian and avian cells. One of these is referred to as Hsp28 although it may range from 25-30 kDa. Hsp28 is expressed constitutively at low levels in normal cells but increases 10- to 20-fold in the cell after stress (Welch, 1992). Hsp28 is normally present in the cytosol but much of the protein moves into the nucleus after heat shock and it is believed to act as a molecular chaperone (Macario, 1995).

1.1.3.6. Heat Shock mRNA and Protein Expression in Farm Animals

The expression of Hsp mRNA and protein has been observed in domestic farm animal species including cattle, horses, chickens, and turkeys. Most studies attempted only to characterize the nature of the stress response, not to measure a response to a particular stressor. Cultured bovine, ovine, equine and chicken lymphocytes have all been shown to respond to heat stress by increasing the expression of both Hsp70 and Hsp90 (Guerriero and Raynes, 1990). For each species, blood was collected and lymphocytes were isolated and suspended in medium minus methionine. Cells were incubated at 37°C or heat-stressed at 42°C (bovine and equine), 43°C (ovine), or 44°C (chicken). After heat stress, the lymphocytes were incubated in the presence of ^{35}S methionine at 37°C. Densitometric analysis of the western blots showed a 3.75-fold increase in Hsp70 synthesis for equine lymphocytes, 7-fold for ovine, 14-fold for bovine, and an estimated 20-fold increase in Hsp70 synthesis for chicken lymphocytes.

In earlier work with chicken reticulocytes and lymphocytes, Morimoto and Fodor (1984) found that chicken reticulocytes respond to increased temperatures by the induction of Hsp70, while chicken lymphocytes respond by the induction of Hsp89, Hsp70, Hsp23, and Hsp22. In both cell types the synthesis of other cellular proteins decreased dramatically. Along with a tissue-dependent pattern of expression, a temperature-dependent pattern of expression was observed. While optimal synthesis of each protein was induced after incubation at 45°C, elevated synthesis of Hsp70 and Hsp89 was first observed after incubation at 42°C. At 47°C only Hsp70 synthesis was detected.

In vitro and *in vivo* heat stress have been shown to induce the synthesis of Hsp90, Hsp70, and Hsp23 in turkey leukocytes (Wang and Edens, 1993). The *in vitro* heat-stressed leukocytes were incubated at temperatures from 37°C to 45°C for 30 minutes or at 43 or 45°C for 180 minutes. After 30 minutes, the synthesis of Hsps reached a maximum at 45°C with the concentration of Hsp90 and Hsp70 increasing 2.5- and 6.75-fold respectively, compared to the control cells at 37°C. Expression of other cellular proteins dropped rapidly during this time. At 43°C, Hsp90 and Hsp70 synthesis reached a maximum after 60 minutes, while only 30 minutes were required to reach the maximum

synthesis at 45°C. The *in vivo* heat-stressed turkeys were kept in a 43°C chamber for 120 minutes. Blood samples were taken at 30-minute intervals. Hsp90, Hsp70, and Hsp23 synthesis were induced by the acute heat stress with levels gradually increasing over the 120 minutes. The synthesis rate of other cellular proteins, represented by measurement of actin synthesis, was found to drop only slightly (5% decrease compared to 74% decrease for *in vitro* heat-stressed cells) over the same time period. This difference was attributed to other protective and homeostatic mechanisms of the body that were not present *in vitro*.

The expression of Hsp70 mRNA in leukocytes from heat-stressed broiler chickens has been studied (Wang and Edens, 1994). The birds were heat-stressed acutely at 41°C for 60 minutes. Blood samples were taken from the ulnar wing vein of the birds, peripheral leukocytes were isolated, and total cellular RNA was isolated and analyzed for Hsp70 mRNA by Northern blot hybridization. Hsp70 mRNA was found to increase significantly in peripheral leukocyte samples taken from the heat-stressed birds. Hsp70 mRNA was not detected in samples taken from birds held at a control temperature of 25°C. In the same experiment birds were exposed to 41°C for one hour per day for 0 (control), 1, 3, or 5 weeks. At six weeks all the birds were exposed to 41°C. Hsp70 mRNA increased significantly for the birds exposed for one and three weeks with Hsp70 mRNA reaching a maximum at three weeks. The birds exposed to five weeks of daily heat-stress had Hsp70 mRNA levels significantly higher than control birds, no different from 1-week exposed birds, and significantly lower than 3-week exposed birds. This may indicate that the chickens had acquired thermotolerance to the environmental conditions after five weeks of daily heat exposure.

Recently, similar work also by Wang and Edens (1998) has shown that peripheral leukocytes from broiler chickens and turkey poults synthesize Hsp90, Hsp70 and Hsp23 in response to *in vitro* and *in vivo* high temperature exposure. The *in vitro* heat-stressed lymphocytes had a greater increase in Hsp expression than in leukocytes heat-stressed *in vivo*, however, in both cases the increase was significant over control samples. As with the previous study, heat conditioning was found to enhance Hsp expression *in vitro* and *in vivo*. The extent of enhancement was much less *in vivo* than *in vitro*.

Gabriel *et. al.* (1996) investigated the synthesis of Hsp70 mRNA and the expression of Hsp70 in the liver of heat-stressed broiler chickens. Five birds were heat-stressed at 35°C for a total of five hours and one bird was kept at a control temperature of 25°C. After each hour, rectal temperatures were measured and one bird was killed. Analysis using western blots showed an increase in the amount of Hsp70 protein from the first up to the fifth hour with the maximum amount observed at five hours. Hsp70 mRNA increased slightly after the first hour and peaked at three hours, after which there was a decline in Hsp70 mRNA. The control bird was found to express a basal level of Hsp70 protein and mRNA. The rectal temperatures increased gradually each hour and the birds remaining at hours 4 and 5 were visibly suffering from heat stress.

A study to determine the relationship between the expression of Hsp70 and pork quality was performed by van Laack *et. al.* (1993). This was the first study to report stress protein expression in pigs. Four pigs of each genotype (NN, Nn, nn) for halothane sensitivity were transported for 35 minutes. Half of the animals were slaughtered immediately upon arrival (stressed group), while half were kept for 18 hours before slaughter. At 45 minutes *post mortem*, samples from cardiac and longissimus muscle, liver, spleen, kidney, and adrenal gland were collected. Hsp70 was found in all tissues examined and the expression in liver and skeletal muscle was greater than in other tissues. There was no effect of transport stress or genotype on the expression of Hsp70. There was no correlation between Hsp70 expression and meat quality. The lack of response due to transport may have been affected by the length of time between slaughter and sample collection.

McComb and Spurlock (1997) conducted a study to determine whether changes in Hsp expression in porcine tissues could be induced by an immunological challenge. *E. coli* lipopolysaccharide was injected into the *sterno hyoideus* muscle of the pigs in the immuno-challenged group. Phosphate buffered saline was injected into control pigs. After four hours all pigs were killed and tissue samples were taken. Hsc70 and Hsp60 were expressed in all tissues (heart, skeletal muscle, mid and hind brain, jejunum, liver, lung, and spiral colon). Hsp60 expression increased only in cardiac muscle and Hsc70 increased only in the jejunum. Hsp70 was found in liver, jejunum and spiral colon. Expression of Hsp70 in the liver did not change, however there was an increase in Hsp70

expression in the spiral colon and expression levels doubled in the jejunum. The authors concluded that Hsps are inducible *in vivo* and may be useful in evaluating the impact of disease and other stressors on the growth performance of commercially reared pigs.

There are a limited number of studies investigating the *in vitro* and *in vivo* response of bovine cells to stress as most studies have focused on the developmental changes in Hsp expression. Kochevar *et. al.* (1991) isolated lymphocytes, neutrophils, and macrophages from *Bos indicus* and *Bos taurus* cattle and heat-stressed the cells at 42°C. A 70kD protein (Hsp70) was enhanced in heat-stressed cells compared to control cells for all three cell types. *Bos indicus* cattle are generally considered to be heat tolerant compared to *Bos taurus* animals, however no significant differences were found between their overall pattern of Hsp expression. It was noted that certain cells from individuals within the *Bos indicus* group had a greater capacity to increase Hsp70 expression over non-stressed control levels which suggests that even within a breed certain individuals may possess a greater ability to respond to stress than others. This raises the question of what is the significance of increased Hsp70 expression? Compared to other animals of the same species facing the same stress, is an animal that has a greater capacity to increase Hsp70 expression more able to cope with a stress? Alternatively, is this animals' Hsp70 expression greater because it is less able to cope with a stress through other physiological and behavioural mechanisms? These questions have not been directly addressed in the literature, however, acquired thermotolerance data for Hsp70 may provide clues.

Hsp70 mRNA and protein has been found in bovine brain, heart, kidney, liver, lung, skeletal muscle, spleen, and testes (Gutierrez and Guerriero, 1995). Solution hybridization and ELISA assays were used to determine the levels of inducible Hsp70 protein and mRNA. Skeletal muscle was found to contain the highest amount of Hsp70 mRNA and protein, while brain tissue contained the lowest. Skeletal muscle was further studied by incubating tissue on ice, at 37°C, or 42°C. Incubating skeletal muscle on ice or at 37°C did not significantly increase Hsp70 mRNA or protein. Incubation of the muscle at 42°C for three hours caused a three-fold increase in Hsp70 mRNA and a two-fold increase in Hsp70 protein.

1.2. REVERSE TRANSCRIPTION – POLYMERASE CHAIN REACTION

Reverse transcription-polymerase chain reaction (RT-PCR) has become the method of choice in many labs for detecting and quantifying mRNA expression of a gene(s) in place of performing Northern blots and *in situ* hybridization. In RT-PCR, RNA is isolated from cells or tissues and then used as a template for reverse transcription of mRNA to complimentary DNA (cDNA). The cDNA now acts as the template for PCR using primers designed to amplify a selected cDNA region, known as the target sequence. The amount of PCR products generated increases exponentially during each PCR cycle until a plateau is reached. The PCR products are usually analyzed by gel electrophoresis and can be identified by the size of the PCR product, which is determined from knowledge of the nucleotide sequence of the gene of interest.

RT-PCR has several advantages over traditional methods of detecting mRNA. The biggest advantage of RT-PCR is its extreme sensitivity. It is possible to detect low abundance mRNAs using RT-PCR that would go unnoticed using traditional techniques. Due to the exponential nature of PCR, only a few molecules of initial target mRNA are required. In addition, mRNAs in small numbers of cells or from small amounts of tissue can be detected using RT-PCR. RT-PCR can be performed in half a day, while Northern blots can take two or more days.

The biggest advantage of RT-PCR can also be its greatest drawback. Due to the exponential nature of PCR, small variations in the efficiency of the RT and/or PCR reactions can lead to huge differences in the amount of PCR product generated from reaction to reaction. This can obscure measurements of initial amount of target sequence and make quantification of mRNA very difficult. Quantification can also be complicated if the PCR is allowed to continue to the point were the amount of PCR product generated reaches a plateau. The plateau effect occurs in the later stages of PCR when one or more of the required components is consumed and limits the reaction.

1.2.1. COMPETITIVE PCR

Competitive PCR uses an internal control in the PCR to compare the efficiency of the PCR in different reactions. This overcomes the problem of variations in efficiency during PCR. In principle, the internal control DNA fragment, or competitor DNA, and the target DNA compete for common substrates and are co-amplified during PCR in proportion to their concentrations. Variation in RT efficiency is not controlled for which leads to under-evaluation of the quantity of mRNA. For this reason competitive PCR is best used for determining relative quantitative differences in mRNA levels between samples. It is possible to introduce an internal control RNA in the RT to account for differences in efficiency during the RT. However, Babu *et. al.* (1993) found that if the RNA standard is added in disproportion to the target RNA, competition between the two templates is too great. A titration study must be performed to estimate the appropriate amount of standard RNA to be added to the RT. This increases the amount of RNA sample required and makes this approach unavailable when sample is limited. Also, Khan *et. al.* (1996) argue that the use of RNA standards do not necessarily accurately quantify mRNA because of inaccuracies in quantifying RNA and of maintaining of RNA integrity. Khan *et. al.* (1996) conclude that, if used appropriately, the use of a competitor DNA allows for meaningful quantification.

Many different variations of the competitive PCR method have been developed. Generally, each method can be grouped into one of the following types: (i) The internal standard is a homologous DNA fragment that has the same primer templates as the target sequence. The internal standard DNA fragment competes with the target DNA for the primers and is designed to generate a PCR product of a different size than the target DNA. It is important that the competitor and target DNA are amplified with equal efficiencies. Since the competitor and target DNA possess the same primer sequences, they are expected to amplify with equal efficiency (Siebert and Larrick, 1992). (ii) Another popular method is to use an additional set of primers to a gene product that is expressed invariably, referred to as a "house-keeping" gene. Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) and β-actin are two genes commonly used. The arguments against this method is that the mRNA levels of these genes do not remain constant under

all conditions and the additional primers can sometimes interfere with the PCR. (iii) The third method, and the one employed in this research, uses a non-homologous DNA fragment that has the same primer templates as the target DNA. In other words, the intervening sequence of the competitor DNA is completely different from the target DNA sequence. The target DNA and competitor DNA are expected to amplify with equal efficiency because they have identical primer templates. Amplification efficiency is believed to be primarily determined by the primer sequences providing that there are no substantial differences in denaturation or polymerase extension characteristics due to high G/C content or secondary structure (Siebert and Larrick, 1992; Raeymaekers, 1995). The competitor DNA used in this method is often referred to as a PCR MIMIC because it "mimics" the primer binding and amplification characteristics of the target.

The PCR MIMIC method has been used by numerous research groups to determine relative changes in mRNA levels of particular genes. Siebert and Larrick (1993) constructed a PCR MIMIC for IL-1β and tested its use in quantitative analysis of changes in IL-1β mRNA. To ensure that the amplification efficiencies of the MIMIC and target DNA were similar, a kinetic analysis of the amplification was performed. Equal quantities of IL-1β cDNA and IL-1β MIMIC were added to a single PCR and aliquots were removed for a total of seven cycles from cycle 24 to 30. They found that the target and MIMIC had very similar amplification kinetics. The IL-1β MIMIC was then used to measure changes in IL-1β mRNA. cDNA was synthesized from 0.5 and 2 μg of human lung total RNA, which imitated an induction of IL-1β mRNA. Competitive PCR was then performed using the Il-1β MIMIC and an average increase of 3.1-fold of IL-1β mRNA was measured. The measured value was close to the predicted 4-fold increase.

Abe *et. al.* (1995) constructed PCR MIMICs for Hsp72 and Hsc73. The right middle cerebral artery of rats was transiently occluded for 30 minutes. The rats recovered for 3 or 8 hours until decapitation. Sham control animals were decapitated immediately after cervical surgery. Total RNA was extracted from cerebral cortices of the rat brains and RT was performed to obtain Hsp72 and Hsc73 cDNA. After competitive PCR with the Hsp72 and Hsc73 MIMICs, the amount of Hsp72 mRNA was determined to be 20.2, 8.8, and 0.5 attomoles/g wet brain for 8-hour recovery, 3-hour recovery and sham control, respectively. The amount of Hsc73 mRNA was determined to be 6.8, 3.1, and 1.6

attomoles/g wet brain for 8-hour recovery, 3-hour recovery, and sham control, respectively. Northern blots were also performed on the same samples. A significant correlation between PCR-based intensities and optical densities detected by Northern blot were found for Hsp72 mRNA (correlation coefficient = 0.93) and Hsc73 mRNA (correlation coefficient = 0.94).

1.3. CONCLUSION AND OBJECTIVES

The relationship between the cellular heat shock response and endocrine responses to whole body stress is not understood although one can speculate that these responses are integrated at some level. Cortisol and other blood hormones have not proven useful as stress tests under field conditions since their blood level changes are transient and variable. Although they can indicate the presence and absence of stress they do not necessarily distinguish between mild, moderate, and severe stress. Stress proteins may provide an integrated measure or index of the overall severity of the stress and its potential impact on the health, welfare, and productivity of the animal. If the expression of heat shock proteins improves the ability of an animal to cope with stress, there may be genetic variations in their expression that contribute to variations in resistance to stress and disease.

Although research has shown that cattle produce heat shock proteins in response to a variety of stressors, the response has not been characterized as a means of evaluating the level of stress the animal is experiencing. The present study tested the following hypotheses: (1) Hsp70 mRNA expression will increase in bovine lymphocytes incubated at 42°C, but not at 38°C or 34°C; (2) Hsp70 mRNA expression will be much greater in bovine fibroblasts incubated at 45°C than at 38°C and Hsp70 mRNA expression at 42°C will be intermediate; (3) Hsp70 mRNA expression will increase in lymphocyte samples taken from cattle in response to handling and/or transport stress; (4) Hsp70 mRNA expression will vary among individual animals; (5) The use of the PCR MIMIC system will result in a reliable measurement of relative changes in Hsp70 mRNA expression.

The present study was undertaken in attempt to better characterize the expression of heat shock protein mRNA in cattle. The objectives of this study were:

1. **To develop a reliable assay for Hsp70 mRNA in bovine cells using RT-PCR technology.**

2. **To determine if the *in vitro* expression of Hsp70 mRNA in bovine cells is indicative of the level of stress, i.e. the degree of heat shock.**

3. **To determine if Hsp70 mRNA expression in an easily accessible cell (blood lymphocytes) will provide a reliable means of assessing stress in cattle during *in vivo* experiments.**

2. MATERIALS AND METHODS

2.1. ISOLATION AND HEAT-SHOCK OF LYMPHOCYTES

In order to develop a reverse transcription-polymerase chain reaction (RT-PCR) system for measuring bovine lymphocyte Hsp mRNA it was necessary to obtain blood samples from cattle. Lymphocytes were chosen for analysis because they were easily isolated from blood samples and blood sampling was minimally invasive. To obtain lymphocyte RNA, approximately 50 ml of blood was taken from a steer or heifer housed at the Metabolic Unit, Edmonton Research Station. The animal was brought into the squeeze and the blood was collected in 10 ml heparinized blood collection tubes and kept on ice for about 30 minutes until the samples were processed. Each blood sample was centrifuged at 1500 x g for 30 minutes at 4°C to prepare a "buffy coat". The plasma layer was discarded and the white layer containing the lymphocytes was removed. All the lymphocytes isolated from the 50 ml blood sample were then split between two sterile 15 ml centrifuge tubes. The cells in each tube were washed twice with 10 ml of phosphate-buffered saline containing 2% (v/v) fetal bovine serum (PBS/2% (v/v) FBS) (Appendix A) and centrifuged at 500 x g for 10 minutes at 4°C. Any contaminating red blood cells were destroyed by adding 10 ml of lysis buffer (Appendix A) and inverting on a rotator for 10 minutes at room temperature. The samples were then centrifuged for 10 minutes at 500 x g at 4°C. The cells were washed twice more with 10 ml PBS/2% (v/v) FCS. The lymphocyte pellets were suspended in 12 ml of RPMI 1640 medium each and placed in culture dishes. One sample was placed in an incubator at physiological temperature (38°C) while the other was placed in an incubator at 42°C for one hour. The cells were somewhat adherent to the bottom of the culture dishes and were gently agitated to free them. The suspended cells were then transferred to sterile 15 ml centrifuge tubes and centrifuged for 3 minutes at 500 x g and ready for RNA isolation.

2.1.2. Isolation of Total RNA from Bovine Lymphocytes

Total RNA was isolated from bovine lymphocyte samples using Trizol™ (Gibco BRL, Burlington, ON, Canada) according to the protocol provided by the manufacturer. Trizol is a total RNA isolation reagent consisting of a mono-phasic solution of phenol and guanidine isothiocyanate. Briefly, pelleted lymphocytes were resuspended in Trizol at a ratio of $3x10^6$ cells per ml and incubated at room temperature for 5 minutes. Chloroform was added (200 µl chloroform per ml Trizol added) and the samples were shaken vigorously for 15 seconds and then incubated at room temperature for 2-3 minutes. Samples were centrifuged for 15 minutes at 12000 x g at 4°C. The supernatant was put into a fresh tube and 500 µl of 100% isopropyl alcohol per ml Trizol was added and mixed. The samples were incubated at room temperature for 10 minutes and then placed in the freezer overnight at -70°C. The following day the samples were centrifuged for 35-45 minutes at 12000 x g at 4°C. The RNA pellet was washed with 1 ml of 75% (v/v) ethanol and centrifuged at 7500 x g at 4°C for 5 minutes. The RNA pellet was air dried for 8-10 minutes and dissolved in 20 µl of DEPC (diethylpyrocarbonate, 0.01% (v/v)) water. The amount of total RNA extracted from each sample was determined by reading the absorbance at 260 nanometers of the sample using the GeneQuant spectrophotometer (Pharmacia, Baie d'Uifé, Quebec). Quartz capillary tubes were used to measure a 3 µl aliquot of each sample. The integrity of the RNA samples were checked by gel electrophoresis for the presence of 18S and 28S bands (Appendix C). The samples were stored at -70°C.

2.2. QUANTIFICATION OF BOVINE HSP MRNA: METHOD DEVELOPMENT

2.2.1. RT-PCR USING HUMAN HSP PRIMERS

RT-PCR was used for the determination of expression of Hsp mRNA. PCR primer pairs were purchased (Stressgen Biotechnologies, Victoria, BC, Canada) for human Hsp70A, Hsp70B, Hsc70, Hsp90(beta) and Hsp27 (Table 2-1). It was believed that these primers would be suitable since there is high sequence homology between the human and bovine Hsp genes (Ang *et al.*, 1991; Grosz *et al.*, 1992).

All RT-PCR reactions were performed in a Perkin Elmer DNA Thermal Cycler using 0.5 ml GeneAmp™ PCR reaction tubes (Perkin Elmer). GeneAmp™ RNA PCR Kit (Perkin Elmer) was used to perform RT-PCR on heat-shocked HeLa cell RNA (human control; supplied with the kit) and on bovine lymphocyte RNA (control and heat-shocked samples) for each primer pair according to the protocol provided by the manufacturer. Briefly, reverse transcription was performed by adding the following components to each reaction tube and each tube was mixed and placed in the thermocycler for 15 minutes at 42°C, 5 minutes at 99°C, followed by 5 minutes at 5°C:

Volume	Component	Final Concentration
4 µl	$MgCl_2$ (25mM)	5 mM
2 µl	10xPCR Buffer II (500mM KCl, 100mM Tris-HCl, pH 8.3)	1 x
2 µl	dNTPs (10mM each)	1 mM
1 µl	RNase Inhibitor (20U/µl)	1 U/µl
1 µl	MuLV Reverse Transcriptase (50 U/µl)	2.5 U/µl
1 µl	Downstream (antisense) primer (15µM)	0.75 U/µl
x µl	sample RNA	1 µg
	add DEPC H_2O up to 20 µl	

A separate reaction was performed for each RNA sample and primer combination. Whenever possible, master mixes of components were used to minimize pipetting error.

At the completion of the reverse transcription reaction the following components were added to each reaction tube (master mix used where possible):

Volume	Component	Final Concentration
4 µl	$MgCl_2$ (25mM)	2 mM
8 µl	10xPCR Buffer II	1 x
66.5 µl	dd H_2O	
0.5 µl	AmpliTaq® DNA Polymerase (5 U/µl)	2.5 U/100µl
1.0 µl	Upstream (sense) Primer (15µM)	0.15 µM
= 80 µl + 20 µl RT reaction = 100 µl total volume		

Each reaction tube was briefly mixed and overlayered with approximately 50 µl of mineral oil. PCR was performed using amplification protocol A for primer pairs for Hsc70, Hsp90(beta), and Hsp27 (Table 2-1) and using amplification protocol B for primer pairs for Hsp70A and Hsp70B (Table 2-1).

Amplification Protocol A:	Amplification Protocol B:
Initial step: 95°C for 2 minutes	Initial step: 95°C for 2 minutes
35 cycles: 95°C for 30 seconds and	35 cycles: 95°C for 30 seconds and
50°C for 30 seconds	58°C for 30 seconds

PCR products were visualized by loading 20 µl of PCR product and 5 µl Orange G dye on a 1-1.2 % (w/v) agarose gel run in 0.0045 M Tris-borate, 1 mM EDTA (TBE) buffer (Appendix B) at 85 mA for 1.5 hours at room temperature. Low DNA Mass™ Ladder (Gibco BRL) was used as a size marker. Each gel was soaked in ethidium bromide solution (2.5 µg/ml) for 20 minutes. The Gel-Doc 1000 (BioRad Laboratories, Hercules, CA) was used to observe the PCR products and gel images were viewed and saved using Molecular Analyst® (Bio-Rad) software.

Table 2-1. Heat shock protein primer pairs used for analysis by RT-PCR.

Hsp Gene (species)	Primer Pair (5' → 3')* Sense (S) DNA strand primer and Antisense (AS) DNA strand primer	PCR Product Length (base pairs)
Hsp70A (human)	S: TGTTCCGTTTCCAGCCCCCAA AS: GGGCTTGTCTCCGTCGTTGAT	360
Hsp70B (human)	S: CTCCAGCATCCGACAAGAAGC AS: ACGGTGTTGTGGGGGTTCAGG	234
Hsc70 (human)	S: TGTGGCTTCCTTCGTTATTGG AS: GCCAGCATCATTCACCACCAT	342
Hsp90β (human)	S: GTCTGGGTATCGGAAAGCAAG AS: CTGAGGGTTGGGGATGATGTC	303
Hsp27 (human)	S: CACGAGGAGCGGCAGGACGAG AS: CAGTGGCGGCAGCAGGGGTGG	285
Hsc70 (bovine)	S: AAGATGCTGGAACTATTGCTGG AS: ATCAACCTCTTCAATGGTGG	1474
Hsp70-1 (bovine)	S: AGGACTTCGACAACAGGCTGGTGAA AS: CTCTTGTGCTCAAACTCGTCCTTCT	1098
Hsp70-2 (bovine)	S: TCATCAACGACGGAGACAAGCCTA AS: ATCGATGTCGAAGGTCACCTCGATCT	1165

* Human-derived primers were purchased from Stressgen Biotechnologies and bovine-derived primers were designed from bovine gene sequence information (DeLuca-Flaherty and McKay, 1990; Grosz and Skow, 1994)

2.2.2. RT-PCR Using Bovine Hsp Primers

PCR primers were designed from sequence data for Hsc70, Hsp70-1 and Hsp70-2 (DeLuca-Flaherty and McKay, 1990; Grosz and Skow, 1994) (Table 2-1) using primer design software, Gene Jockey 2.0 and Amplify 1.2. Primers for Hsc70, Hsp70-1 and Hsp70-2 were designed to amplify a 1474, 1098 and 1165 base pair (bp) sequence, respectively. All primer sequences were synthesized at the DNA Synthesis Lab, Department of Biological Sciences at the University of Alberta. The lengths of the PCR products using the bovine primers were beyond the recommended lengths for using the GeneAmp™ Kit. Expand™ Reverse Transcriptase and Expand Long Template PCR System (Roche) were used to perform RT-PCR on bovine RNA samples (control and heat-shocked) using the bovine primers for Hsc70, Hsp70-1, and Hsp70-2 according to the protocol supplied by the manufacturer. Briefly, sample RNA was denatured by adding the following components to each reaction tube, mixing briefly and placing each tube into the thermocycler for 10 minutes at 65°C and then cooling immediately on ice:

Volume	Component	Final Concentration
x µl	sample RNA	1 µg
5.83 µl	downstream primer (8.57µM)	50 pmoles
add DEPC H_2O up to 11.0 µl		

RT was performed by adding the following components as a master mix and placing each tube in the thermocycler for 60 minutes at 42°C and then cooling on ice:

Volume	Component	Final Concentration
4 µl	5 x Expand™ reverse transcriptase buffer (250mM Tris-HCl, 200mM KCl, 25mM $MgCl_2$, 2.5% Tween 20 (v/V), pH 8.3 (25°C))	1 x
2 µl	DTT (100mM)	10 mM
2 µl	dNTPs (10mM)	1 mM
1 µl	Expand™ Reverse Transcriptase (50 U/µl)	2.5 U/µl
20 µl	Total volume	

A separate reaction was performed for each RNA sample and primer combination. Following the reverse transcription reaction, PCR was performed using the Expand™ Long Template PCR System. The following components were added to each reaction tube using master mixes where possible:

Volume	Component	Final Concentration
1.75 µl	dNTPs (10mM)	350 µM
1.75 µl	upstream primer (8.57µM)	300 nM
1.75 µl	downstream primer (8.57µM)	300 nM
5 µl	template cDNA (from RT)	
5 µl	10 x PCR buffer-system 1 (17.5mM $MgCl_2$, system 2 (22.5mM $MgCl_2$), or system 3 (22.5mM $MgCl_2$ and detergents)	1.75 or 2.25 mM
0.75 µl	Expand Long Template enzyme (3.5 U/µl)	2.6 units
add dd H_2O up to 50 µl.		

Each reaction tube was mixed briefly and 50 µl of mineral oil was added as an overlay. PCR was performed using the following amplification protocol:

Initial step: 94°C for 2 minutes
10 cycles: 94°C for 30 seconds, 68°C for 30 seconds and 72°C for 90 seconds
15 cycles: 94°C for 30 seconds, 68°C for 30 seconds and 72°C for 90 seconds with 20 seconds added per cycle
Final step: 72°C for 7 minutes

All RT-PCR reactions were performed in a Perkin Elmer DNA Thermal Cycler using 0.5 ml GeneAmp™ PCR reaction tubes (Perkin Elmer). PCR products were visualized as previously described (see 2.2.1.).

2.2.3. Quantification of Relative Differences in Hsp mRNA

A competitive PCR method was chosen to quantify relative differences in bovine lymphocyte Hsp70 mRNA abundance between samples. This competitive PCR approach used a non-homologous internal standard known as a PCR MIMIC that consisted of a heterologous DNA fragment with primer templates that were recognized by gene-specific primers (Figure 2-1). The gene-specific primers had to meet certain requirements in order to be used in the construction of the PCR MIMIC. (i) *Size of the target gene amplified fragment.* The size of the PCR MIMIC can range from 200-650 base pairs. Differences in amplification efficiency between the PCR MIMIC and target gene are minimized when the size of the target and PCR MIMIC fragments are similar. However, at least a 150 bp difference in size is required in order to distinguish the two fragments on an agarose gel. (ii) *Properties of the primer pairs.* It was recommended that the gene-specific primers be 25-30 nucleotides in length, have a G/C content of 50-70%, and a melting temperature of at least 60°C. To meet these requirements new primers were designed for Hsc70 and Hsp70 from sequence data (DeLuca-Flaherty and McKay, 1990; Grosz and Skow, 1994) (Table 2-2). The Hsp70 primer pair was designed to amplify both Hsp70-1 and Hsp70-2 mRNA.

PCR MIMICs were constructed for Hsp70 and Hsc70 using PCR MIMIC™ Construction Kit (Clontech Laboratories, Inc., Palo Alto, CA) according to the manufacturer's protocol with a few modifications. Briefly, composite primers for Hsc70 and Hsp70 were designed (Table 2-2). These primers consisted of the target gene (i.e. Hsc70 or Hsp70) primer sequence attached to a 20-nucleotide segment designed to hybridize to opposite strands of the MIMIC DNA fragment (DNA fragment and sequence supplied with the kit). The composite primers for Hsc70 and Hsp70 were designed to amplify products of 650 base pairs. All primer sequences were synthesized at the DNA Synthesis Lab, Department of Biological Sciences at the University of Alberta.

Two rounds of PCR were required to construct the MIMICs. The first round of PCR used the composite primers to incorporate the target gene primer sequences onto the MIMIC DNA fragment (Figure 2-1). The following components were added to 0.5 ml GeneAmp™ reaction tubes:

Volume	Component	Final Concentration
37.4 µl	dd H_2O	
5.0 µl	10 x PCR Buffer 2 (2.25mM $MgCl_2$)	2.25 mM
1.0 µl	dNTPs (10mM each)	200 µM
4.0 µl	neutral DNA fragment	2 ng
1.0 µl	upstream Hsp70 or Hsc70 composite primer (20µM)	400 nM
1.0 µl	downstream Hsp70 or Hsc70 composite primer (20 µM)	400 nM
0.6 µl	Expand™ Long Template enzyme mixture (3.5 U/µl)	2.1 units
50 µl	final reaction volume	
+ 50 µl	mineral oil overlay	

The Expand™ Long Template enzyme was substituted for AmpliTaq® DNA Polymerase in the protocol. PCR was performed on the Perkin Elmer DNA Thermal Cycler according to the manufacturers protocol. Twenty cycles of 94°C for 45 seconds, 50°C for 45 seconds followed by 72°C for 90 seconds were performed. Upon completion, PCR products were visualized as previously described (see 2.2.1.) except 5 µl of PCR products were loaded on the gel instead of 20 µl. Strong bands of the expected size (650 bp) were observed (see results section 3.1.3, Figure 3-5) and construction of the MIMICs proceeded to the second round.

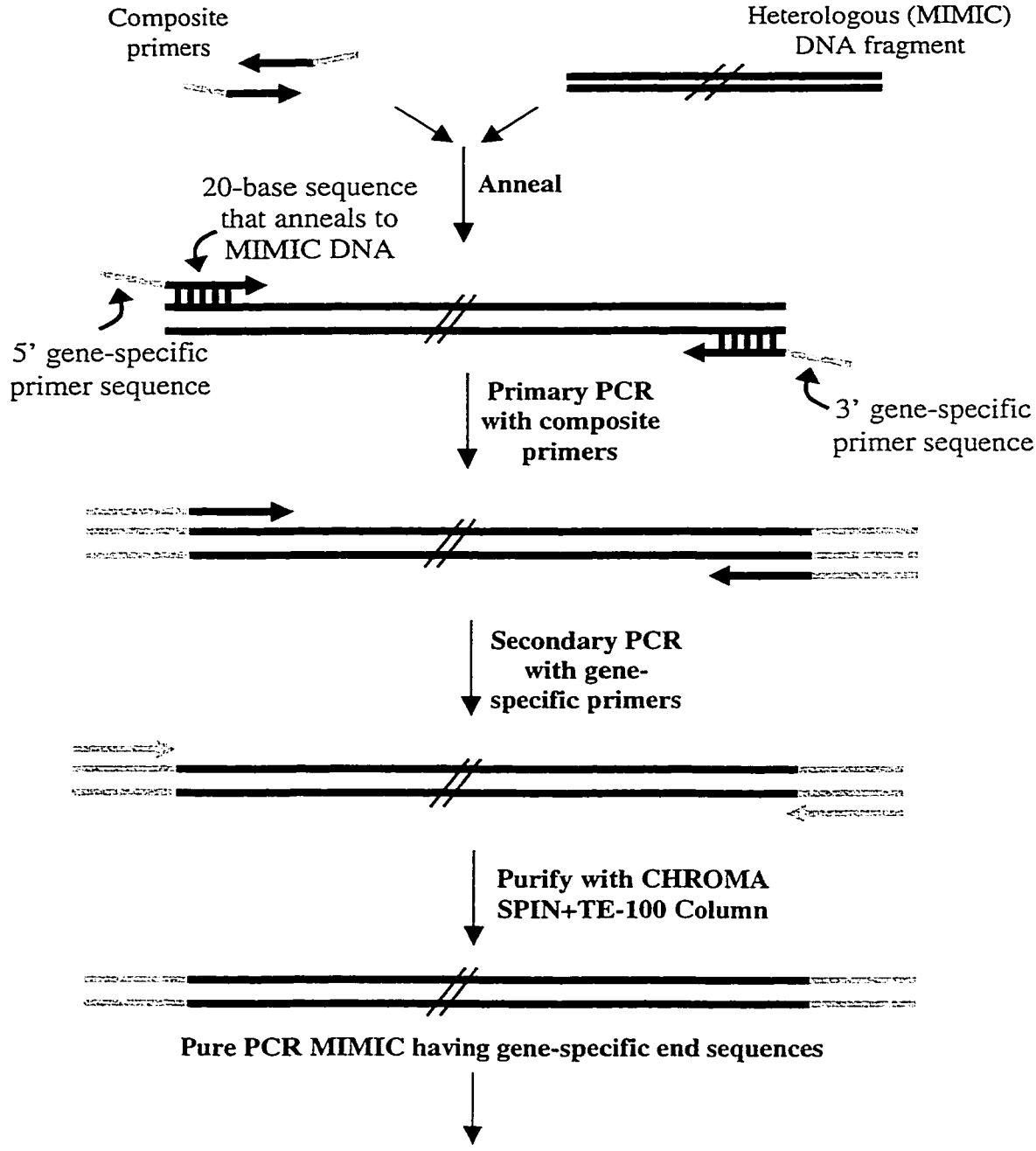

Figure 2-1. Construction of PCR MIMICs. The composite primers annealed to the MIMIC DNA fragment and generated PCR templates with gene-specific 5' and 3' ends. Subsequent PCR with the gene-specific primers generated many copies of this template. The resulting PCR MIMIC was purified using CHROMA SPIN+TE-100 Columns and yield was estimated by comparison of the intensity of electrophoretic bands generated by the PCR MIMIC against those generated by known quantities of size markers. (Adapted from Clontech PCR MIMIC™ Construction Kit User Manual)

Table 2-2. PCR primer pairs used for MIMIC construction and analysis for heat shock protein mRNA.

Hsp Gene	Primer Pair (5' → 3')** Sense (S) DNA strand primer and Antisense (AS) DNA strand primer	PCR Product Length (base pairs)
Hsc70 (bovine)	S: GGCACCCTGGACCCTGTGGAGAAGGCCCTTA AS: GGCATTCCCCCAGGCATGCCTCCTGCTGT	934
Hsp70 (bovine)	S: AGGGCGCGGTTCGAGGAGCTGTGCTCCGA AS: CAGCCAAGGTGTTGGCGTCCAGCCAGGAA	870
Hsc70 Composite Primers*	S: GGCACCCTGGACCCTGTGGAGAAGGCCCTTAC GCAAGTGAAATCTCCTCCG AS: GGCATTCCCCCAGGCATGCCTCCTGCTGTTTGA GTCCATGGGGAGCTTT	
Hsp70 Composite Primers*	S: AGGGCGCGGTTCGAGGAGCTGTGCTCCGAC GCAAGTGAAATCTCCTCCG AS: CAGCCAAGGTGTTGGCGTCCAGCCAGGAAT TGAGTCCATGGGGAGCTTT	

* Underlined nucleotides indicate the sequence of the gene-specific primers included in the composite primer sequences.

** Hsp70 and Hsc70 bovine primers derived from gene sequence information (DeLuca-Flaherty and McKay, 1990; Grosz and Skow, 1994) and composite primers derived from sequence information of the MIMIC DNA fragment supplied with the PCR MIMIC Construction kit.

The second round of PCR was performed to ensure that all PCR MIMIC molecules had the entire gene-specific primer sequence and to amplify copies of the MIMICs (Figure 2-1). The components for the second round of PCR were added to the reaction tubes as follows:

Volume	Component	Final Concentration
75.74 µl	dd H_2O	
10 µl	10 x PCR Buffer 2 (2.25mM $MgCl_2$)	2.25 mM
2 µl	dNTPs (10mM each)	200 µM
2 µl	100x dilution of primary PCR reaction	
4.68 µl	upstream Hsp70 or Hsc70 primer (20µM)	400 nM
4.68 µl	downstream Hsp70 or Hsc70 primer (20µM)	400 nM
0.9 µl	Expand™ Long Template enzyme mixture(3.5 U/µl)	3.2 units
100 µl	final reaction volume	
+ 50 µl	mineral oil overlay	

PCR was performed according to the manufacturer. Twenty cycles of 94°C for 45 seconds, 50°C for 45 seconds followed by 72°C for 90 seconds were performed. PCR products were visualized as described and once again strong product bands of the expected size were observed (see Results 3.1.3., Figure 3-5).

Each PCR MIMIC was purified by passing each of two 45 µl aliquots of the secondary PCR reaction through separate CHROMA SPIN+TE-100 columns that were supplied with the kit. The columns were inverted to resuspend the gel matrix and placed into one of the 17 x 100 mm polypropylene tubes provided. The columns were centrifuged for 3 minutes at 700 x g. The column was removed from the tube and the buffer discarded. The tip of the column was placed in a microcentrifuge tube inside the polypropylene tube. The 45 µl aliquots of the secondary PCR reaction (two aliquots for each reaction) were applied to the centre of the gel bed flat surface and centrifuged for 5 minutes at 700 x g. The purified MIMICs were verified by gel electrophoresis as previously described.

The yield of each PCR MIMIC was estimated by comparison of intensity of electrophoretic bands. Three concentrations of the 100 ng/µl ϕX174/*Hae* III digest DNA supplied with the kit were used as DNA size markers. Five microlitres of each PCR

MIMIC and the three concentrations (100, 200, and 400 ng) of the DNA size marker were run on a 1% (w/v) agarose gel run in 0.045 M Tris-borate, 1 mM EDTA (TBE) buffer for 1.5 hours at room temperature. The gel was stained with ethidium bromide (2.5 µg/ml) for 20 minutes. PCR products were observed using the Gel-Doc 1000(Bio-Rad). For each MIMIC the ϕX174 DNA band that was close to the intensity of the MIMIC was chosen. By knowing the amount of ϕX174 DNA loaded in that lane and the ratio of the size of the band with respect to the total size of the ϕX174 DNA (5386 bp), the yield in nanograms of PCR MIMIC per microlitre could be estimated. The yield of the MIMIC was estimated by the equation:

$$\text{ng PCR MIMIC/µl} = \frac{[\text{size of } \phi\text{X174 DNA fragment (bp)}][\text{amount of } \phi\text{X174 DNA (ng)}]}{[\text{sum of all } \phi\text{X174 DNA fragments (bp)}][\text{vol. of MIMIC loaded (µl)}]}$$

Nanograms was converted to molar quantities and the concentration of the PCR MIMICs were expressed in attomoles/µl (1 attomole = 10^{-18} moles). The Hsc70 and Hsp70 PCR MIMICs were diluted to a concentration of 100 attomole/µl with the MIMIC dilution solution provided with the kit.

Conditions for RT-PCR using the new Hsc70 primers could not be optimized using the Expand™ RT-PCR system because of the presence of non-specific product bands. The gene-specific primers were designed to anneal only to a specific sequence of mRNA of the Hsc70 gene (i.e. target sequence). The RT temperature was too low and the primers were able to anneal to other sequences of mRNA that were similar in sequence but not identical to the target sequence. This resulted in the amplification of PCR products other than products corresponding to Hsc70, referred to as non-specific products. Tth DNA polymerase (Roche) allowed for a higher reverse transcription temperature and this enzyme system was used to optimize RT-PCR reactions using primers for Hsp70, however conditions for the Hsc70 primers still could not be optimized using this system (see Results, Figure 3-11). Further work with Hsc70 primers was put on hold due to time constraints.

Before analysis with the Hsp70 MIMIC continued, the sensitivity and linearity of the amplification with the Hsp70 primers was tested. Reverse transcription reactions were performed using 200 ng total RNA in each reaction (see below). The RNA sample used

was from bovine lymphocytes incubated at 38°C (control). PCRs of 15, 20, 25, 30, 35, or 40 cycles were performed (see below). Furthermore, RT-PCR was performed with 100, 200, or 400 ng RNA and 35 cycles of PCR. PCR products were visualized as described. The ability of the Hsp70 primers to detect differences in Hsp70 mRNA expression in total RNA taken from bovine lymphocytes incubated at the control temperature (38°C) or heat shock temperature (42°C) was also tested. RT-PCR was performed on the control and heat-shocked RNA samples. Reverse transcription was performed using Tth DNA polymerase according to the two-step RT-PCR protocol provided by the manufacturer as follows:

Volume	Component	Final Concentration
10.3 µl	DEPC H_2O	
2.0 µl	10xRT Buffer (100mM Tris-HCl, pH 8.9, 900mM KCl)	1 x
2.0 µl	$MnCl_2$ (9 mM)	0.9 mM
3.5 µl	Hsp70 antisense primer (8.57µM)	750 nM
0.4 µl	dNTPs (10mM each)	200 µM
1.0 µl	RNA (or equivalent to 200ng)	200 ng
0.8 µl	Tth DNA Polymerase (5 U/µl)	4 units
20.0 µl Total volume		

Each tube was incubated for 30 minutes at 60°C in the thermocycler. Then 80 µl of the PCR master mix was added to each 20 µl RT reaction and overlayered with 50 µl of mineral oil. The PCR master mix was prepared as follows:

Volume	Component	Final Concentration
8.0 µl	10xPCR Buffer (100mM Tris-HCl, pH 8.9, 1M KCl, 15mM $MgCl_2$, 500 µg/µl bovine serum albumin, 0.5% Tween 20 (v/v))	0.8 x
10.0 µl	EGTA (7.5 mM)	0.75 mM
3.5 µl	Hsp70 sense primer (8.57 µM)	150 nM
58.5 µl	dd H_2O	
80.0 µl	total volume	

PCR was performed using the following amplification protocol:

Initial step: 94°C for 2 minutes
15 cycles: 94°C for 60 seconds, 65°C for 60 seconds and 72°C for 90 seconds
20 cycles: 94°C for 30 seconds, 65°C for 60 seconds and 72°C for 90 seconds with 5 seconds added per cycle
Final step: 72°C for 5 minutes

The results were visualized as previously described. Gel images were viewed and saved using Molecular Analyst® (Bio-Rad) software.

The amount of Hsp70 MIMIC to be added to each PCR reaction was determined for each experiment. This was done by making a series of ten-fold dilutions of the 100 attomole/µl stock solution from 10 to 10^{-4} attomole/µl. Six separate RT-PCR reactions were performed using an RNA sample that represented a control sample for a particular experiment (e.g. RNA from lymphocytes incubated at 38°C). The RT-PCR reactions were performed as described above except 2 µl of Hsp70 MIMIC was added to the PCR master mix in the place of water. The results were visualized as described. Gel images were viewed and saved using Molecular Analyst® software.

The MIMIC dilution that produced Hsp MIMIC and target Hsp bands of approximately equal intensity was chosen. In order to more closely estimate the MIMIC dilution that resulted in target and MIMIC bands of equal intensity, two-fold dilutions of the MIMIC dilution ten-fold less dilute than the one chosen were made. For example, if the 10^{-4} dilution resulted in MIMIC and target bands of equal intensity, the two-fold serial dilution series were begun with the 10^{-3} dilution. RT and PCR were performed in the same manner as above. The results were visualized as described. The two-fold MIMIC dilution that produced MIMIC and target bands of equal intensity was determined. This dilution of MIMIC was used in the RT-MIMIC PCR analysis of relative abundance of Hsp70 mRNA in the lymphocyte RNA samples from control (38°C) and heat-shocked (42°C) bovine lymphocytes using the same RT-PCR protocol as above. PCR products were visualized as described.

The Gel-Doc 1000 was used to observe the PCR products on the gel and gel images were viewed and saved in Molecular Analyst®. Using the profile option in Molecular Analyst it was possible to determine the area (counts*mm) of all the bands on

the gel. Electrophoresis of the Low DNA Mass™ ladder results in bands containing a known concentration of DNA. Standard curves of DNA concentration versus area were plotted from this information (Appendix D). The concentration of Hsp70 target and Hsp70 MIMIC bands were calculated using the equation generated by the standard curve. The relative abundance of Hsp70 mRNA was determined by calculating the ratio of Hsp70 DNA concentration to Hsp70 MIMIC DNA concentration.

2.3. *IN VITRO* THERMAL SHOCK OF BOVINE LYMPHOCYTES

An *in vitro* thermal shock experiment was performed on lymphocyte samples isolated from blood samples taken from six different steers. The steers were housed at the Metabolic Unit, Edmonton Research Station under normal management conditions. Each animal was brought into the squeeze (one animal per day) and an approximately 300 ml blood sample was taken by jugular venipuncture. The blood sample was immediately taken to the lab for lymphocyte isolation. Using sterile techniques, 8 ml of blood was layered over 12 ml of Histopaque-1077 (Sigma) in 50 ml centrifuge tubes. A total of sixteen tubes were used for each animal. The tubes were centrifuged at 1000 x g for 30 minutes at room temperature with no brake in order to prevent the layers formed from re-mixing. The lymphocyte band was recovered and placed in a new sterile 50 ml centrifuge tube. The lymphocytes were washed twice by adding 12 ml of 2% (v/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS) (Appendix A) and centrifuging at 1000 x g for 10 minutes at room temperature. The supernatant was discarded and the lymphocyte pellet was resuspended in 4 ml of complete culture media (CCM) (Appendix A). The lymphocytes were then diluted to $3x10^6$ cells/ml with CCM and placed in sterile culture dishes. Five samples were placed in each incubator at 38°C (physiological temperature), 42°C (heat-stress), and 34°C (cold-stress). One sample was removed from each incubator at 30 minutes, 1, 2, 4, and 24 hours. At each time point, the lymphocytes were pelleted and total RNA was extracted using Trizol™. In addition, one lymphocyte sample from each animal was not incubated and RNA was extracted immediately after lymphocyte isolation. All RNA samples were frozen at -70°C until analysis of Hsp70 mRNA was performed by RT-PCR with PCR MIMIC.

2.4. *IN VIVO* CONTROLLED ENVIRONMENT EXPERIMENTS

2.4.1. EXPERIMENT ONE - TEMPERATURE EXPOSURE

A large collaborative study was conducted to determine the effect of environmental temperature and feeding level on various stress measurements on yearling heifers. The research presented in this thesis is only a small part of a larger experiment involving collaboration with H. Harrison, Graduate Student, Laval University, Dr. S.L. Scott, Agriculture and Agri-food Canada, Brandon, Manitoba, Dr. A.D. Kennedy, University of Manitoba, Dr. A.L. Schaefer, Agriculture and Agri-food Canada, Lacombe, and Dr. R.J. Christopherson, University of Alberta (unpublished).

A total of 27 heifers were subdivided evenly into subgroups (n=3) and assigned to one of the following treatment groups:

WR: warm environment (18°C) and restricted feed intake (1.3 x maintenance)
WAL: warm environment (18°C) and unrestricted feed intake
CAL: cold environment (-18°C) and unrestricted fee intake

The experiment consisted of three trials with three animals from each group per trial. Two trials were conducted during the summer and another in November of 1995 at the Metabolic Unit, Edmonton Research Station. In each trial, nine cross-bred heifers (body weight ranging from 325-450 kg) were initially housed in a pre-treatment pen to which they were adapted prior to movement into either of two new pens in a thermoneutral (18°C) or in a cold environment (-18°C). The heifers in all treatment groups were fed a pelleted alfalfa-based ration (75% alfalfa, 25% barley) with an estimated 88.7% DM, 2.87 MJ NE.kg^{-1} DM, and 12% CP content.

Blood samples were collected from each animal in each treatment group before and 1-2 hours after movement to their new pen. At each collection time, the animals were brought into the squeeze and approximately 50 ml of blood was collected into five 10 ml heparinized tubes by jugular venipuncture. Lymphocytes were isolated using the method outlined in section 2.1 and frozen at -70°C for future analysis of Hsp70 mRNA by RT-

MIMIC PCR according to the method developed in section 2.2.3. Blood plasma was also collected and frozen at -70°C. Cortisol, T_3, and T_4 assays were performed on blood plasma samples by radioimmunoassay using Coat-a-Count kits (Diagnostic Products Corporation, Los Angeles, CA) after validation with bovine plasma.

2.4.2. Experiment Two - Transport Stress

After the heifers had acclimated to their treatments for three weeks, all the cattle were shipped to slaughter. Blood samples were taken prior to loading for shipment and after unloading at the slaughter plant. The animals were put into a squeeze and approximately 50 ml of blood was collected into five 10 ml heparinized tubes by jugular venipuncture. Lymphocytes were isolated using the method outlined in section 2.1 and frozen at -70°C for future analysis of Hsp70 mRNA by RT-MIMIC PCR according to the method developed in section 2.2.3. Blood plasma was also collected and frozen at -70°C. Cortisol, T_3, and T_4 assays were performed on blood plasma samples. The other research group involved in the experiment took various other measurements, two of which, recordings of core body temperature by radiotelemetry and neutrophil-to-lymphocyte ratios from blood samples, were included with this study.

2.5. *IN VITRO* HEAT SHOCK OF BOVINE FIBROBLAST CELL-LINE

Bovine fibroblasts stored in liquid nitrogen were thawed and cultured at 38°C until the cells became confluent. At this time, three large culture flasks containing the bovine fibroblasts was transferred to a 42°C incubator, three to a 45°C incubator, and one flask remained at 38°C. One flask was removed from both the 42°C and 45°C temperatures at 30 minutes, one, and two hours. The flask incubated at 38°C was removed after two hours. The media was poured from each flask and total RNA was extracted from the cells using 7 ml of Trizol™/flask. RT-PCR with PCR MIMIC was performed on the RNA samples to determine relative differences in Hsp70 mRNA abundance as described. These reactions were carried out using a different thermocycler than the one used in the previous experiment. A Perkin Elmer 2400 thermocycler was used. The reactions were performed in 0.2 ml thin-walled PCR reaction tubes (Perkin Elmer) for 35 cycles using the same components and volumes as previously described. The only exception being that mineral oil was not necessary as an overlay in these PCR reactions.

2.6. STATISTICAL ANALYSIS

Statistical analyses were performed by SAS (Version 6.12, SAS Institute, Cary, N.C.). The level of significance was set at $p<0.05$. Unpaired student t-test was used to determine differences between treatments.

3. RESULTS

3.1. QUANTIFICATION OF BOVINE HSP MRNA: METHOD DEVELOPMENT

3.1.1. RT-PCR USING HUMAN HSP PRIMERS

RT-PCR was performed using the GeneAmp™ RNA PCR kit (Perkin Elmer) with the human-derived primer pairs for Hsp70A, Hsp70B, Hsc70, Hsp90(beta), and Hsp27 on RNA from heat-shocked HeLa cells (human control). Bands of the appropriate size corresponding to each primer pair were observed. RT-PCR was performed using the same primer pairs on total RNA from control (38°C) and *in vitro* heat-shocked (42°C) bovine lymphocytes. Bands were not seen for primers Hsc70, Hsp70A, Hsp90B, and Hsp27. A faint band appeared for both the control and heat-shocked bovine RNA samples when the Hsp70B primer pair was used, however the band was not of the appropriate size.

3.1.2. RT-PCR USING BOVINE HSP PRIMERS

RT-PCR was performed on bovine control (38°C) and *in vitro* heat-shocked (42°C) lymphocyte RNA using the bovine Hsc70 primer pairs and Expand™ Reverse Transcriptase and Expand™ Long Template PCR System (Roche). Product bands of the expected size corresponding to Hsc70 (1474 bp) were observed for both the control and *in vitro* heat-shocked bovine lymphocyte RNA (Figure 3-1). RT-PCR analysis of control and *in vitro* heat-shocked lymphocyte RNA for Hsc70, Hsp70-1, and Hsp70-2 was performed. The lymphocyte RNA in this analysis originated from a different steer than in the previous analysis for Hsc70 mRNA. Product bands of the expected sizes corresponding to Hsc70 (1474 bp), Hsp70-1 (1098 bp), and Hsp70-2 (1165 bp) were observed for the control (38°C) and *in vitro* heat-shocked (42°C) bovine lymphocyte

RNA (Figure 3-2). PCR product bands corresponding to Hsp70-1 and Hsp70-2 mRNA expression were more pronounced in the heat-shocked sample than in the control sample. A smaller prominent product band (approximately 900 bp) appearing below Hsp70-1 was also more pronounced after heat shock. This band may correspond to another closely related gene, perhaps a heat shock protein, amplified by the Hsp70-1 primers.

Two sets of lymphocyte RNA samples from the cattle involved the *in vivo* controlled environments were analyzed for Hsc70, Hsp70-1, and Hsp70-2 mRNA expression to further test the RT-PCR system. One set consisted of lymphocyte RNA from blood samples taken before and after transport (transport), and the other set from before and one-hour after movement to a thermoneutral temperature pen in a new room (movement). Hsc70 mRNA was detected in only one of the four samples. Analysis of the sample taken one hour after movement to a new pen resulted in a strong band of the expected size corresponding to Hsc70 (Figure 3-3). Detection of Hsp70-1 mRNA expression in these samples was not successful. Hsp70-2 mRNA expression was not detected in samples taken before movement or before transport (Figure 3-4). Hsp70-2 mRNA expression was present in samples taken after movement or after transport (Figure 3-4).

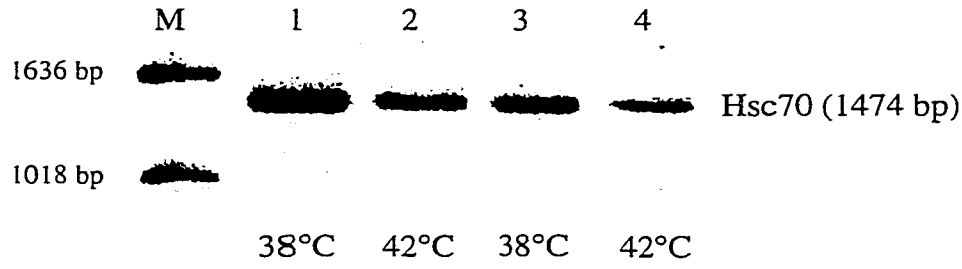

Figure 3-1. RT-PCR analysis of control (38°C; lanes 1 and 3) and *in vitro* heat-shocked (42°C; lanes 2 and 4) bovine lymphocyte RNA for Hsc70 mRNA. The PCR reaction volume was 100 µl in lanes 1 and 2, and 50 µl in lanes 3 and 4.

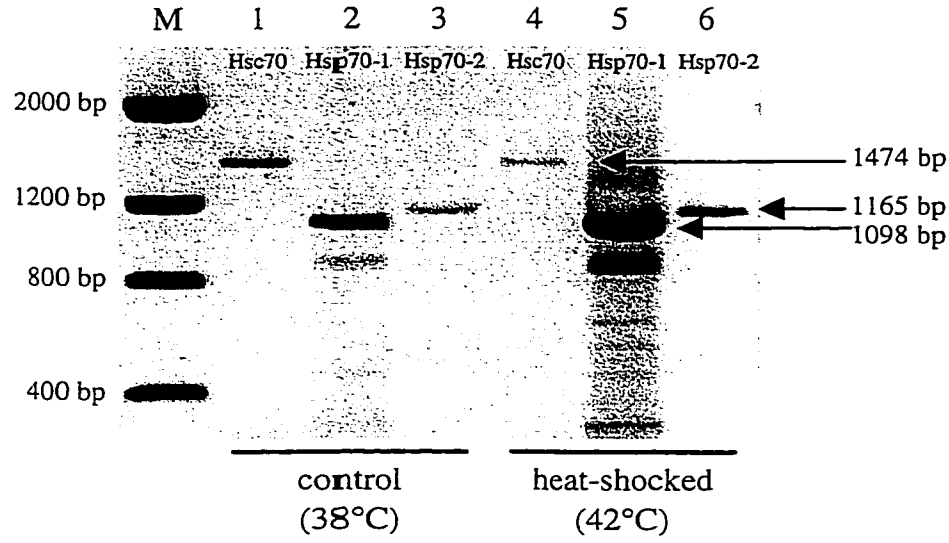

Figure 3-2. RT-PCR analysis of control (38°C) and *in vitro* heat-shocked (42°C) bovine lymphocyte RNA for Hsc70 (lanes 1, 4; 1474 bp), Hsp70-1 (lanes 2, 5; 1098 bp) and Hsp70-2 (lanes 3, 6; 1165 bp) mRNA.

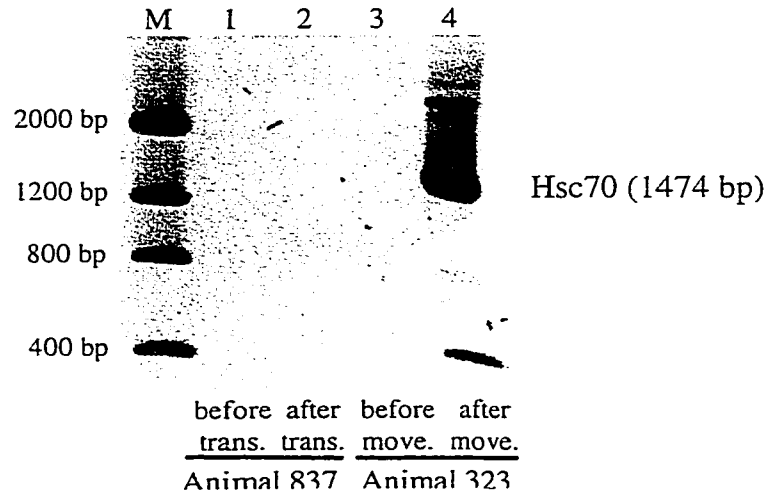

Figure 3-3. Expression of Hsc70 mRNA in lymphocyte samples taken from animal 323 before and after movement to a new pen (lanes 1 and 2), and from animal 837 before and after transport for one hour (lanes 3 and 4).

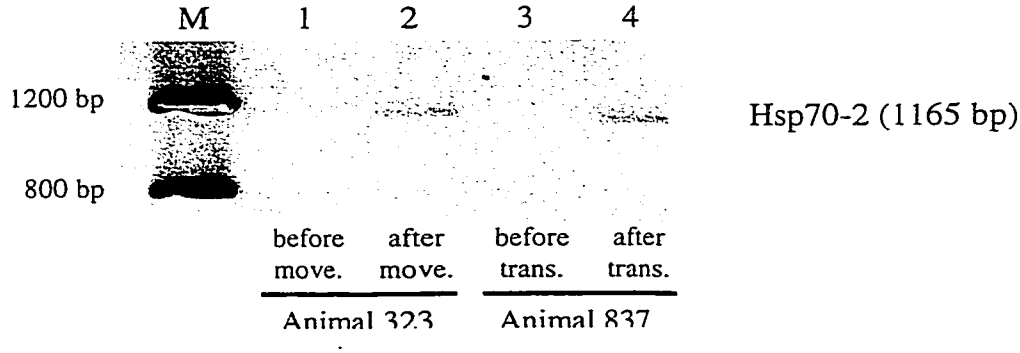

Figure 3-4. Expression of Hsp70-2 mRNA in lymphocyte samples taken from animal 323 before and after movement to a new pen (lanes 1 and 2), and from animal 837 before and after transport for one hour (lanes 3 and 4).

3.1.3. Quantification of Relative Differences in Hsp mRNA Using PCR MIMICs

PCR MIMICs were successfully constructed for bovine Hsp70 and Hsc70. The target gene primer sequences were incorporated into the MIMIC DNA fragment during the first round of PCR (Figure 3-5a). A larger product band also appeared, however this band was not seen after the final MIMIC product was purified (see below). The second round of PCR resulted in the amplification of copies of the MIMIC fragments (Figure3-5b). Purification of the PCR MIMICs removed any remaining primers and reaction components (Figure 3-6). The Hsp70 primers generated a band that was slightly larger than the Hsp70 MIMIC band. This band was not expected to interfere in the analysis of Hsp70 mRNA expression. The estimated yield of each MIMIC was calculated to be 4.685×10^4 attomole/µl (Figure 3-7).

The sensitivity and linearity of the amplification with the Hsp70 primer set was tested. A band at the expected size (870 bp) was detected after 30, 35, and 40 cycles of PCR and the intensity of the PCR products increased in proportion to the increase of PCR cycles (Figure 3-8). Similarly, the intensity of Hsp70 product bands increased with an increase in the amount of RNA (100, 200, or 400 ng) added to the RT reaction (Figure 3-9). The bovine Hsp70 and Hsc70 primers were tested in RT-PCR reactions (without MIMIC) to detect Hsp70 and Hsc70 mRNA in RNA samples from heat-shocked and control bovine lymphocytes. PCR product bands corresponding to Hsp70 were more prominent from the heat shocked samples indicating that expression of Hsp70 mRNA in the *in vitro* heat-shocked (42°C) lymphocyte RNA was greater than in a RNA sample from lymphocytes incubated at control temperature (38°C) (Figure 3-10). Hsc70 PCR product bands were more prominent in the heat-shocked samples than in control samples (Figure 3-11) indicating an increase in Hsc70 mRNA expression. An internal control was not present in the assay for Hsp70 and Hsc70 mRNA, so these results could not be quantified. The Hsc70 primers generated non-specific products that would interfere with quantification using the PCR MIMIC (Figure 3-11). Attempts to increase the specificity of synthesis of the desired product by increasing the RT and annealing temperatures were unsuccessful. Further attempts to optimize the reaction for Hsc70 were not pursued.

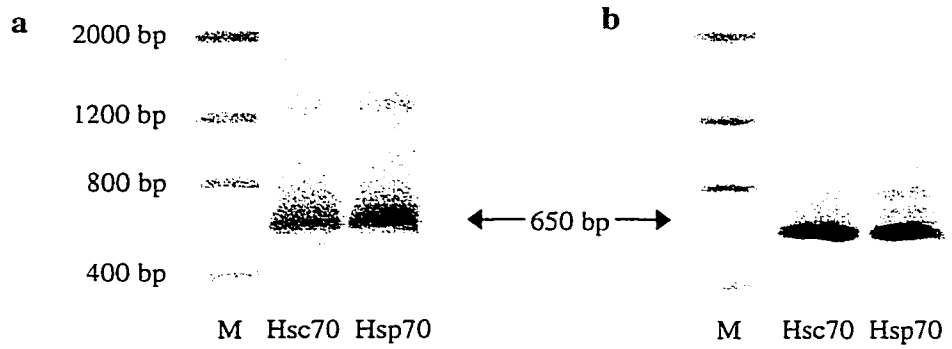

Figure 3-5. First (**a**) and second (**b**) rounds of PCR in the construction of the Hsp70 and Hsc70 PCR MIMICs. **a**: The target gene primer sequences were successfully incorporated into the MIMIC DNA fragments using the composite primers. **b**: Amplification of copies of the MIMIC DNA fragments with the gene-specific primers.

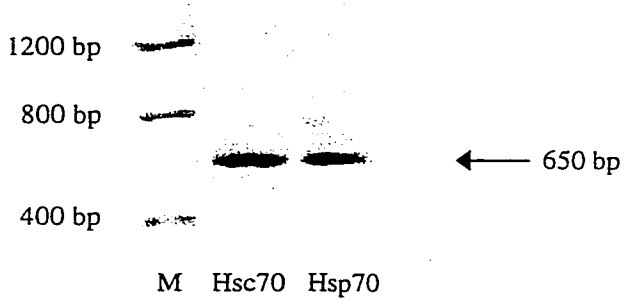

Figure 3-6. Gel electrophoresis of purified Hsc70 and Hsp70 PCR MIMICS. The MIMICs were purified by passing aliquots of the secondary PCR reaction through CHROMA SPIN+TE-100 columns.

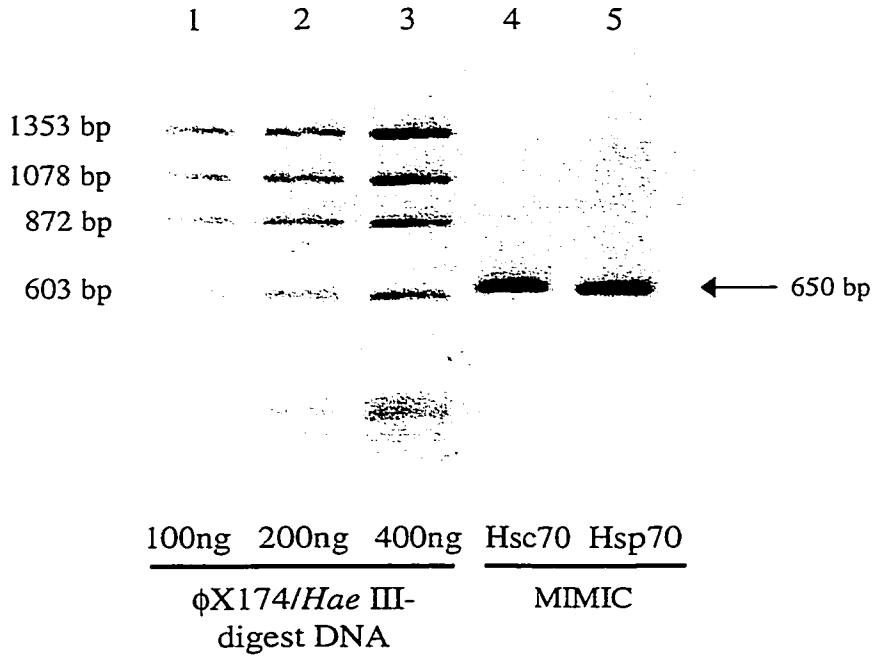

Figure 3-7. Estimation of Hsc70 and Hsp70 PCR MIMIC yield. The MIMIC bands (lanes 4,5) had an intensity closest to the intensity of the 1353 base pair φX174/*Hae* III-digest DNA at a concentration of 400ng.

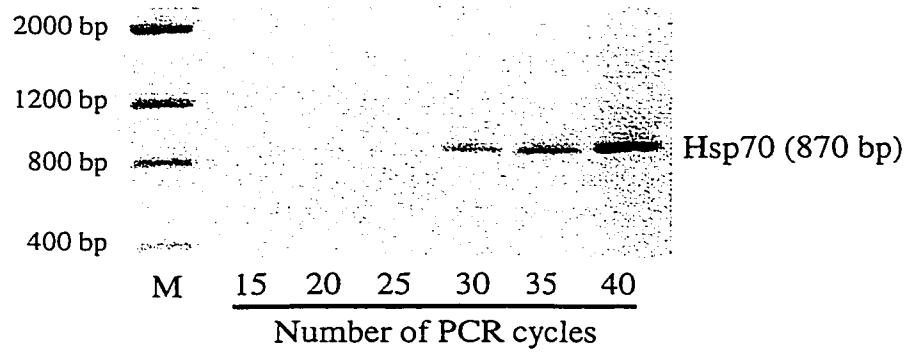

Figure 3-8. RT-PCR analysis for Hsp70 mRNA of control lymphocyte RNA for 15, 20, 25, 30, 35, or 40 PCR cycles.

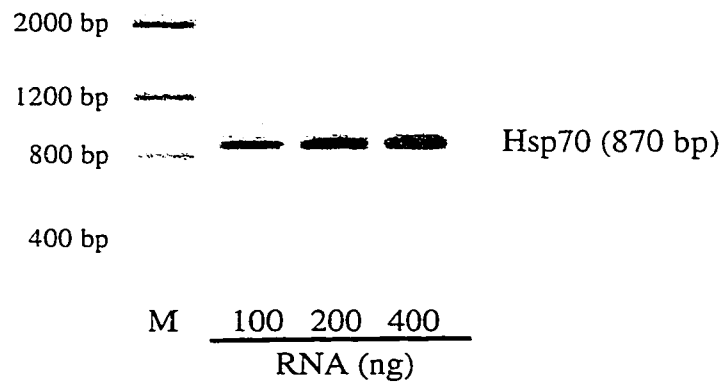

Figure 3-9. RT-PCR analysis for Hsp70 mRNA starting with 100, 200, or 400 ng of total RNA.

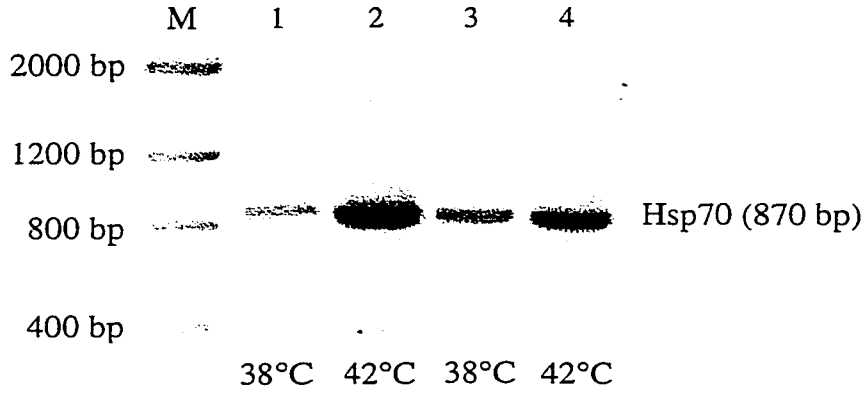

Figure 3-10. Results from an optimized RT-PCR reaction using new primers designed to amplify a 870 base pair fragment corresponding to Hsp70 mRNA. Lanes 1 and 3 represent Hsp70 mRNA expression in lymphocytes incubated at the control temperature (38 °C) and lanes 2 and 4 represent Hsp70 mRNA expressed in *in vitro* heat-shocked (42°C) lymphocytes.

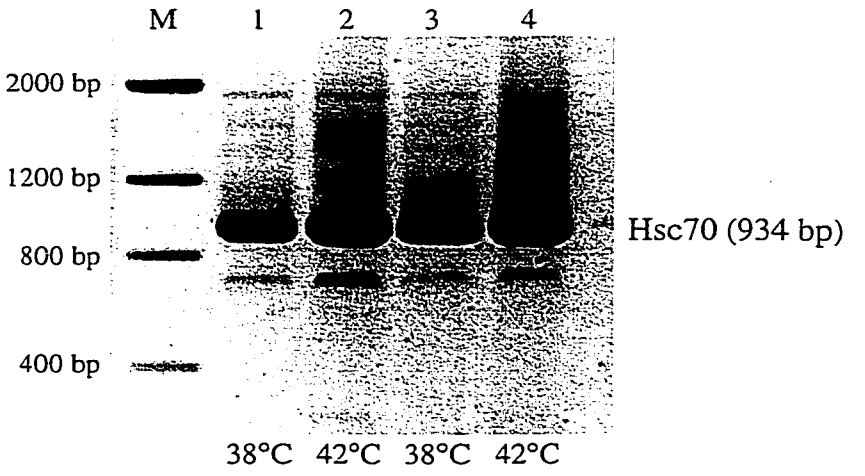

Figure 3-11. Results from a RT-PCR reaction using new primers designed to amplify a 934 base pair fragment corresponding to Hsc70 mRNA. Lanes 1 and 3 represent Hsc70 mRNA expression in lymphocytes incubated at the control temperature (38°C) and lanes 2 and 4 represent Hsc70 mRNA expressed in *in vitro* heat-shocked (42°C) lymphocytes. Bands corresponding to Hsc70 are dominant, however there are many non-specific product bands.

The concentration of Hsp70 MIMIC required in each reaction was determined by performing RT-PCR on the control temperature RNA sample with 2 µl of either 10, 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, or 10^{-5} attomole Hsp70 MIMIC/µl added to the PCR reaction. At a MIMIC concentration of 10^{-2} attomole/µl, PCR product band intensities were approximately equal for Hsp70 MIMIC and Hsp70 target (Figure 3-12). To better approximate the amount of MIMIC required in each reaction, two-fold dilutions of 10^{-1} attomole/µl were made and 2 µl of each dilution were added to separate PCR reactions (Figure 3-13). The intensity of PCR product bands for Hsp70 and Hsp70 MIMIC were approximately equal at 1.25 x 10^{-5} attomole/µl. No product band appeared for Hsp70 when 0.625 x 10^{-5} attomole/µl was added which was most likely due to an unsuccessful RT reaction.

Figure 3-14 shows the results of RT-MIMIC PCR on RNA samples from bovine lymphocytes cultured at the control temperature, 38°C, or heat-shocked at 42°C for one hour. The ratio of Hsp70:MIMIC for the control samples were calculated to be 5.085 and 6.794. The ratio of Hsp70:MIMIC for heat-shocked samples were 12.874 and 9.340. This represents an almost 2-fold increase in Hsp70:MIMIC in the heat-shocked lymphocytes compared to the control lymphocytes which represents an increase in the relative expression of Hsp70 mRNA.

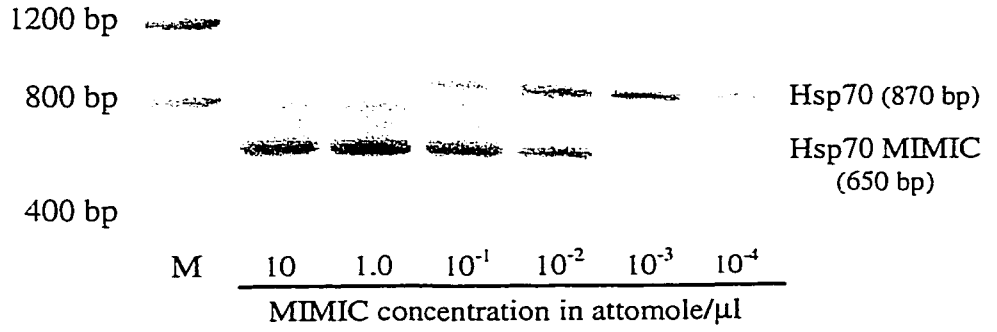

Figure 3-12. Ten-fold dilution series of 100 attomole/µl Hsp70 MIMIC for the determination of the amount of Hsp70 MIMIC required in subsequent PCR reactions to quantify relative amounts of Hsp70 mRNA. Hsp70 MIMIC and Hsp70 band intensities are approximately equal at 10^{-2} attomole/µl.

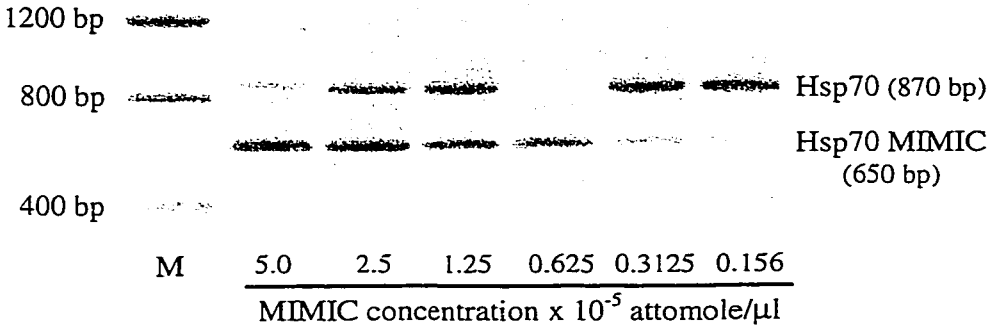

Figure 3-13. Two-fold dilution series of 10^{-1} attomole/µl Hsp70 MIMIC for the determination of the amount of Hsp70 MIMIC required in subsequent PCR reactions to quantify relative amounts of Hsp70 mRNA. Hsp70 MIMIC and Hsp70 band intensities are approximately equal at 1.25×10^{-5} attomole/µl.

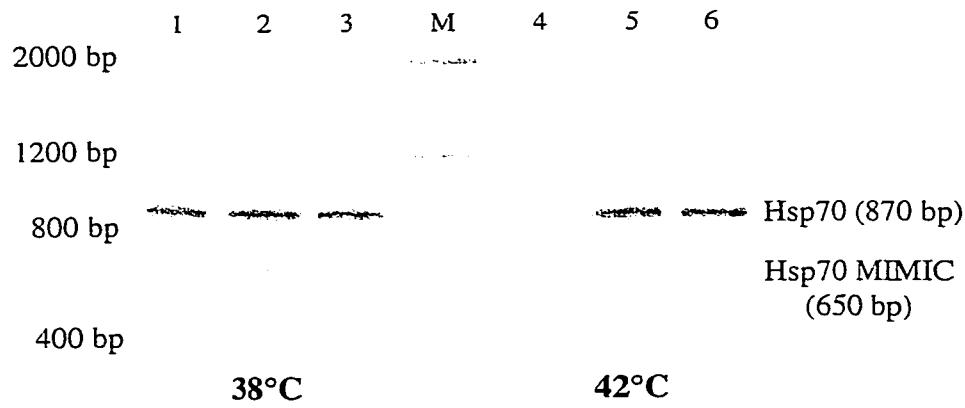

Figure 3-14. RT-PCR using PCR MIMICs to determine the relative expression of Hsp70 mRNA in RNA from bovine lymphocytes incubated at 38°C (lanes 1-3) or 42°C (lanes 4-6) for one hour. PCR MIMIC was not added to the RT-PCR reactions in lanes 1 and 4.

3.2. *IN VITRO* THERMAL SHOCK OF BOVINE LYMPHOCYTES

Lymphocytes were isolated from six blood samples taken from six different steers. The six lymphocyte samples, identified according to the animal number from which they were taken, were each subdivided into 16 sub-samples. Five sub-samples from each animal were placed in an incubator at 38°C, 42°C, and 34°C. One sample representing each animal at each temperature was removed after 30 minutes, 1, 2, 4, and 24 hours of incubation and total RNA was isolated from each sample. The remaining sub-sample from each animal was not subjected to incubation and total RNA was isolated from these samples immediately following lymphocyte isolation. These RNA samples served as control samples for determining the concentration of Hsp70 MIMIC to be added in the analysis for determining relative abundance of Hsp70 mRNA. A 24 hour incubated sample of lymphocytes from animal 44 was not taken. Also, a four-hour sample was not taken for animal 28.

3.2.1. DETERMINATION OF HSP70 MIMIC CONCENTRATION

RT-PCR was performed in duplicate on the control lymphocyte RNA from animals 44, 41, 6, and 26 to ensure that the RT-PCR parameters for detecting Hsp70 mRNA were correct. Control RNA from animal 101 was not analyzed due to low RNA yield and a control lymphocyte sample was not obtained for animal 28. RT-PCR analysis of RNA from animals 41, 6, and 26 resulted in PCR products corresponding to Hsp70, while no products appeared for animal 44 (Figure 3-15). The lack of PCR products for animal 44 may have been due to lack of Hsp70 mRNA expression, RT or PCR failure, degradation of RNA.

RT was performed on control RNA from animal 6 and 26. The resulting cDNA were used in the ten- and two-fold serial dilutions of Hsp70 MIMIC. Gel electrophoresis of the PCR products from the ten-fold serial dilution of MIMIC showed that the intensity of Hsp70 MIMIC and Hsp70 target bands were approximately equal at a MIMIC concentration of 10^{-2} and 10^{-3} attomole/μl for sample 26 and 6, respectively (Figure 3-

16). A MIMIC concentration of 10^{-2} attomole/µl was used to make up the two-fold serial dilutions. Visualization of PCR products showed equal intensities of Hsp70 target and Hsp70 MIMIC at a MIMIC concentration of $2.5x10^{-3}$ attomole/µl (Figure 3-17).

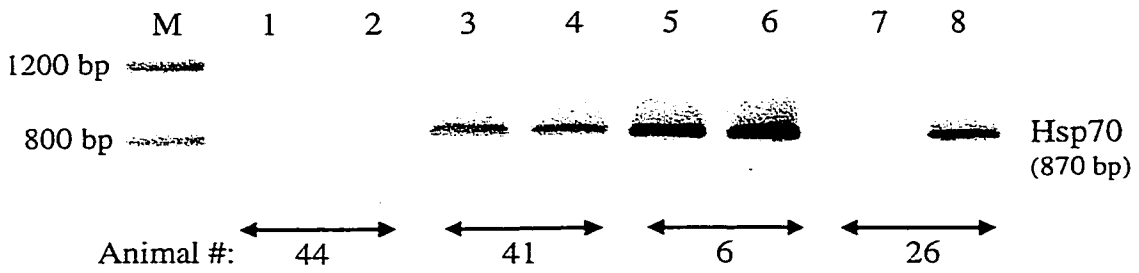

Figure 3-15. RT-PCR analysis for Hsp70 mRNA of control RNA from animals 44, 41, 6, and 26 without Hsp70 MIMIC.

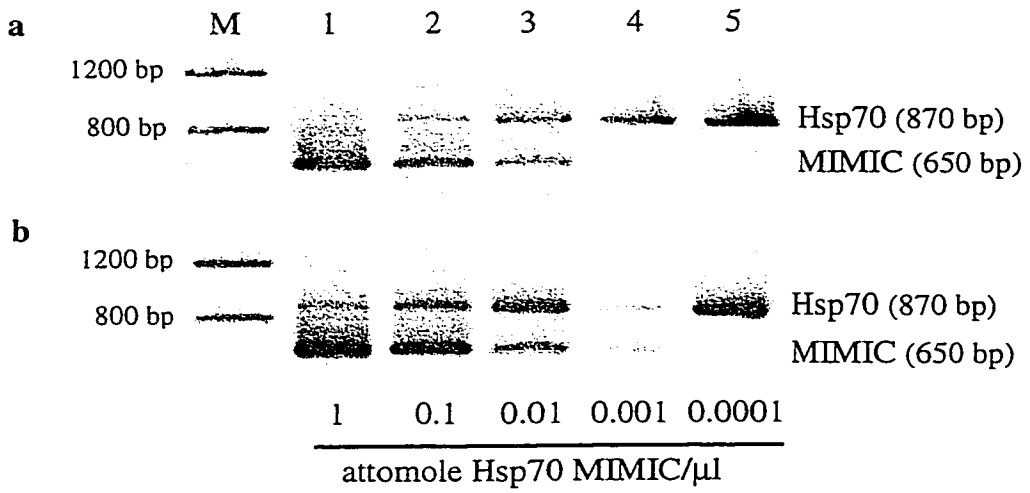

Figure 3-16. Ten-fold serial dilutions of Hsp70 MIMIC with control RNA from animal 26 (**a**) and 6 (**b**). Hsp70 and Hsp70 MIMIC bands were approximately equal in intensity at a MIMIC concentration of 10^{-2} attomole/µl for animal 26 (**a**, lane 3) and 10^{-3} for animal 6 (**b**, lane 4).

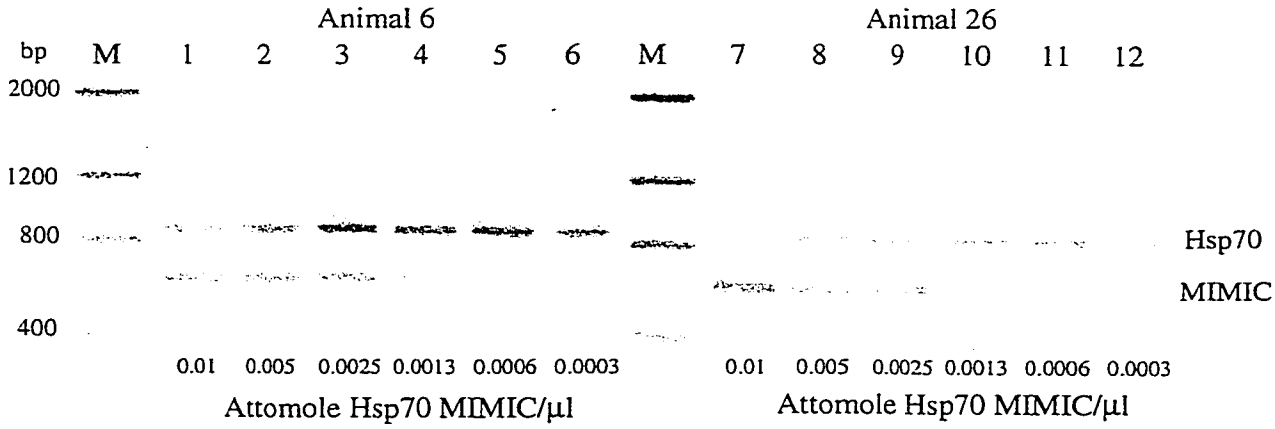

Figure 3-17. Two-fold serial dilutions of Hsp70 MIMIC with control RNA from animal 6 (lanes 1-6) and 26 (lanes 7-12). Hsp70 (870 bp) and Hsp70 MIMIC (650 bp) bands were approximately equal in intensity at a MIMIC concentration of 2.5×10^{-3} attomole/µl for animal 26 (lane 9) and 5.0×10^{-3} attomole/µl for animal 6 (lane 2).

3.2.2. Relative Expression of Hsp70 mRNA in Bovine Lymphocytes Exposed to *In Vitro* Thermal Shock

RT-PCR was performed in triplicate on RNA samples from the incubated lymphocytes with Hsp70 MIMIC added to the PCR at a concentration of $2.5x10^{-2}$ attomole/µl (Figures 3-18 to 3-22). Tables 3-1 through 3-5 summarize the results obtained for each animal. The results are presented as the ratio of Hsp70 target to Hsp70 MIMIC DNA concentration. RT-PCR analysis for the expression of Hsp70 mRNA in samples taken from animal 101 all gave a negative result, i.e. bands corresponding to Hsp70 were absent and MIMIC bands were present (Figure 3-23).

The results do not follow the expected heat shock induction of expression of Hsp70 mRNA. An increase in relative expression of Hsp70 mRNA was not consistently observed. Also, there were large variations in the calculated ratio for the three measurements made for each sample. In an attempt to determine where the variation was originating, the reproducibility of RT-PCR reactions in various wells of the thermocycler was tested. A RT master mix was aliquoted into one of 14 PCR tubes and the tubes were placed in the thermocycler. Following the completion of the RT step, the components of the PCR reaction, excluding the MIMIC, were added and the tubes were placed in the same well in which the RT reaction was performed. Gel electrophoresis of the resulting PCR products showed that there was variation among the wells of the thermocycler (Figure 3-24). The intensity of the products from each tube should be similar since each tube contained the same components. However, this was not observed. Tubes placed in the top two rows of the thermocycler had relatively strong product bands. Those placed in the middle two rows results in moderate intensity bands, while no products were observed from tubes placed in the bottom two rows of the thermocycler.

The thermocycler block was further tested by running RT-PCR reactions in the top two rows of the thermocycler. The results are shown in figure 3-25. A band corresponding to Hsp70 was seen for each tube, however there was variation in the intensity of each band.

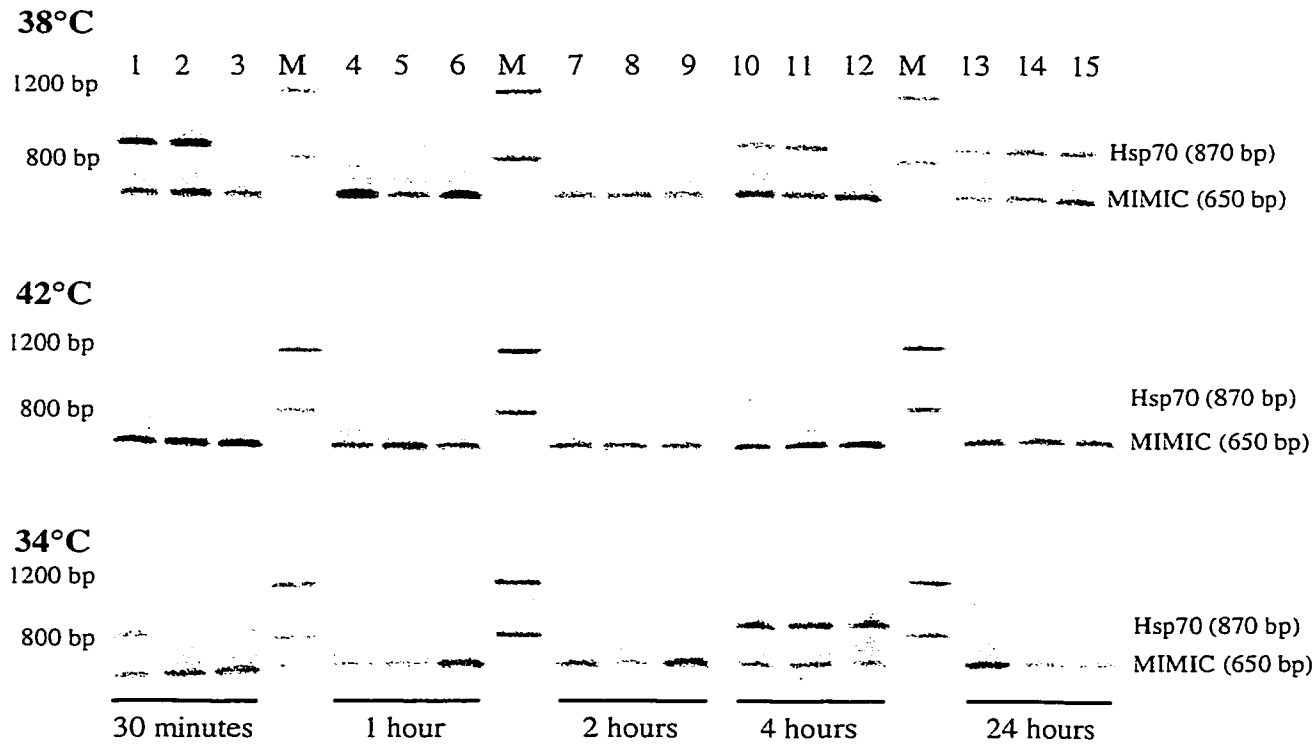

Figure 3-18. RT-MIMIC PCR analysis for relative expression of Hsp70 mRNA in lymphocyte samples from animal 6 incubated at 38°C, 42°C, and 34°C. Lanes 1-3, 4-6, 7-9, 10-12, and 13-15 correspond to samples incubated for 30 minutes, 1, 2, 4, and 24 hours, respectively.

Table 3-1. Relative expression of Hsp70 mRNA in bovine lymphocytes from animal #6 incubated at 38°C, 42°C, or 34°C as determined by RT-MIMIC PCR. Results are expressed as the ratio of Hsp70 to Hsp70 MIMIC DNA concentration.

	TEMPERATURE						
TIME (hrs.)	38°C	Mean ± SEM	42°C	Mean ± SEM	34°C	Mean ± SEM	Avg. ratio by time
0.5	1.79	1.26	0.16	0.05	1.14	0.45	0.59
0.5	1.63	± 0.45	0.00	± 0.05	0.00	± 0.35	± 0.24
0.5	0.36		0.00		0.21		
1	0.00	0.04	0.24	0.15	0.00	0.00	0.07
1	0.12	± 0.04	0.00	± 0.08	0.00	± 0.00	± 0.03
1	0.00		0.23		0.00		
2	0.00	0.00	0.24	0.08	0.00	0.00	0.03
2	0.00	± 0.00	0.00	± 0.08	0.00	± 0.00	± 0.03
2	0.00		0.00		0.00		
4	0.37	0.37	0.24	0.11	2.74	2.78	1.08
4	0.74	± 0.21	0.00	± 0.07	2.30	± 0.29	± 0.44
4	0.00		0.09		3.29		
24	0.80	0.79	0.13	0.04	0.00	0.28	0.37
24	0.94	± 0.09	0.00	± 0.04	0.40	± 0.14	± 0.12
24	0.63		0.00		0.43		
Avg. ratio by temp.	0.49 ± 0.15		0.09 ± 0.03		0.70 ± 0.29		

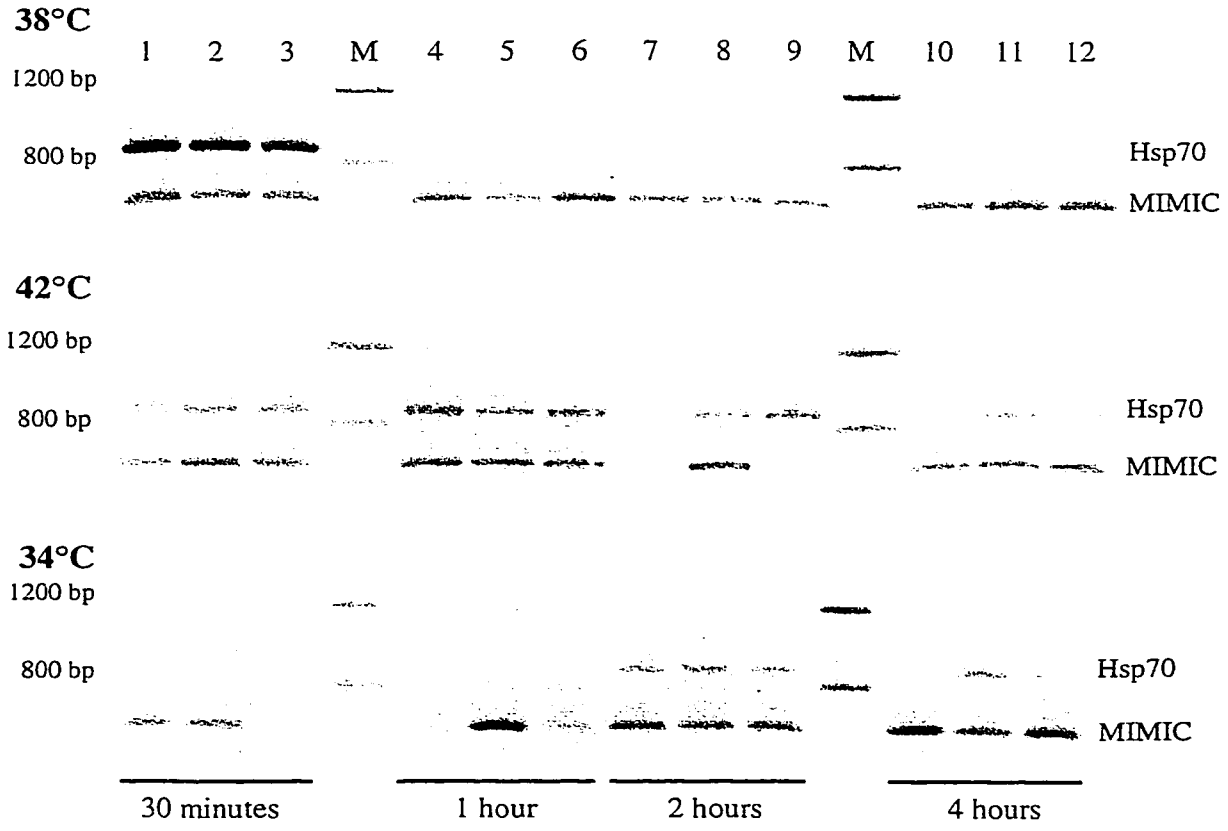

Figure 3-19. RT-MIMIC PCR analysis for relative expression of Hsp70 mRNA in lymphocyte samples from animal 44 incubated at 38°C, 42°C, and 34°C. Lanes 1-3, 4-6, 7-9, 10-12, and 12-15 correspond to samples removed from the incubator 30 minutes, 1, 2, and 4 hours after temperature exposure, respectively.

Table 3-2. Relative expression of Hsp70 mRNA in bovine lymphocytes from animal #44 incubated at 38°C, 42°C, or 34°C as determined by RT-MIMIC PCR. Results are expressed as the ratio of Hsp70 to Hsp70 MIMIC DNA concentration.

	TEMPERATURE						
TIME (hrs.)	38°C	Mean ± SEM	42°C	Mean ± SEM	34°C	Mean ± SEM	Avg. ratio by time
0.5	3.10	3.66	0.86	0.86	0.00	0.00	1.70
0.5	4.37	± 0.37	0.76	± 0.05	0.00	± 0.00	± 0.60
0.5	3.52		0.95		**		
1	0.00	0.00	0.99	0.86	**	0.03	0.30
1	0.00	± 0.00	0.80	± 0.06	0.10	± 0.04	± 0.14
1	0.00		0.78		0.00		
2	0.00	0.00	**	0.40	0.38	0.45	0.25
2	0.00	± 0.00	0.40		0.52	± 0.04	± 0.09
2	0.00		**		0.45		
4	**	0.00	0.45	0.48	0.00	0.21	0.30
4	0.00		0.58	± 0.05	0.50	± 0.15	± 0.09
4	**		0.42		0.14		
Avg. ratio By temp.	1.10 ± 0.57		0.70 ± 0.07		0.19 ± 0.07		

** indicates a reaction in which the Hsp70 MIMIC did not amplify.

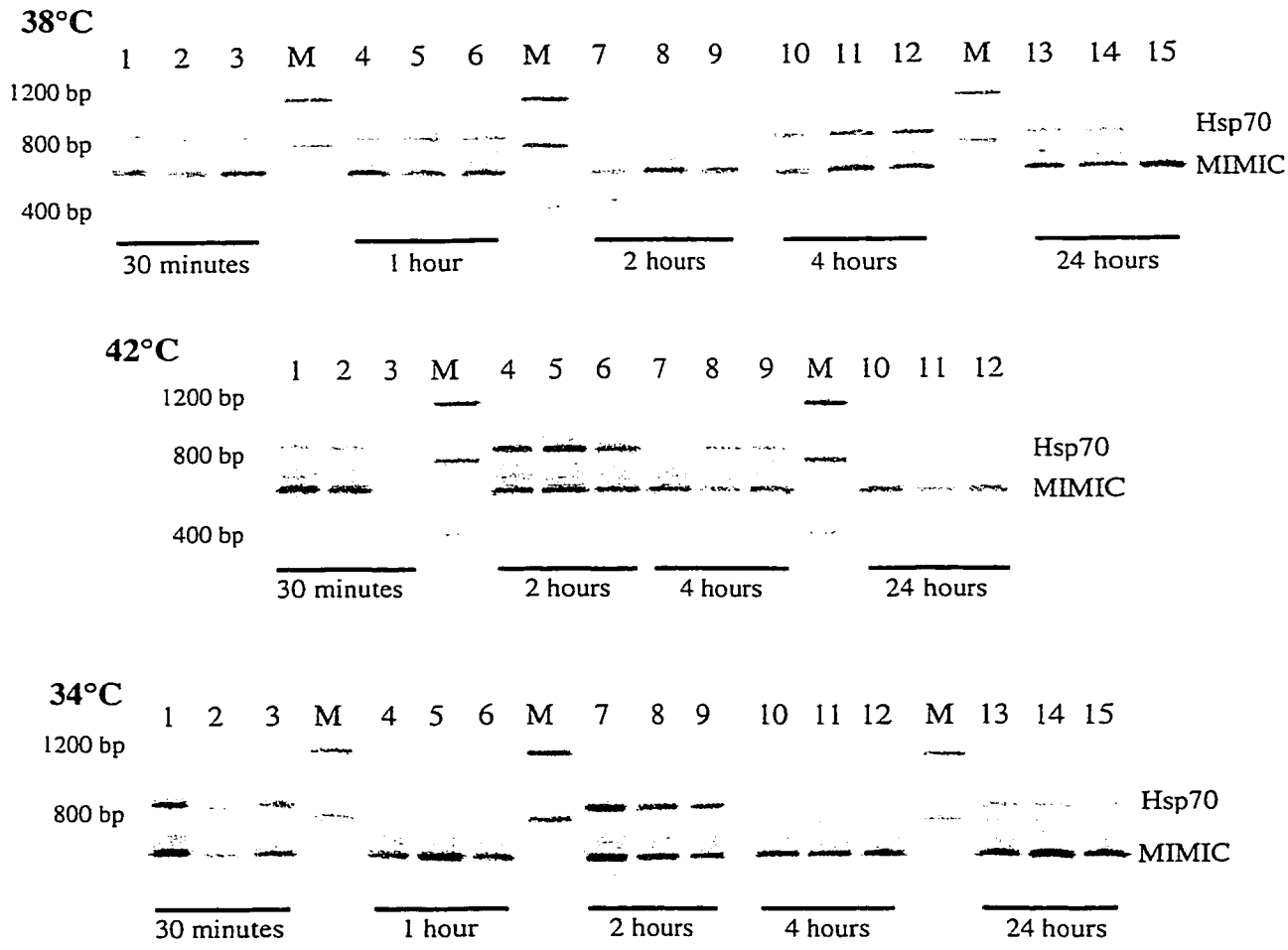

Figure 3-20. RT-MIMIC PCR analysis for relative expression of Hsp70 mRNA in lymphocyte samples from animal 41 incubated at 38°C, 42°C, and 34°C for the specified times.

Table 3-3. Relative expression of Hsp70 mRNA in bovine lymphocytes from animal #41 incubated at 38°C, 42°C, or 34°C as determined by RT-MIMIC PCR. Results are expressed as the ratio of Hsp70 to Hsp70 MIMIC DNA concentration.

	Temperature						
Time (hrs.)	38°C	Mean ± SEM	42°C	Mean ± SEM	34°C	Mean ± SEM	Avg. ratio by time
0.5	0.63	0.51	0.21	0.16	0.77	0.72	0.46
0.5	0.60	± 0.11	0.26	± 0.08	0.69	± 0.03	± 0.09
0.5	0.30		0.00		0.70		
1	0.26	0.45	ND	ND	0.00	0.00	0.23
1	0.53	± 0.10	ND		0.00	± 0.00	± 0.11
1	0.56		ND		0.00		
2	0.34	0.31	0.77	0.90	0.86	0.79	0.67
2	0.23	± 0.04	1.05	± 0.08	0.70	± 0.05	± 0.10
2	0.36		0.87		0.82		
4	0.84	0.74	0.25	0.49	0.00	0.00	0.46
4	0.61	± 0.07	0.71	± 0.13	0.00	± 0.00	± 0.12
4	0.77		0.51		0.00		
24	0.29	0.22	0.00	0.00	0.18	0.13	0.12
24	0.33	± 0.09	0.00	± 0.00	0.11	± 0.02	± 0.04
24	0.05		0.00		0.11		
Avg. ratio By temp.	0.45 ± 0.06		0.39 ± 0.11		0.33 ± 0.09		

ND = not determined

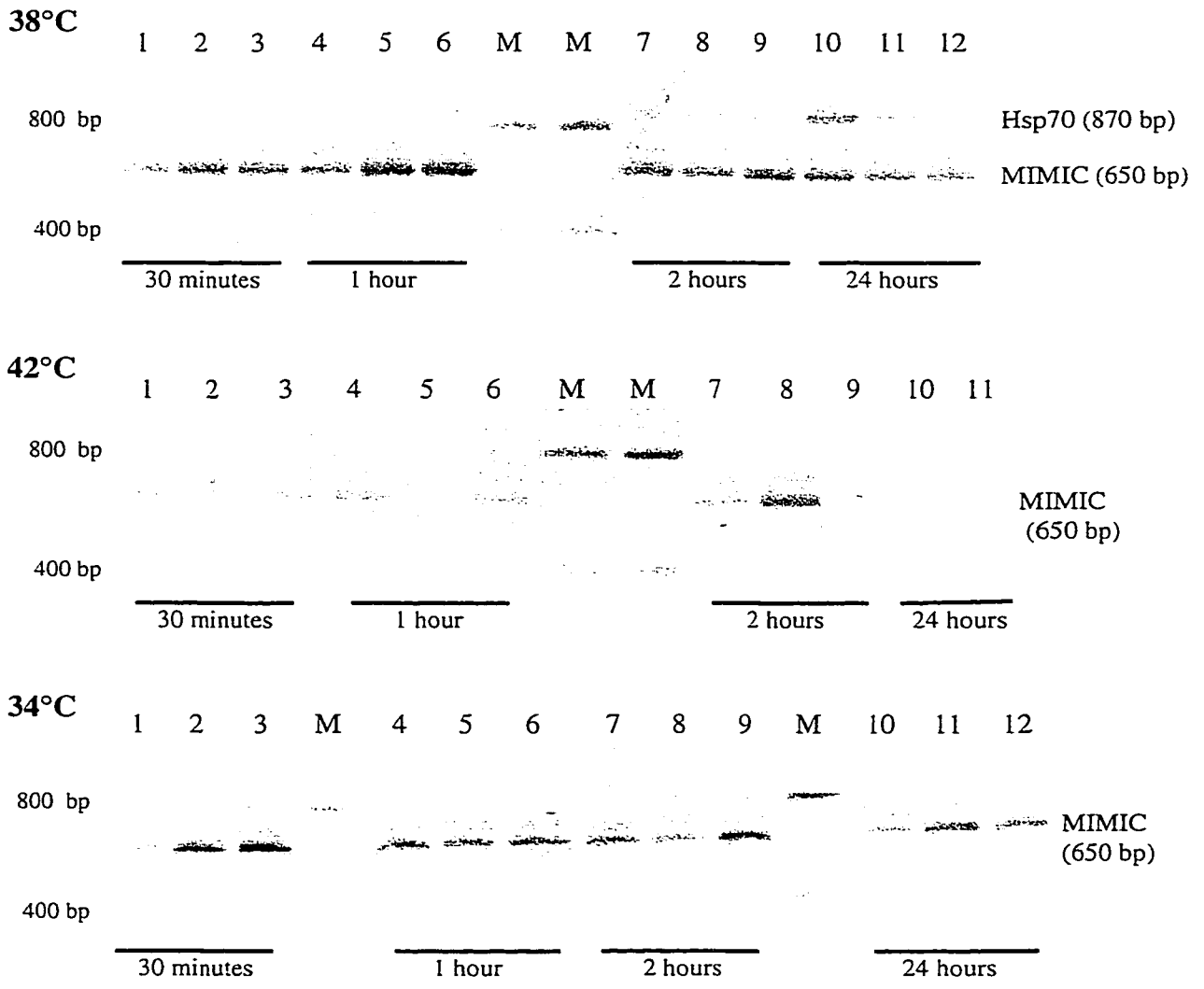

Figure 3-21. RT-MIMIC PCR analysis for relative expression of Hsp70 mRNA in lymphocyte samples from animal 28 incubated at 38°C, 42°C, and 34°C for the specified times.

Table 3-4. Relative expression of Hsp70 mRNA in bovine lymphocytes from animal #28 incubated at 38°C, 42°C, or 34°C as determined by RT-MIMIC PCR. Results are expressed as the ratio of Hsp70 to Hsp70 MIMIC DNA concentration.

	TEMPERATURE						
Time (hrs.)	**38°C**	**Mean ± SEM**	**42°C**	**Mean ± SEM**	**34°C**	**Mean ± SEM**	**Avg. ratio By time**
0.5	0.00	0.08	0.00	0.00	0.00	0.00	0.03
0.5	0.14	± 0.04	0.00	± 0.00	0.00	± 0.00	± 0.02
0.5	0.11		0.00		0.00		
1	0.13	0.08	0.00	0.00	0.00	0.000	0.03
1	0.07	± 0.02	**	± 0.00	0.00	± 0.00	± 0.02
1	0.05		0.00		0.00		
2	0.30	0.22	0.00	0.000	0.00	0.00	0.08
2	0.21	± 0.04	0.00	± 0.00	0.00	± 0.00	± 0.04
2	0.14		**		0.00		
24	0.70	0.44	**	**	0.00	0.00	0.22
24	0.35	± 0.13	**		0.00	± 0.00	± 0.11
24	0.27		**		0.00		
Avg. ratio by temp.	0.21 ± 0.05		0.00 ± 0.00		0.00 ± 0.00		

** indicates a reaction in which the Hsp70 MIMIC did not amplify.

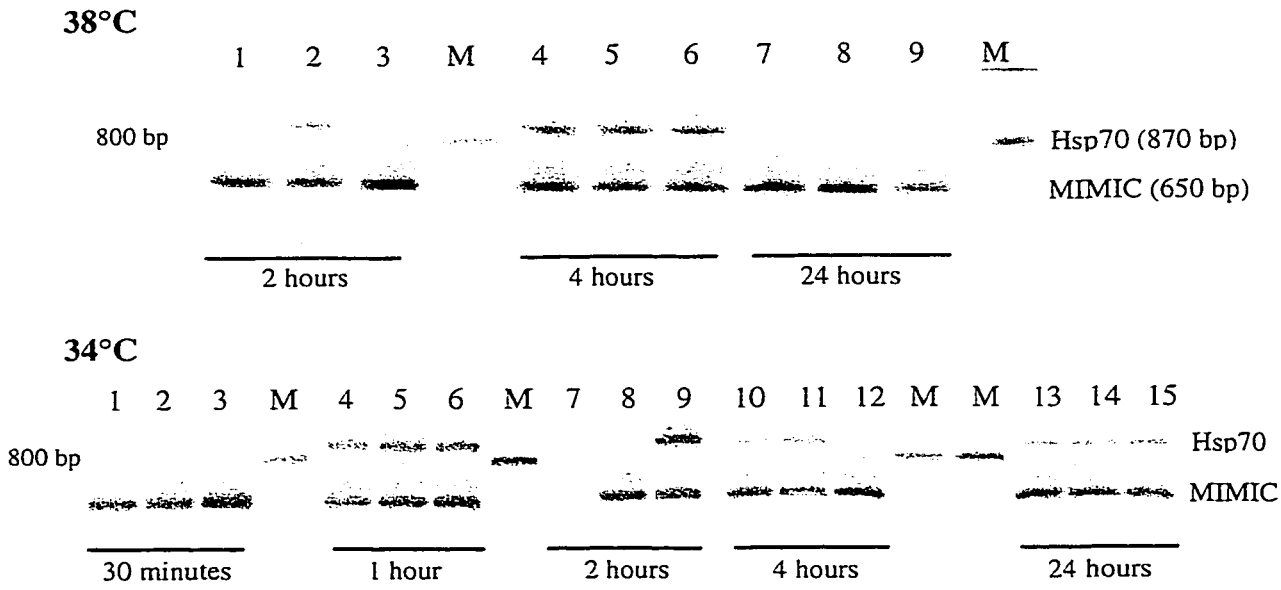

Figure 3-22. RT-MIMIC PCR analysis for relative expression of Hsp70 mRNA in lymphocyte samples from animal 26 incubated at 38°C and 34°C for the specified times.

Table 3-5. Relative expression of Hsp70 mRNA in bovine lymphocytes from animal #26 incubated at 38°C, 42°C, or 34°C as determined by RT-MIMIC PCR. Results are expressed as the ratio of Hsp70 to Hsp70 MIMIC DNA concentration.

	Temperature						
Time (hrs.)	**38°C**	**Mean ± SEM**	**42°C**	**Mean ± SEM**	**34°C**	**Mean ± SEM**	**Avg. ratio by time**
0.5	ND	ND	0.00	0.00	0.00	0.04	0.02
0.5	ND		0.00	± 0.00	0.11	± 0.04	± 0.02
0.5	ND		0.00		0.00		
1	ND	ND	0.00	0.00	0.56	0.54	0.267
1	ND		0.00	± 0.00	0.61	± 0.54	± 0.12
1	ND		0.00		0.43		
2	0.00	0.12	0.00	0.00	**	0.48	0.16
2	0.35	± 0.12	0.00	± 0.00	0.00	± 0.48	± 0.12
2	0.00		0.00		0.96		
4	0.53	0.58	0.00	0.00	0.17	0.18	0.26
4	0.65	± 0.04	0.00	± 0.00	0.38	± 0.11	± 0.09
4	0.57		0.00		0.00		
24	0.00	0.00	0.00	0.00	0.15	0.21	0.07
24	0.00	± 0.00	0.00	± 0.00	0.16	± 0.06	± 0.04
24	0.00		0.00		0.33		
Avg. ratio By temp.	0.23 ± 0.10		0.00 ± 0.00		0.28 ± 0.08		

** indicates a reaction in which the Hsp70 MIMIC did not amplify.

ND = not determined

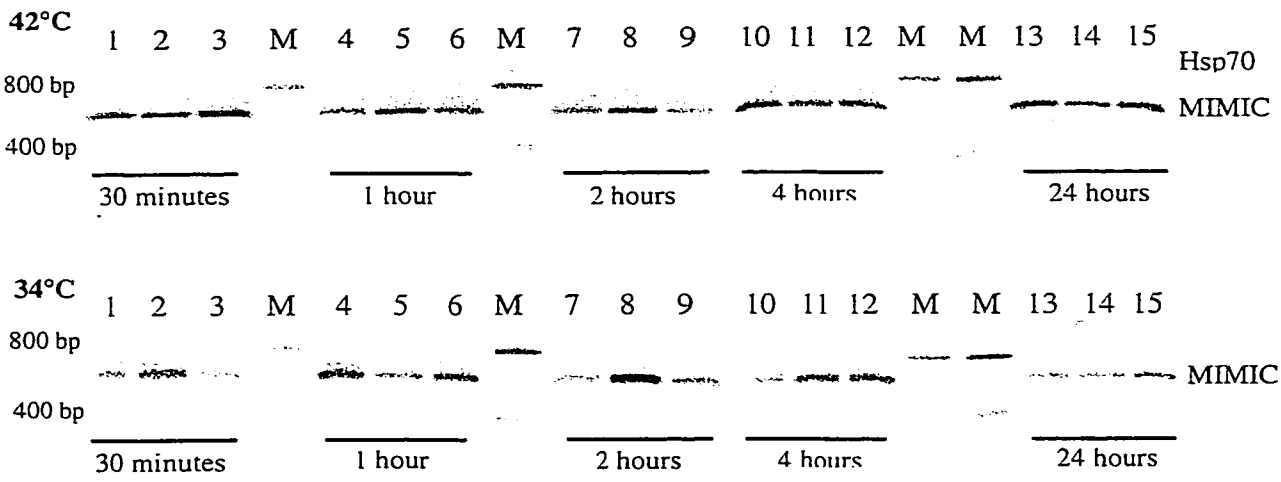

Figure 3-23. RT-MIMIC PCR analysis for relative expression of Hsp70 mRNA in lymphocyte samples from animal 101 incubated at 42°C and 34°C for the specified times.

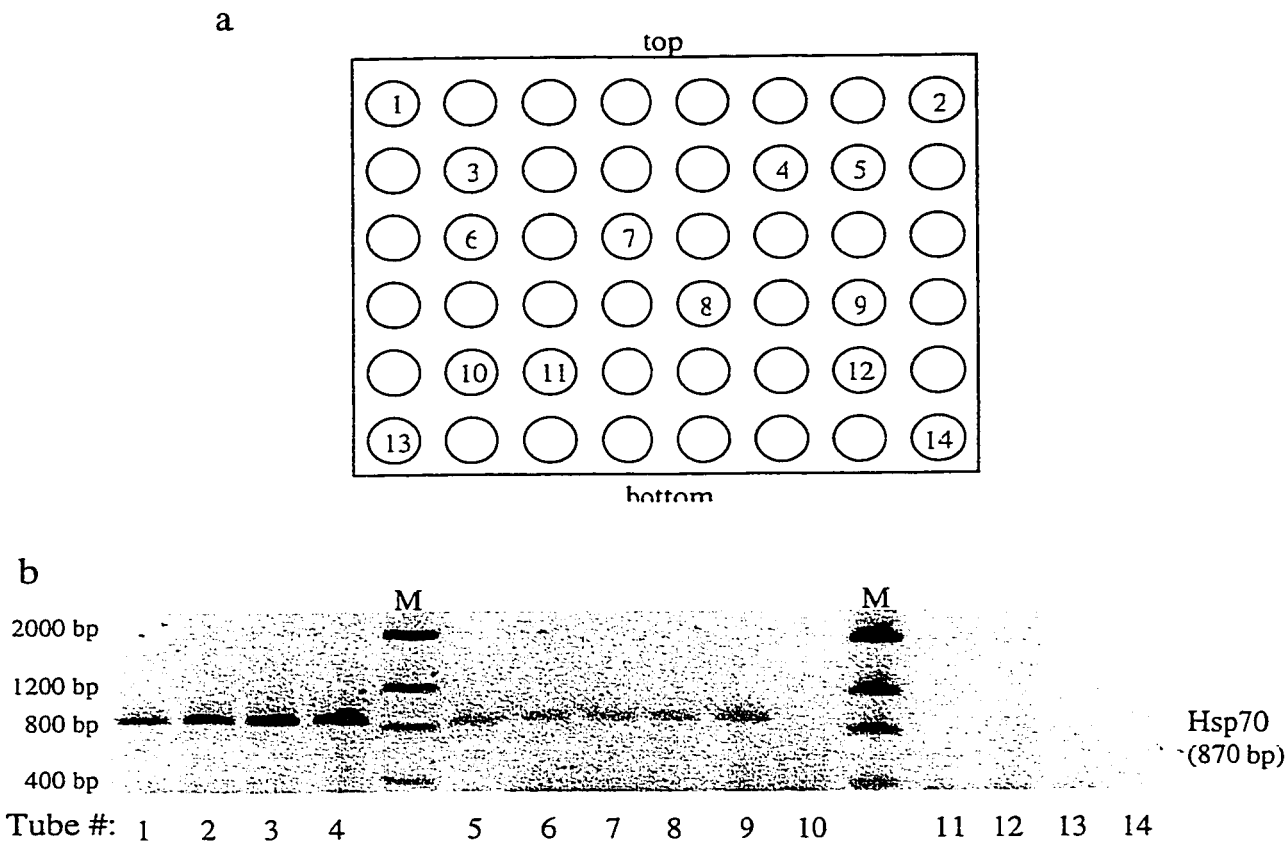

Figure 3-24. a: Diagram of the thermocycler block showing the placement of tubes 1-14 during the RT-PCR reaction. **b**: Gel electrophoresis showing the products of RT-PCR for tubes 1-14.

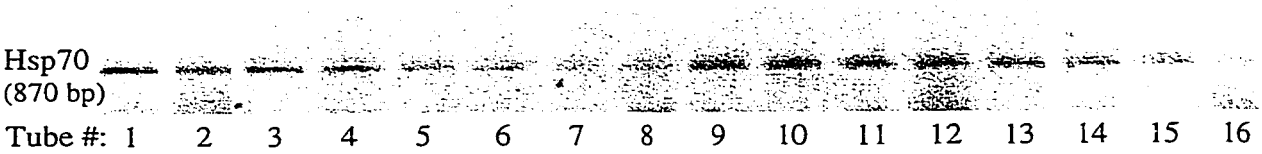

Figure 3-25. Gel electrophoresis of RT-PCR reactions from tubes placed in the top two rows of the thermocycler. Tubes 1-8 were placed in the first row left to right. Tubes 9-16 were placed in the second row left to right.

3.3. *IN VIVO* CONTROLLED ENVIRONMENT EXPERIMENT

3.3.1. EXPERIMENT ONE – MOVEMENT/TEMPERATURE EXPOSURE

Blood samples were taken from 27 heifers prior to and 1-2 hours after movement into a new pen in either a thermoneutral (18°C) or cold (-18°C) environment. At each blood sampling, the heifers were placed in a squeeze and blood was obtained by jugular venipuncture. Lymphocyte RNA was extracted from each blood sample.

Samples from eight animals were available for RNA analysis before and after movement to pens. Samples from several additional animals were tested and rejected due to apparent degradation of RNA. Analysis of RNA samples that resulted in smeared lanes were considered to be the result of RNA degradation. RT-MIMIC PCR was performed on lymphocyte RNA isolated from blood taken from the heifers before and one to 2 hours after movement to a new pen in a climate controlled area (Figure 3-26). Table 3-6 summarizes the results of the RT-MIMIC PCR analysis for the expression of Hsp70 mRNA.

Of the eight samples tested, relative Hsp70 mRNA expression was not different before and after movement to a new pen. Also, there was no effect of pen temperature on the relative level of Hsp70 mRNA expression after movement. As with the *in vitro* experiment results, there was some variation between triplicate measurements of Hsp70 mRNA level for some of the samples. This is believed to be due in part to the problem identified with the thermocycler heating block.

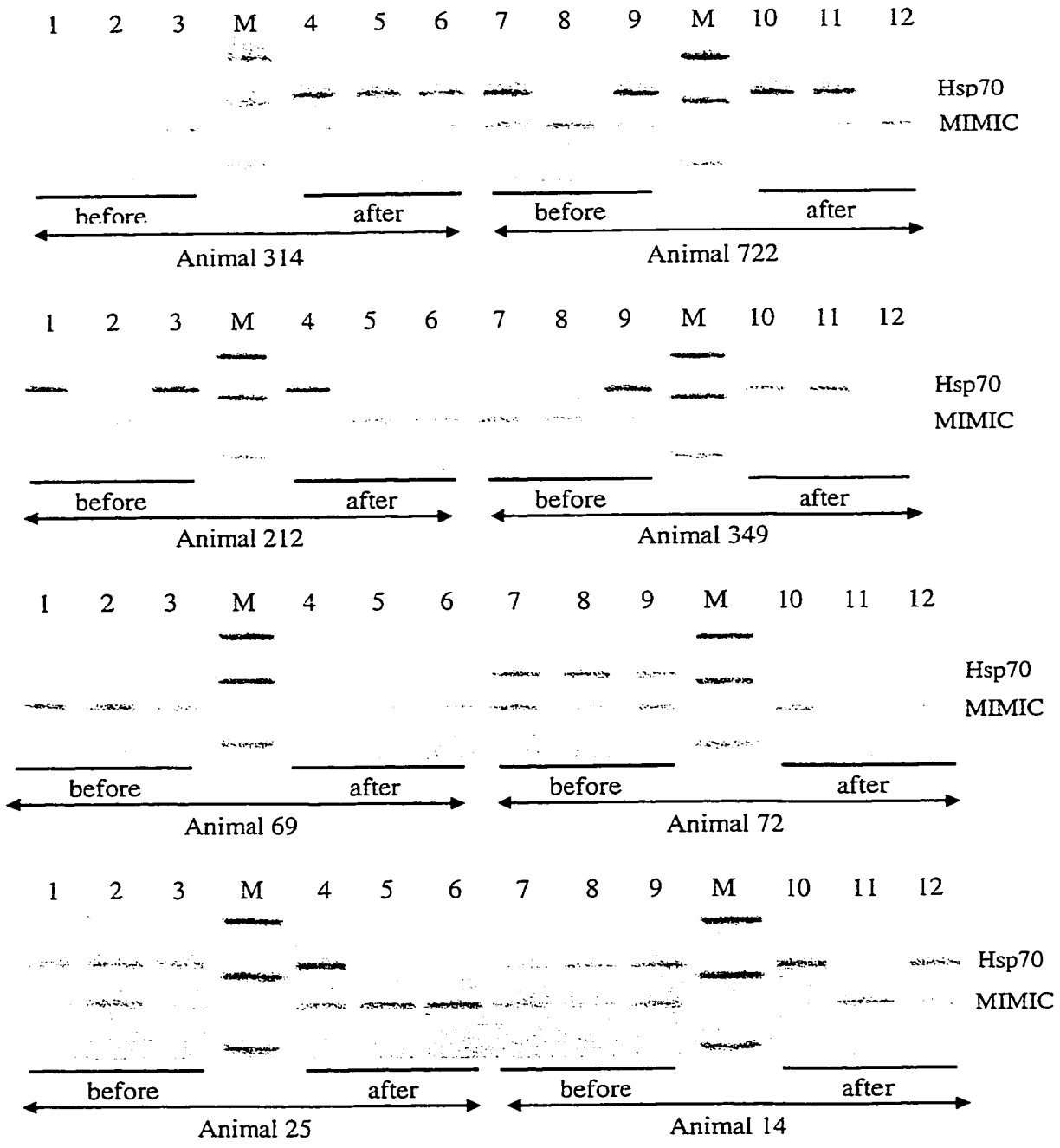

Figure 3-26. RT-MIMIC PCR analysis for relative expression of Hsp70 mRNA in lymphocyte samples from animals 314, 722, 212, 349, 69, 72, 25, and 14 before and after movement to a new pen.

Table 3-6. Relative expression of Hsp70 mRNA in bovine lymphocytes from blood samples taken from cattle before and after movement to a new pen in either a thermoneutral room (warm) or a below thermoneutral room (cold). Results are expressed as the ratio of Hsp70 to Hsp70 MIMIC DNA concentration.

Animal #	Pen Temp.	Before Movement	Mean ± SEM	After Movement	Mean ± SEM
314	**Warm**	0.00	0.00	4.06	4.46
314	**Warm**	0.00	± 0.00	6.05	± 0.83
314	**Warm**	0.00		3.28	
722	**Warm**	4.32	3.42	7.36	4.19
722	**Warm**	0.00	± 1.77	5.22	± 2.24
722	**Warm**	5.93		0.00	
212	**Warm**	**	6.28	10.85	3.62
212	**Warm**	0.00	± 6.28	0.00	± 3.62
212	**Warm**	12.56		0.00	
349	**Warm**	0.00	2.20	4.29	2.35
349	**Warm**	0.00	± 2.20	2.76	± 1.26
349	**Warm**	6.61		0.00	
69	**Warm**	0.00	0.16	1.58	0.53
69	**Warm**	0.00	± 0.16	0.00	± 0.53
69	**Warm**	0.47		0.00	
72	**Cold**	1.31	1.61	0.00	0.00
72	**Cold**	2.44	± 0.42	0.00	± 0.00
72	**Cold**	1.07		0.00	
25	**Cold**	2.46	2.42	3.34	1.11
25	**Cold**	0.94	± 0.85	0.00	± 1.11
25	**Cold**	3.87		0.00	
14	**Cold**	0.81	1.49	6.09	3.03
14	**Cold**	2.05	± 0.36	0.00	± 1.76
14	**Cold**	1.61		3.01	
Average by treatment			2.02 ± 0.63		2.41 ± 0.62
Average ratio warm			2.14 ± 1.03		3.03 ± 0.86
Average ratio cold			1.84 ± 0.33		1.38 ± 0.75

** indicates a reaction in which the Hsp70 MIMIC did not amplify.

3.3.2. EXPERIMENT TWO – TRANSPORT STRESS

After the heifers from had adjusted to their treatments for three weeks, all the animals were shipped for slaughter. Blood samples were taken by jugular venipuncture before loading and after unloading at the slaughter plant. The samples were analyzed in triplicate for relative expression of Hsp70 mRNA in lymphocyte RNA samples. In addition, cortisol, T3, T4, N:L, and CBT measurements were taken as alternative stress measurements to better evaluate the effect of transport on the animals and to determine the relationship between Hsp70 mRNA expression and these more commonly used indicators of stress.

Samples from six animals before and after transport were available for RNA analysis. Samples from several additional animals were tested and rejected due to apparent degradation of RNA. RT-MIMIC PCR was performed on lymphocyte RNA isolated from blood taken from the heifers before and after transport (Figures 3-27 and 3-28). Table 3-7 summarizes the results of the RT-MIMIC PCR analysis for the relative expression of Hsp70 mRNA.

Transport did not have a significant effect on the relative Hsp70 mRNA expression. The change in relative Hsp70 mRNA expression of individual animals after transport was variable Once again variations in triplicate measurements were seen, presumably caused by the inconsistent thermocycler block.

Other data obtained for these animals is shown in Table 3-8. The neutrophil-to-lymphocyte ratio (N:L) increased significantly ($P<0.05$) after the animals were transported for one hour. Cortisol values were significantly lower following transport ($P<0.05$). Similarly, T3 and T4 levels decreased significantly after transport ($P<0.01$ and $P<0.001$, respectively). The core body temperature (CBT) increased significantly ($P<0.01$) after transport.

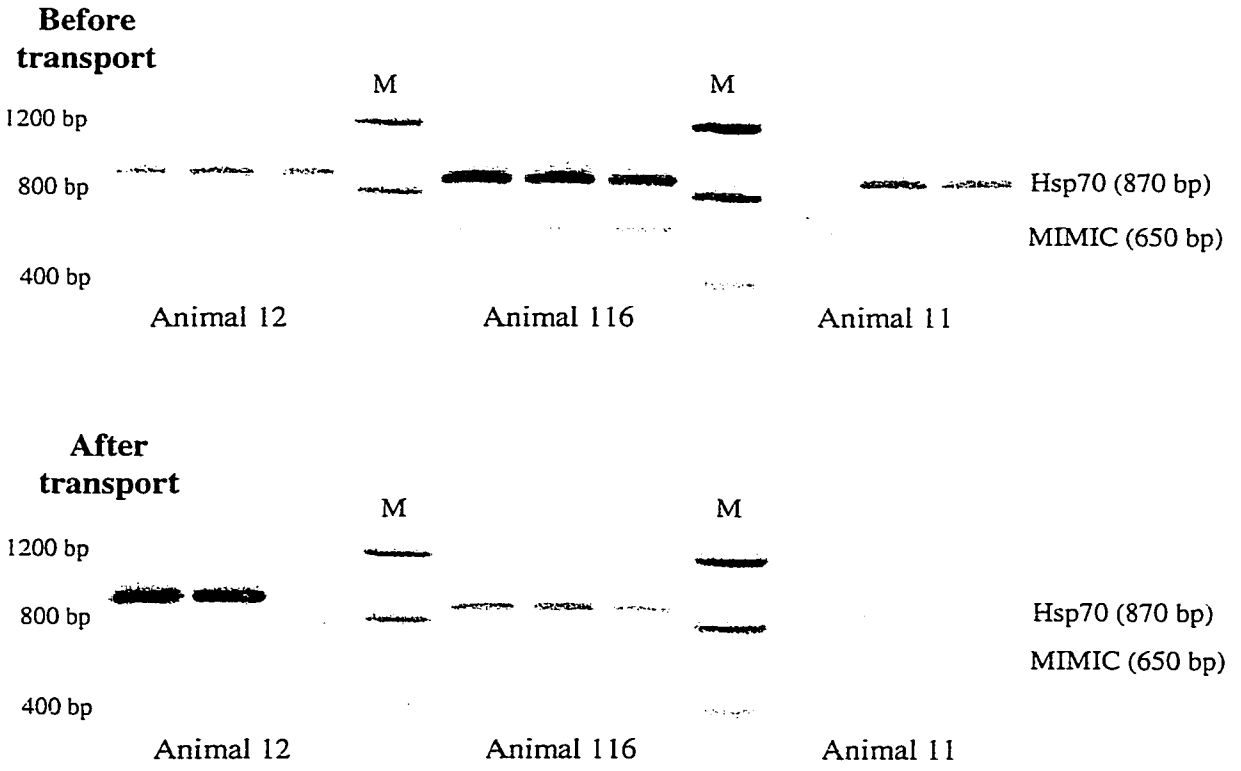

Figure 3-27. RT-MIMIC PCR analysis for relative expression of Hsp70 mRNA in lymphocyte samples from animals 12, 116, and 11 before transport and after transport.

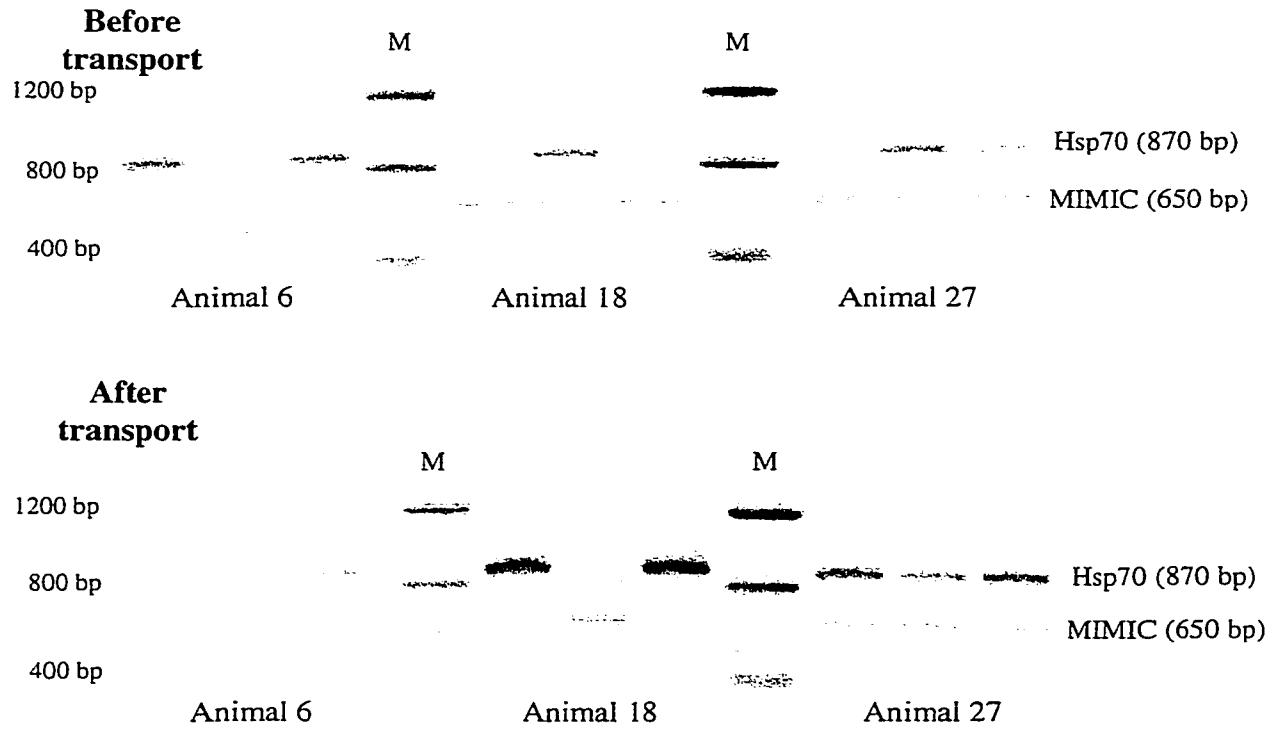

Figure 3-28. RT-MIMIC PCR analysis for relative expression of Hsp70 mRNA in lymphocyte samples from animals 6, 18, and 27 before transport and after transport.

Table 3-7. Relative expression of Hsp70 mRNA in bovine lymphocytes from blood samples taken from cattle before transport and after transport for one hour. Results are expressed as the ratio of Hsp70 to Hsp70 MIMIC DNA concentration.

Animal #	Before transport	Mean ± SEM	After transport	Mean ± SEM
12	2.33	2.58	22.87	10.15
12	3.01	± 0.22	7.60	± 6.72
12	2.38		0.00	
116	9.64	8.00	4.47	3.84
116	9.54	± 1.60	4.19	± 0.50
116	4.81		2.85	
11	0.00	2.82	**	0.00
11	4.01	± 1.41	**	
11	4.44		0.00	
6	4.54	2.82	**	2.26
6	0.00	± 1.42	**	
6	3.93		2.26	
18	0.00	0.72	10.34	7.06
18	2.15	± 0.72	0.000	± 3.53
18	0.00		10.83	
27	0.00	0.94	3.89	3.74
27	1.75	± 0.51	1.53	± 1.23
27	1.06		5.78	
Overall average:		2.98 ± 0.70		5.47 ± 1.64

Table 3-8. Core body temperature (CBT), neutrophil to lymphocyte ratio (N:L), plasma cortisol, plasma T_3 and plasma T_4 concentrations before and after transport.

Animal	N:L before	N:L after	Cortisol Before (µg/dL)	Cortisol After (µg/dL)	T_3 before	T_3 after	T_4 Before	T_4 after	CBT (°C) before	CBT (°C) after
12	0.19	0.81	2.97	0.75	199	135	8.45	4.94	38.24	38.79
116	ND	ND	1.50	0.41	184	156	7.97	6.67	ND	ND
11	0.54	1.36	4.61	0.51	162	129	8.24	4.38	38.38	39.46
6	0.92	2.87	1.70	1.38	224	174	10.19	6.51	38.86	39.09
18	0.43	1.80	0.83	1.06	170	161	9.51	6.81	38.48	39.34
27	0.37	0.79	2.19	1.10	209	160	9.15	7.17	39.03	39.25
Mean ± SEM	0.49^{b} ± 0.29	1.53^{a} ± 0.29	2.30^{a} ± 0.40	0.87^{b} ± 0.40	191^{a} ± 8.4	153^{b} ± 8.4	8.92^{a} ± 0.41	6.08^{b} ± 0.41	38.60^{b} ± 0.13	39.19^{a} ± 0.13

ND = not determined

$^{a, b}$ = Values within each measurement with different letters are significantly different at $P<0.05$

3.4. *IN VITRO* HEAT SHOCK OF BOVINE FIBROBLAST CELL-LINE

RT-MIMIC PCR was performed on total RNA extracted from bovine fibroblasts cultured at 38°C (control), 42°C (heat-shock), and 45°C (severe heat shock) for one and two hours. The reactions were performed in the Perkin Elmer 2400 thermocycler for two reasons: (i) the older thermocycler was shown to give inconsistent results due to a heating block problem, and (ii) to verify the usefulness of the MIMIC analysis without the compounding problems of the thermocycler block. RT-MIMIC PCR was performed twice using a different dilution of MIMIC. Results using 6.25×10^{-4} attomole/μl of Hsp70 MIMIC are shown in Figure 3-29. MIMIC bands were too faint to measure, so quantitative analysis could not be performed on these results.

Figure 3-30 illustrates the same RNA samples analyzed with a higher MIMIC concentration, 1.25×10^{-3} attomole/μl. The results show a large stress response to the 45°C heat shock but no response to the 42°C heat shock (Table 3-9). The Hsp70:MIMIC ratio of lymphocytes incubated at 45°C were significantly higher than lymphocytes incubated at 38°C and 42°C. The relative expression of Hsp70 mRNA in the fibroblast sample incubated at 42°C for two hours was lower than expected and did not reflect the increase seen in Figure 3-29. Also, the fibroblast RNA sample that was heat-shocked at 45°C for one hour did not produce a band corresponding to Hsp70, although bands were observed for this sample in the previous reactions (Figure 3-29).

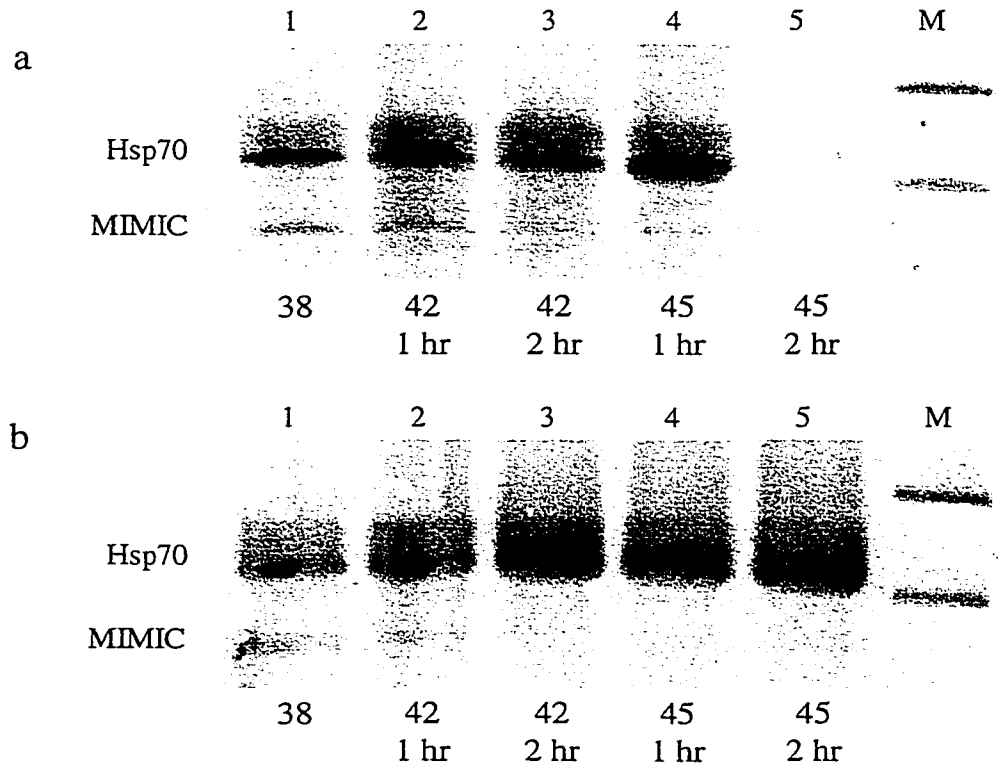

Figure 3-29. Hsp70 mRNA expression in total RNA extracted from bovine fibroblasts incubated at 38°C (control; lane 1, a and b), 42°C (lanes 2 and 3, a and b), or 45°C (lanes 4 and 5, a and b) for one and two hours.

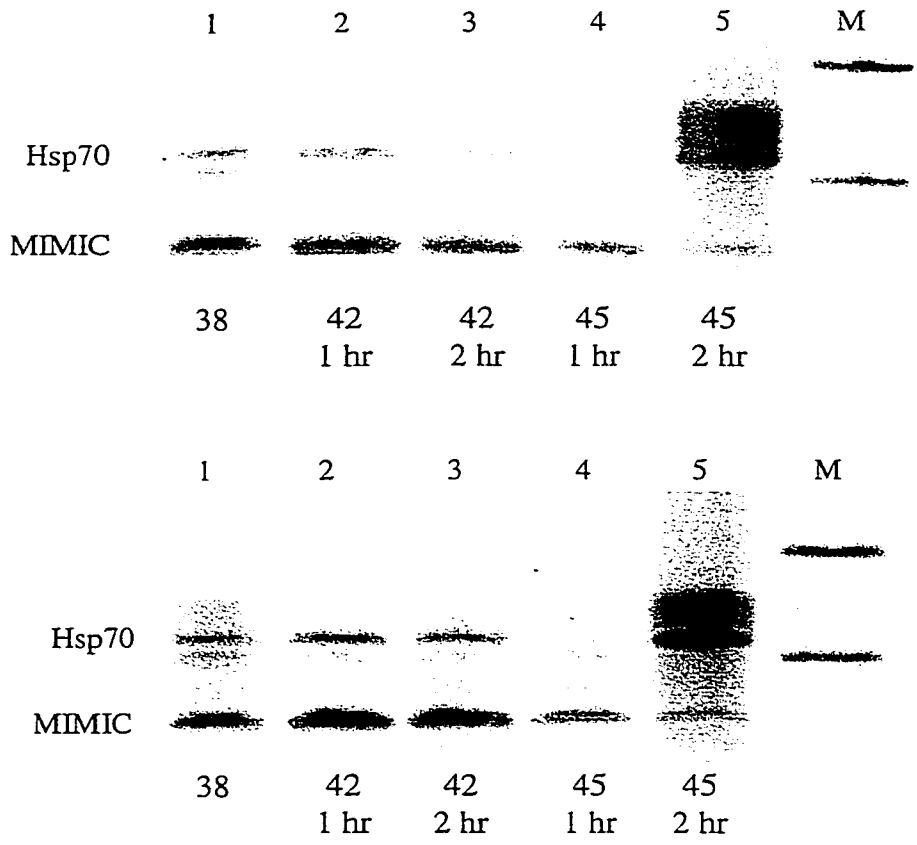

Figure 3-30. Hsp70 mRNA expression in total RNA extracted from bovine fibroblasts incubated at 38°C (control; lane 1), and 42°C (lanes 2 and 3), or 45°C (lanes 4 and 5) for one and two hours. RT-MIMIC PCR reactions were performed in duplicate.

Table 3-10. Relative expression of Hsp70 mRNA in bovine fibroblasts measured using RT-MIMIC PCR.

Temperature (°C)	Time (hours)	Hsp70 Concentration (ng)	Ratio (Hsp70:MIMIC concentration)	Mean Ratio ± SEM	Mean Ratio ± SEM by temp
38	-	11.00 13.74	0.24 0.21	0.22 ± 0.01	0.22 ± 0.01 [b]
42	1	31.36 31.32	0.28 0.30	0.29 ± 0.01	0.25 ± 0.02 [b]
42	2	17.31 14.47	0.20 0.22	0.21 ± 0.01	
45	1	- -	- -	-	13.15 ± 2.45 [a]
45	2	111.24 165.66	10.69 15.60	13.15 ± 2.45	

[a, b] Values with different letters are significantly different at $P<0.001$

4. DISCUSSION

4.1. RT-PCR USING HUMAN HSP PRIMERS

Human sequence-derived primers for various Hsps genes were initially used in an attempt to measure their expression in bovine lymphocyte RNA samples. These primers had already been tested using human RNA samples. It was believed that sequence similarities between the human and bovine Hsp genes might allow these primers to be suitable for performing RT-PCR on bovine RNA. However, all attempts to amplify bovine target sequences failed. Sequence analysis software (Gene Jockey 2.0 and Amplify 1.2) became available and analysis of available bovine Hsp genes revealed that the downstream (anti-sense) primers were suitable for proper priming. However, the upstream (sense) primers were not able to find an appropriate match in the bovine Hsp sequences and this prevented successful RT-PCR. Therefore, the commercial primer sets were of no value for performing RT-PCR on bovine RNA samples. Instead, primer sets for the bovine Hsp genes had to be developed from the available bovine gene sequence information.

4.2. RT-PCR USING BOVINE HSP PRIMERS

Although it was more time consuming than using available primers, it was necessary to develop primers from available bovine Hsp sequence information. At the time this research began, there were only sequences available for a limited number of bovine Hsp genes. Two of the most important Hsps, Hsp70 and Hsc70, were chosen as targets for study. RT-PCR of bovine lymphocyte RNA samples showed that *in vitro* Hsc70 mRNA expression was detected in control (38°C) and heat-shocked (42°C) lymphocytes. This was expected since Hsc70 is constitutively expressed, and has also been shown to be slightly heat-inducible (Lindquist and Craig, 1988).

Hsp70-1 and Hsp70-2 mRNA expression was detected in *in vitro* heat-shocked and control bovine lymphocyte RNA samples. The sensitivity of RT-PCR made it possible to easily detect the low basal expression of Hsp70-1 and Hsp70-2 mRNA in the lymphocytes incubated at physiological (control) temperature.

Analysis of two sets of samples from the *in vivo* controlled environment experiment showed mixed results. Analysis for Hsc70 and Hsp70-1 mRNA did not reveal consistent expression of these products. Hsc70 mRNA was detected only in a sample taken from animal 323 one hour after movement to a new pen. The expression of Hsc70 mRNA should have been apparent in all four samples because Hsc70 is normally constitutively expressed and slightly heat-inducible (Lindquist and Craig, 1988). Perhaps Hsc70 mRNA was not detected in this set of samples because of RNA degradation, RT failure, or inadequate amplification during PCR. Hsp70-1 mRNA expression was not detected in any sample. The movement of the animals to a new pen and transport of the animals may not have been sufficient stressors to induce increased expression of Hsp70-1 mRNA. Alternatively, RNA degradation, RT failure, or inadequate amplification during PCR may explain the lack of Hsp70-1 mRNA expression. Hsp70-2 mRNA was not detected in samples taken before movement to a new pen or before transport for one hour. However, Hsp70-2 mRNA expression was detected in samples taken one hour after movement to a new pen and after transport for one hour, suggesting that both animals were subjected to sufficient stress from both treatments to induce at least a mild heat shock response.

This RT-PCR system was capable of detecting the *in vitro* expression of Hsp70-1, Hsp70-2, and Hsc70 mRNA. However, because an internal control was not used in this set of experiments, it was not possible to accurately quantify differences in expression of Hsp70-1, Hsp70-2, and Hsc70 mRNA. For this reason a competitive PCR approach was adapted to measure relative differences in Hsp mRNA expression.

4.3. QUANTIFICATION OF RELATIVE DIFFERENCES IN HSP MRNA USING PCR MIMICS

Several different methods have been reported that incorporate an internal standard into the PCR. The method of using a non-homologous DNA fragment that has the same primer templates as the target DNA, termed a PCR MIMIC, was chosen for this study. The PCR MIMIC method was chosen because the MIMICs were easy to construct, requiring only one additional set of primers, the composite primers, and two rounds of PCR. The use of a housekeeping gene was ruled out because heat shock is known to cause a decrease in expression of cellular proteins other than the heat shock proteins, especially in *in vitro* heat-shocked cells or tissues (Wang and Edens, 1993).

The PCR MIMIC allowed for semi-quantitative measurement of Hsp70 mRNA. Due to their identical primer templates, the Hsp70 MIMIC fragment and the Hsp70 target template, theoretically, have identical PCR efficiencies, although this was not directly tested. Semi-quantitative measurements between samples were made by comparing the ratio of the amount of DNA in the Hsp70 target product band to Hsp70 MIMIC product band. The MIMIC, which was added during the PCR step in a known concentration, acted as an internal control. Tube-to-tube differences in PCR efficiency could be thus eliminated.

4.4. *IN VITRO* THERMAL SHOCK OF BOVINE LYMPHOCYTES

Preliminary RT-PCR analysis (without MIMIC) of lymphocyte RNA from blood samples taken from different animals immediately following lymphocyte isolation revealed that Hsp70 mRNA was expressed in three out of the four samples analyzed. Hsp70 mRNA and protein can be expressed at low levels under normal physiological conditions and the RT-PCR system was able to detect this basal expression. It is unlikely that the lack of Hsp70 mRNA expression in the one animal (animal #44) was due to failure of the RT and or PCR as the samples were run in duplicate and later repeated for confirmation of results. However, degradation of the RNA sample was possible. Also, it is possible that the lack of Hsp70 mRNA expression in the sample from this animal represented a genetic and/or behavioural difference in this particular animal. This animal may have had an extremely low or a lack of basal Hsp70 mRNA expression.

In vitro heat shock of bovine lymphocytes was expected to increase the expression of Hsp70 mRNA. Expression was expected to increase rapidly (i.e. within the first 30 minutes), to maintain that level of expression for the next few hours, and to return to basal levels 24 hours after heat shock. Guttierrez and Guerriero (1995) reported a 3-fold increase in Hsp70 mRNA and a 2-fold increase in Hsp70 protein in bovine skeletal muscle incubated at 42°C for three hours. Guerriero and Raynes (1990) reported a 14-fold increase in Hsp70 protein synthesis in lymphocytes heat-shocked at 42°C compared to lymphocytes incubated at 37°C. The results obtained in the current study did not follow the expected pattern. Incubation at 42°C of lymphocyte samples from blood from two of the six animals did not show Hsp70 mRNA expression at any time point.. Expression was not detected at any time point or temperature for one of these animals (Animal #101). The measurements of relative expression of Hsp70 mRNA were highly variable for most samples, even within triplicate measurement of the same sample. RNA samples from lymphocytes incubated at 34°C also showed inconsistent results and no meaningful pattern of expression Hsp70 mRNA was observed.

Degradation of sample RNA may explain results where bands corresponding to Hsp70 were not observed in triplicate measurements. During the extraction of RNA, Trizol protects the RNA molecules from degradation by RNases present in the cell and

the RNases are presumably no longer present at the completion of the extraction. However, it is possible that RNase was introduced from an exogenous source. The RNA samples were diluted in DEPC water (RNase-free), however, it is possible that RNase introduced during pipetting of the RNA sample whether the RNase was present on the pipette tip or in the PCR tube.

Testing of the heating block of the thermocycler revealed the likely source of the inconsistency in triplicate measurements. Although diagnostic self-testing of the thermocycler did not reveal any problem, RT-PCR of 14 identical reactions in various wells throughout the heating block indicated that there was substantial variation in generation of PCR products among wells. Nine reactions generated PCR products of varying intensity while the remaining five reactions did not generate any PCR products. Because all the reactions were identical at the start of RT-PCR, the lack of products for these reactions were false negative results. Further testing of the thermocycler block revealed that the wells in the middle and bottom of the block were the main problem areas. Identical RT-PCR reactions performed in the top two rows of the thermocycler (16 reactions total) all resulted in the generation of PCR products of varying intensity. In this case most of the variation could be explained by tube-to-tube variation in RT and PCR efficiency, however, PCR products from reactions run in the right side of each row tended to be less intense than those run on the left.

In consideration of the results from the *in vitro* experiment, the malfunction of the thermocycler could explain the lack of detection of expression of Hsp70 in some of the samples. This was especially likely where PCR products corresponding to Hsp70 mRNA were seen for one or two of the three measurements on each sample. The fact that the Hsp70 MIMIC DNA fragment was amplified consistently in almost all of the reactions indicated that the RT step was most affected by the malfunctioning machine. Unfortunately an internal control was not present during the RT step and the tube-to-tube RT efficiency variation was greater than one would expect. These factors likely obscured treatment differences and probably account for the variability in the results.

4.5. *IN VIVO* CONTROLLED ENVIRONMENT EXPERIMENT

4.5.1. EXPERIMENT ONE – MOVEMENT/TEMPERATURE EXPOSURE

Movement of the heifers to a new pen in either a thermoneutral or cold environment room had mixed effects on the level of relative expression of Hsp70 mRNA. Overall, differences in relative Hsp70 mRNA expression before and after movement were not significant. Pen temperature had no significant effect on relative Hsp70 mRNA expression. Relative Hsp70 mRNA levels were highly variable between individual animals. Grandin (1997) and Zavy *et. al.* (1992) have suggested that the reaction of the animal to stress can be influenced by previous experiences and the perception of the stressor to the animal. Also some animals may be better equipped genetically to deal with stress. These factors may account for the variation in relative Hsp70 mRNA expression from animal to animal. The thermoneutral (warm) environment room was not expected to impose any heat stress on the animal, but the movement of the animal from a familiar pen to a new pen in a new room might have been perceived as a stress to the animal. Although no physical threat was imposed, a psychological stress, fear, may have been sufficient to affect the expression of Hsp70 mRNA in some animals. The psychological stress of exposing rats to the emotional responses of foot-shocked rats, such as jumping and screaming, was shown to increase the level of Hsp70 mRNA in rat aorta (Isosaki and Nakashima, 1998). The mechanism by which a psychological stress triggers an increase in Hsp70 mRNA expression is unknown. The association of Hsp70 with Hsp90 and Hsp56 in the glucocorticoid receptor complex may point to a possible connection between the hormonal and heat shock protein responses to a stressor. The method of obtaining the blood samples may have imposed stress on some animals, whether it was from being in the squeeze or being handled. If the animal perceived the first blood sampling as stressful, the animal may have been more fearful during the second sampling after movement resulting in an increase in Hsp70 mRNA expression. Some animals may not have perceived the situation as stressful or may have been better equipped to adapt through behavioural or other physiological mechanisms.

Movement to a pen in the cold environment (-18°C) was expected to impose stress on the animal, however, nom significant change in Hsp70 mRNA expression was observed.

There was a large variation for some of the samples in triplicate measurements of the ratio of Hsp70:MIMIC band DNA concentrations. Unfortunately, due to small sample size and low RNA yield, there was not sufficient sample remaining to repeat analysis of all the samples. It is unclear whether this variation is due in part, or entirely, to malfunctioning of the heating block identified during the subsequent *in vitro* experiments. RT-MIMIC PCR on the samples from this experiment was performed a few months prior to analysis of the *in vitro* samples. Other analyses performed at this time using the same RT-MIMIC PCR system were consistent. It is believed that the variation in triplicate measurements for this experiment is in part due to the thermocycler heating block. It seems that the heating block problem gradually worsened between the time the *in vivo* experiment samples were analyzed and the time when the *in vitro* lymphocyte samples were analyzed.

4.5.2. Experiment Two - Transport Stress

Transport did not have a significant effect on the relative expression of Hsp70 mRNA in bovine lymphocytes. Although it is uncertain exactly when the heating block problem arouse, malfunction of the thermocycler heating block may have contributed to problems with the analysis of these *in vivo* samples. Transport of animals has been shown to be a significant stressor as shown by increases in cortisol levels (Zavy *et. al.*, 1992; Warriss *et. al.*, 1995). Transport was expected to induce a stress response in the cattle and it is believed that increase in relative Hsp70 mRNA expression would have been apparent if not for the heating block malfunction.

Other measurements taken at the same time indicated that the animals had been subjected to a stressor. Mean core body temperatures and mean N:L were significantly higher after transport, indicating that the animals had undergone some level of stress. Kegley *et. al.* (1997) reported an increase in N:L in blood samples taken from steers that

were transported for 6 hours. An increase N:L is often associated, although not necessarily correlated with, with an increase in cortisol concentration (Stull *et. al.*, 1999). However, cortisol concentration was significantly lower after transport. Warriss *et. al.* (1995) reported that cortisol concentration in steers transported for 5, 10, or 15 hours were all significantly higher over control levels. Mean serum cortisol concentration in intensely exercised horses was found to increase initially at 30 minutes and to decrease significantly at 6 hours after exercise, while N:L was found to increase significantly at 6 hours after exercise (Wong *et. al.*, 1992). The cattle in this experiment were transported for only 1.5 hours, so the decrease in cortisol concentration was unexpected. It may have been associated with diurnal variations in cortisol concentration, since the fist blood sample was taken in the morning when baseline cortisol levels have been shown to be highest. Exhaustion of adrenal cortisol output (MacDonald, 1969) also seems unlikely due to the relatively short duration of the transport.

The data from the two in vivo experiments indicate that variation in the magnitude of response in Hsp70 mRNA expression among animals was quite large and may reflect the different abilities of each animal to adapt to stress.

4.6. *IN VITRO* HEAT SHOCK OF BOVINE FIBROBLAST CELL-LINE

The fibroblast RNA samples were analyzed by RT-MIMIC PCR on a Perkin Elmer 2400 (PE2400) thermocycler instead of the original Perkin Elmer thermocycler. Duplicate measurements of the samples resulted in consistent products indicating that the original thermocycler was likely the cause of the inconsistencies observed in the earlier experiments.

Fibroblasts cultured at the control temperature, 38°C, were found to express Hsp70 mRNA. Expression of Hsp70 mRNA and protein is very low under normal physiological conditions (Lindquist and Craig, 1988). The extreme sensitivity of RT-PCR may be the reason Hsp70 mRNA was so easily detected in these cells. An increase in relative Hsp70 mRNA expression was not seen at 42°C. Other research has shown 42°C to be a sufficient heat shock to induce Hsp70 mRNA and/or protein expression in bovine cells

(Guerriero and Raynes, 1990; Kochevar *et. al*, 1991). The bovine fibroblasts responded to the 45°C heat shock by increasing their expression of Hsp70 mRNA. This presumably led to an increase in Hsp70 protein in the cells. The high temperature triggered a heat shock response likely due to the formation of abnormally folded proteins in the cell. The presence of abnormally folded proteins is believed to be the main inducer of a heat shock response (Kelley and Schlesinger, 1978; Anathan *et. al.*, 1986; Goff and Goldberg, 1987). A much larger response to the heat shock was seen at 45°C probably due to the higher temperature causing more damage to cellular proteins. A greater concentration of Hsp70 was likely required to bind to and repair the damaged proteins.

It was unclear why in initial measurements of relative Hsp70 mRNA expression by RT-MIMIC PCR that the MIMIC bands were faint. The amount of MIMIC added had been determined to result in 1:1 ratio of Hsp70 to MIMIC PCR product band concentration. When a greater concentration of MIMIC was added to the PCR reaction, both Hsp70 and MIMIC bands were present. Calculation of Hsp70:MIMIC demonstrated that relative Hsp70 mRNA expression was significantly greater in the fibroblasts heat-shocked at 45°C compared to fibroblasts heat-shocked at 42°C or the 38°C control fibroblasts. An increase in the relative expression of Hsp70 mRNA was not seen for the fibroblasts heat-shocked at 42°C. This result did not agree with previous results obtained with these samples. Also, a band corresponding to Hsp70 was not detected from the fibroblasts incubated at 45°C for one hour, although strong Hsp70 product bands were observed using this sample in the previous analysis. The reason for the lack of heat shock response at 42°C and the lack of products at 45°C for one hour was believed to be due to partial or complete degradation of the RNA samples. Although preventative measures were taken, it is possible that RNase contamination occurred from contaminated latex gloves, pipettes, pipette tips, or tubes.

5. CONCLUSIONS

Although there were problems with the assay, some important observations were made. Hsp70 mRNA expression was detected in lymphocyte RNA samples from 19 out of 20 animals examined (including *in vitro* and *in vivo* experiments). The *in vitro* heat shock of bovine lymphocytes did not show the expected pattern of Hsp70 mRNA induction, but this was believed to be due to inconsistencies caused by the thermocycler malfunction. The *in vivo* experiments indicated that there was variation in the response of individual animals to the handling treatments with regards to the relative levels of Hsp70 mRNA expression. The *in vitro* heat shock of bovine fibroblast showed that there is potential to use relative changes in Hsp70 mRNA as an indicator of the level of stress (i.e. degree of heat shock) to which cells have been exposed.

6. FUTURE CONSIDERATIONS

The current study demonstrated the potential for quantification of stress protein mRNA as an indicator of stress in cattle, however, further study is required to fully evaluate its application. Future studies should consider the following points: (i) the animals should be of a common genetic and environmental background, (ii) the use of catheters for blood sampling, since some animals may respond to the stress of being placed in the squeeze, and (iii) the use of an internal standard for the RT and PCR by either modifying the MIMIC PCR system or adapting a new system. Consideration of these factors will lessen the variation of individual animal responses to the same stressor and modification of the PCR system will ensure that quantification of Hsp70 mRNA level is accurate.

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8. APPENDICES

APPENDIX A. LYMPHOCYTE ISOLATION BUFFERS

10 x PBS	PBS/2%FBS	Lysis Buffer
80 g NaCl	50 ml 10 x PBS	4.01 g NH Cl
2.0 g KCl	10 ml fetal bovine serum	0.42 g $NaHCO_3$
14.4 g Na_2HPO_4	440 ml d.d. H_2O	0.1 ml EDTA (0.5 M)
2.4 g KH_2PO_4		in 500 ml d.d. H_2O
in 1 L H_2O		
adjust pH to 7.4		

2% Bovine Serum Albumin (BSA) in phosphate-buffered saline (PBS):

PBS

8.0 g NaCl
0.2 g KCl
1.15 g Na_2HPO_4
0.2 g KH_2PO_4
in 1 L H_2O

- dissolve chemicals and adjust pH to 7.4
- autoclave solution at 121°C for 20 minutes

2% BSA

- make a stock solution of 10% BSA
- sterile filter
- dilute the sterile 10% BSA solution with sterile PBS to make to 2% BSA
- add 1 ml antibiotic/antimycotic to each 100 ml of sterile buffer

Complete Culture Media (CCM)(500 ml):

445 ml RPMI-1640 with glutamine
50 ml fetal bovine serum (FBS)
5 ml antibiotic/antimycotic

APPENDIX B. GEL ELECTROPHORESIS RUNNING BUFFER

0.0045 M Tris-borate, 1 mM EDTA (0.5xTBE):

Concentrated 5xTBE stock solution

54 g Tris base
27.5 g Boric acid
20 ml 0.5 M EDTA (pH 8.0)
in 1 L d.d. H_2O
- autoclave solution at 121°C for 20 minutes
- dilute 1:10 with d.d. H_2O to make O.5xTBE running buffer

APPENDIX C. RNA QUALITY

The quality of the RNA samples following extraction was examined by gel electrophoresis of the RNA samples (see Figure C-1 for example). Briefly, the agarose gel was made using 0.35 g of agarose dissolved in 30 mL d.d. H_2O by heating. After the solution had cooled, 3.5 mL 10xMOPS and 1.9 mL of formaldehyde were added and the gel was poured. 1 x MOPS was used as the running buffer. Five microliters of RNA (1 µg/µl) and 10 µl loading buffer (see below) were mixed, boiled for 2 minutes and cooled on ice. The samples were loaded and run at 100 mv for 1.5 hours.

Loading Buffer:

48 µl deionized formamide
17.3 µl 37% formaldehyde
34.7 µl loading dye

Loading Dye:

160 µl 10 x MOPS
175 µl H_2O
25 µl ethiduim bromide (10 mg/mL)
80 µl glycerol
80 µ saturated bromothymol blue

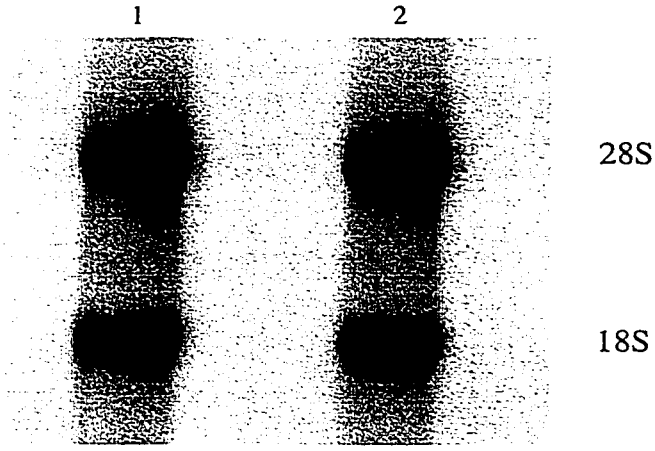

Figure C-1. Gel electrophoresis of RNA samples. Lane 1 is RNA isolated from bovine lymphocytes incubated at 38°C and lane 2 is RNA isolated from bovine lymphocytes incubated at 42°C for one hour.

APPENDIX D. DETERMINATION OF DNA CONCENTRATION IN PCR PRODUCT BANDS AND OF HSP70:MIMIC RATIOS

In Molecular Analyst, the profile option was used to determine the area (counts*mm) of each product band including the molecular size and DNA concentration standard. The area of each standard band was plotted against the square root of the known amount of DNA in each band. Table D-1 shows typical area values used to generate standard plots. The standard plot generated by this data is shown in Figure D-1. The equation of the line was determined and used in calculating the amount of DNA in the HSP70 and MIMIC bands. In this example the amount of DNA in each band was determined by the equation:

$$\text{Amount of DNA (ng)} = [(\text{area} + 41.784)/24.784]^2$$

The Hsp70:MIMIC ratio for each sample was determined by dividing the Hsp70 band concentration by the MIMIC band concentration. Even though each gel was run under the same conditions, a standard plot had to be generated for each gel due to slight differences in standard areas.

Table D-1. Typical values used to generate standard plot of area vs. square root DNA.

Standard molecular size (bp)	Amount of DNA (ng)	Square root DNA (ng)	Area (counts*mm)
2000	100	10	200.431
1200	60	7.745967	154.861
800	40	6.324555	120.125
400	20	4.472136	68.895
200	10	3.162278	32.725

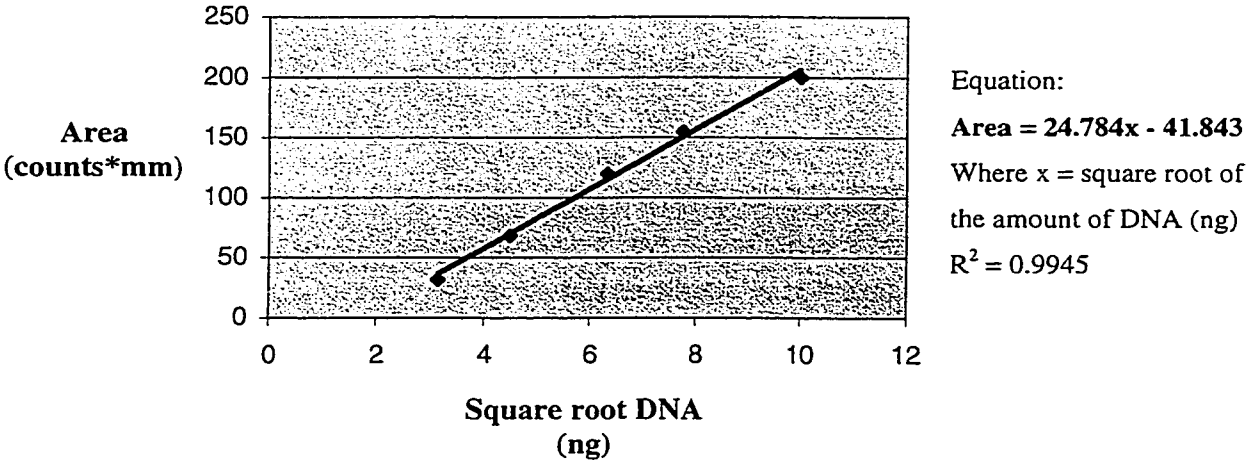

Figure D-1. Standard plot of area versus square root of the amount of DNA.