MOLASSES AS A POSSIBLE CAUSE OF "ENDOCRINE DISRUPTIVE SYNDROME" IN CATTLE

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MOLASSES AS A POSSIBLE CAUSE OF "ENDOCRINE DISRUPTIVE SYNDROME" IN CATTLE

By

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LIST OF ABBREVIATIONS

γ-HCH:	Gamma-hexachlorocyclohexane
γ-IFN:	Gamma-interferon
A/G:	Albumin/Globulin ratio
AbBaso:	Absolute basophils
AbEos:	Absolute eosinophils
AbLymp:	Absolute lymphocytes
AbMono:	Absolute monocytes
AbNmat:	Absolute neutrophils (mature)
AbNimm:	Absolute neutrophils (immature)
ALB:	Albumin
ALP:	Alkaline phosphatase
ANOVA:	Analysis of variance
ARC:	Agricultural Research Council
AST:	Aspartate aminotransferase
Bisphenol A:	4,4' isopropylidenediphenol
Ca:	Calcium
CCN:	Cerebrocortical necrosis
C Fat:	Crude fat
CFT:	Complement fixation test
Con-A:	Concanavilin A
CP:	Crude protein
Cpm:	Counts per minute
Creat:	Creatinine
Cu:	Copper
DCHP:	Dicyclohexyl phthalate
DDE:	Dichloro-diphenyl-dichloroethylene
DDT:	Dichloro-diphenyl-trichloroethane
DEHP:	Di(2-ethylhexyl)phthalate

DM:	Dry matter
EDCs:	Endocrine disruptive compounds
EDS:	Endocrine disruptive syndrome
EM:	Electron microscopy
FA:	Fluorescent antibody
Fe:	Iron
GGT:	Gamma-glutamyltransferase
GLOB:	Globulins
Hb:	Haemoglobin
Ht:	Haematocrit
IBR:	Infectious Bovine Rhinotracheitis
IgG:	Immunoglobulin G
IgM:	Immunoglobulin M
IL-6:	Interleukin-6
ISCW:	Institute for Soil, Climate and Water
K:	Potassium
KP:	Molasses collected at Komatipoort Sugar Mill
LDH:	Lactate dehydrogenase
MCF-7:	Human breast cancer cell line
MCHC:	Mean corpuscular haemoglobin concentration
MCV:	Mean corpuscular volume
ME:	Metabolisable energy
MEHP:	Monoethylhexyl phthalate
Mg:	Magnesium
MJ:	Mega Joules
Mn:	Manganese
mRNA:	Messenger ribonucleic acid
Na:	Sodium
NO:	Molasses collected at Noodsberg Sugar Mill
NP:	4-Nonylphenol; *p*-nonylphenol

OBP:	Onderstepoort Biological Products
OD:	Optical density
OVI:	Onderstepoort Veterinary Institute
P:	Molasses collected at Pongola Sugar Mill
P:	Phosphorous
PBBs:	Polybrominated biphenyls
PCBs:	Polychlorinated biphenyls
PCDDs:	Polychlorinated dibenzo-*p*-dioxins
PCDFs:	Polychlorinated dibenzofurans
PEM:	Polioencephalomalacia
RCC:	Red cell count
RDW:	Red cell distribution volume
RT-PCR:	Reverse transcription–polymerase chain reaction
SD:	Standard deviation
SI:	Stimulation Index = mean counts per minute (cpm) of tested sample/mean cpm of unstimulated control
SMRI:	Sugar Milling Research Institute
T_3:	Tri-iodo thyronine
T_4:	Tetra-iodo thyronine (thyroxine)
TDS:	Total dissolved solids
TH:	Thyroid hormone
Thr C:	Thrombocyte count
TRs:	Thyroid nuclear receptors
TRβ:	Thyroid hormone receptor Beta
TSP:	Total serum proteins
UK:	Molasses collected at Umzimkulu Sugar Mill
UPBRC:	University of Pretoria Biomedical Research Centre
US EPA:	Environmental Protection Agency of the United States
Vtg:	Vitellogenin
WCC:	White cell count

WM:	Wet mass
Zn:	Zinc

SUMMARY

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By

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Promoter:	**Prof. C.J. Botha**
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The objective of this study was to evaluate the suspected endocrine disruptive effect of molasses included in cattle feed. During the mid 1990´s a potentially serious, chronic syndrome was reported in well-managed beef and dairy herds from unrelated parts of South Africa. Farmers reported that it manifested as various combinations of seriously decreased production,

increased reproductive disorders, apparent immune incompetence in previously immune competent animals, various mineral imbalances in non-deficient areas and goitre, perceptible by enlarged thyroids. Farmers related this syndrome to certain batches of sugarcane molasses and molasses-based products. Their opinion was based on observations that dramatic improvements in health and productivity often followed the withdrawal of the molasses component of the diet.

The syndrome had all the characteristics of an "endocrine disruptive syndrome". Endocrine disrupting compounds (EDCs) are exogenous substances that have the potential to alter the functions of the endocrine system and consequently cause ill health. The EDCs comprise a diverse group of compounds that are widely used in detergents, in the paint and plastics industries and as wetting agents in agricultural remedies. Contamination of molasses with these compounds could occur as a result of uptake of chemicals by growing sugarcane or by the use of contaminated river water in the extraction of sugar from chopped sugarcane.

It was essential to investigate the safety of molasses, currently available as stock feed in South Africa and the rest of the world, to allay any fears created by the farmers. Four batches of molasses previously collected from four different sugar mills located in different parts of the country were screened for potential endocrine disruptive activity, including oestrogen and

thyroid activity and immune suppression. Two batches of molasses were selected to be used in a calf feeding trial. Thirty-two, 4 to 6 week-old Holstein bull calves of approximately the same weight, and supplied by the same rearing facility, were included in a single phase, three treatment, parallel design experiment. Calves from the three experimental groups were housed in the same facilities, under the same conditions and fed diets with similar composition. Two of the groups had molasses (Umzimkulu and Komatipoort) included in their rations and the control group was fed a ration where no molasses was added, but fermentable sugars and minerals were included. The weight gain of the calves was recorded over a six-month study period. Regular clinical examinations were conducted and clinical pathology parameters, immune responses and endocrine effects were evaluated.

Even though endocrine disrupting effects were detected in *in vitro* screening tests, these could not be reproduced in calves under experimental conditions. The two batches of molasses utilized in the calf feeding trial did not induce major differences in any of the parameters measured, except a lower weight gain in the Umzimkulu molasses fed group which tended towards significance.

It appears improbable that these two batches of molasses had any endocrine disruptive or immunosuppressive effects. Therefore, these two batches of molasses *per se* were not deleterious when fed to calves.

CHAPTER 1

INTRODUCTION

A potentially serious, chronic syndrome was reported in well-managed beef and dairy herds from unrelated parts of South Africa. Farmers reported that it manifested as various combinations of:

- Seriously decreased production (weaning weights, milk yield and loss in condition particularly of dairy cows).
- Increased reproductive disorders with poor conception, resorptions and abortions.
- Apparent immune deficiency/incompetency/breakdown resulting in calf diarrhoea, mastitis, pneumonia caused by various pathogens, foot-rot, verminosis in adult stock and relapses of protozoal diseases in animals considered to be immune competent.
- Various mineral imbalances, copper-deficiency in non-deficient areas, selenium-deficiency in spite of a kidney selenium excess and high kidney lead concentrations.
- Goitre manifesting as enlarged and/or cystic thyroids in both adults and aborted foetuses.

Farmers reported this syndrome widely from various areas, in particular the Natal midlands, Eastern Cape Province, North-West Province and Gauteng.

The occurrence of this syndrome seemed probable, based on conception figures and weaning weights received from especially Bonsmara breeders, as it is compulsory for Bonsmara breeders to keep these records. According to certain farmers the syndrome first appeared in the mid 1990's and they related it to certain batches of sugarcane molasses and molasses-based products. Their opinion was based on observations that dramatic improvements in health and productivity often followed the withdrawal of the molasses component of the diet.

The syndrome has all the characteristics of an "endocrine disruptive syndrome" (EDS), a relatively new concept in toxicology that has been described in a variety of wildlife species, but never in cattle. It has, however, been predicted by Rhind (2002) that when it occurs it will be characterised by infertility and immune suppression. Furthermore, there is increasing evidence of causal relationships between endocrine disrupting compounds (EDCs) and the health and reproductive function of farm animals (Rhind 2005).

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

During the past 60 years, environmental pollution research has become extremely important (i.e.; over 5 000 entries in ScienceDirect up to date). The exponential increase in the human population exerts profound effects on the environment and has created much awareness amongst toxicologists, endocrinologists, veterinarians, environmentalists, ecologists, nutritionists, oncologists and other specialized disciplines.

Environmental pollution due to anthropogenic organic compounds causes accumulation of potentially toxic compounds in the food chain (Ap Dewi, Axford, Fayez, Marai & Omed 1994). A group of exogenous compounds has been the target of extensive studies in the past decades due to the potential hazard they present for human and animal health (Gandolfi, Pocar, Brevini & Fischer 2002). These compounds are commonly termed "endocrine disrupting compounds" (EDCs) or "endocrine disruptors".

Endocrine disruptive syndromes have been described in a variety of wildlife species and also in humans. Strong evidence exists, that relates the effects seen in these species to their exposure to environmental contaminants (Lamb 1996; Guillette 2000(a)).

Extensive literature on endocrine disruptors is available and is summarised by Colborn, Dumanoski and Myers (1996) in their definitive book "Our Stolen Future" and by Norris and Carr (2006) in their book "Endocrine Disruption - Biological basis for health effects in wildlife and humans".

2.2 ENDOCRINE DISRUPTING COMPOUNDS (EDCs)

An EDC has been defined as an exogenous substance or mixture that alters the functions of the endocrine system and consequently causes adverse health effects in an intact organism or its progeny (IPCS Steering Group 1998).

The biological actions of hormones synthesized within an organism are mediated by high-affinity receptor proteins, which are located in the target cells. When hormones interact with these receptors, a complex sequence of events occurs and finally a sequence of effects associated with that particular hormone starts (Amaral Mendes 2002). Hormones, therefore, influence a particular biological reaction and consequently an excess of a particular hormone may be as detrimental as a deficiency (McDonald 1977).

Most EDCs have the ability to act as either hormone agonists or antagonists and/or disrupt production, secretion, transportation, metabolism, receptor binding and excretion of natural hormones which regulate developmental processes and support endocrine homeostasis in the organism (Kavlock, Daston, DeRosa, Fenner-Crisp, Gray, Kaattari, Lucier, Luster, Mac, Maczka, Miller, Moore, Rolland, Scott, Sheehan, Sinks & Tilson 1996). This ability of EDCs may, amongst others, result in oestrogenic, anti-oestrogenic, androgenic and anti-androgenic effects, which will affect the normal reproductive functions of animals. Furthermore, there is also evidence that some of these compounds may act through mechanisms that do not directly involve receptor binding (Li & Hansen 1996).

Endocrine disrupting effects are not confined to the steroidal hormones; they can also influence thyroid hormone secretion patterns with associated physiological consequences (Hansen 1998). It has been demonstrated that a number of these environmental agents can alter thyroid hormone levels in humans and animals (Langer, Tajtakova, Fodor, Kocan, Bohov, Michalek & Kreze 1998; Ishihara, Sawatsubashi & Yamauchi 2003). An increasing number of reports are showing that some EDCs can affect the thyroid hormone regulated gene expression (Zoeller 2005) and cause disruption of the thyroid function (Rhind 2002). Thyroid hormones play a key role in the maintenance of body homeostasis. When environmental chemicals alter the status of thyroid hormones, this may lead to changes in basal metabolic rate, lipid metabolism, as well as cardiovascular, gastrointestinal and muscle function (Amaral

Mendes 2002). Thyroid hormones are also essential during growth and development (Dussault & Ruel 1987). Hypothyroidism in rodents has been reported after exposure to polychlorinated biphenyls (PCBs) and chlorinated pesticides (Crisp, Clegg, Cooper, Wood, Anderson, Baetcke, Hoffman, Morrow, Rodier, Schaeffer, Tovart, Zeeman & Patel 1998).

A similar group of compounds, referred to as "immunotoxicants", are pollutants often classified with EDCs and are thought to affect the immune system, mainly by disrupting B- and T-cell homeostasis (Sweeny 2002). Changes in immune competency can result in sub-clinical disease and consequently reduced production levels (Rhind 2002). The observations that exposure of humans to PCBs, carbamates, organochlorines, organometals and certain heavy metals alter immune function, indicate potential immune-suppression and thus increased disease susceptibility (Davis & Safe 1990). Experimental animal studies support these observations (Loose, Pittman, Benitz & Silkworth 1977).

The EDCs are widely used in detergents, in the paint and plastic industries and as wetting agents in agricultural remedies (Warhurst 1995). Some of the most important compounds included in this classification are (Colborn *et al.* 1996):

- Organohalogen compounds
- Phthalates
- Bisphenol A
- Alkylphenols

- Other compounds such as phyto-oestrogens (naturally occurring plant oestrogens)

2.2.1 Organohalogen compounds

Organohalogen compounds are of major interest because of their long half-life in the organism and the environment. This category of compounds comprises a large group of substances, e.g. pesticides, such as dichloro-diphenyl-trichloroethane (DDT) and its metabolites and gamma-hexachlorocyclohexane (γ-HCH); PCBs, polybrominated biphenyls (PBBs), polychlorinated dibenzofurans (PCDFs) and polychlorinated dibenzo-*p*-dioxins (PCDDs) (Pflieger-Bruss, Schuppe & Schill 2004).

The majority of these compounds are environmental pollutants. Since these compounds are chemically very stable and strongly lipophilic, they have low degradation rates and can cause endocrine disruption, mainly oestrogenic effects (Muñoz de Toro, Beldoménico, García, Stoker, De Jesús, Beldoménico, Ramos & Luque 2006). Metabolites of DDT, such as dichloro-diphenyl-dichloroethylene (DDE), have the ability to inhibit androgen receptors affecting reproductive development (Kelce, Gray & Wilson 1998) and also have the potential to produce immunotoxicity (Thomas 1995).

Polychlorinated biphenyls are persistent organic compounds consisting of a pair of benzene rings with different degrees of chlorination (Chana, Concejero,

De Frutos, Gonzalez & Herradon 2002). These compounds have the potential to induce endocrine disruption by reducing circulating levels of thyroxin (T_4) in animals such as rats (Aldridge 1996; Li *et al.* 1996; Zoeller, Downling & Vas 2000), affect oocyte maturation (Gandolfi *et al.* 2002; Brevini, Vassena, Paffoni, Francisci, Fascio & Gandolfi 2004) and also have deleterious effects on male rat reproduction, including reduced number of matings and impaired fertility in post-natally exposed rats (Ahlborg, Hanberg & Kenne 1992).

Polychlorinated biphenyls are also included in the group of environmental immunotoxicants amongst dibenzofurans (PCDFs), dibenzo-*p*-dioxins (PCDDs), oxidant gases, benzene, toluene, xylol, dimethylnitrosamine, asbestos, lead, mercury and cadmium (Vos & Van Loveren 1995).

The PCDDs (dioxins) and PCDFs are groups of compounds with similar chemical and toxicological properties. Principal environmental sources of dioxins are emissions from industrial chlorination processes and combustion of materials containing chlorine (Fries 1995). Dioxins can cause abnormal development of the immune system and the brain and reduce thyroid hormone concentrations in rat neonates following pre-natal exposure (Morse, Groen, Veerman, Amerongen, van Koeter, Smits-van-Prooije, Visser, Koeman & Brouwer 1993).

2.2.2 Phthalates

Phthalate esters are plasticizers used in toys, vinyl floors, food and beverage wrappings and cosmetic and medical products and are therefore ubiquitous in our environment (Jobling, Reynolds, White, Parker & Sumpter 1995; Nakai, Tabira, Asai, Yakabe, Shimyozu, Noguchi, Takatsuki & Shimohigashi 1999). Certain phthalates have oestrogenic properties and can accumulate in fat and liver tissue of ruminants (Boerjan, Freijnagel, Rhind & Meijer 2002). Phthalates have deleterious effects on testicular physiology, not only in rodent species, but also guinea pigs, rabbits, frogs and ferrets (Parks, Ostby, Lambright, Abbott, Klinefelter, Barlow & Gray 2000).

Di(2-ethylhexyl)phthalate (DEHP) and its toxic metabolite monoethlyhexyl phthalate (MEHP), have been shown to cause a wide range of undesirable adverse effects in animals. These effects include non-reproductive problems such as hepatotoxicity, hepatic and renal malignancies as well as reproductive toxicity (teratogenicity, testicular atrophy, hypospermia and ovarian dysgenesis) (Luban, Rais-Bahrami & Short 2006). Di(2-ethylhexyl)phthalate can alter the expression of genes that are involved in testis development and steroid hormone synthesis (Wong & Gill 2002). Gray and Foster (2003) also described in detail the "phthalate syndrome" in rats where deleterious effects on reproductive development include, amongst others, a decrease in foetal testicular testosterone.

2.2.3 Bisphenol A

Bisphenol A (4,4' isopropylidenediphenol) is mainly used in the manufacture of plastics such as dentures; polycarbonate plastics and epoxy resins used to coat food and beverage containers (Lewis, Rueggeberg, Lapp, Ergle & Schuster 1999; Maffini, Rubin, Sonnenschein & Soto 2006). Bisphenol A is also commonly halogenated (chlorinated or brominated) to produce flame retardants (Thomsen *et al.* 2002).

Even though there is strong evidence that brominated flame retardants can affect thyroid hormone metabolism (Ghisari & Bonefeld-Jorgensen 2005), a number of studies have also indicated that these compounds may interfere with endogenous oestrogen (Legler & Brouwer 2003). They can exert an oestrogen-mimic effect (Staples, Dorn, Klecka, O'Block & Harris 1998; Maffini *et al.* 2006) by binding to the oestrogen receptor and inducing oestrogen receptor-mediated gene expression (Matthews, Twomey & Zacharewski 2001).

2.2.4 Alkylphenols

Alkylphenols and their ethoxylates are non-ionic surfactants, used worldwide in household products, agricultural chemical products and in paper and plastic manufacturing (Nimrod & Benson 1996). They consist of a phenol group and an alkane. Commercially available alkylphenols usually consist of a mixture of alkylphenols with various degrees of branching, but with the same number of

carbon atoms. Alkylphenol ethoxylates are not oestrogenic *per se,* but upon degradation during sewage treatment they may release oestrogenic alkylphenols (Sonnenschein & Soto 1998).

4-nonylphenol (para-nonylphenol, NP) is commercially the most prevalent of the alkylphenol family (Pflieger-Bruss *et al.* 2004). Nonylphenol exerts toxic effects on testes and epididymi of male rats exposed during early post-natal life (De Jager, Bornman & Oosthuizen 1999). Certain alkylphenols (e.g. 4-nonylphenol, 4-octylphenol) exhibit the potential to exert thyroid hormone disruption (Ghisari *et al.* 2005).

2.2.5 Other EDCs

Numerous plants contain naturally occurring substances with oestrogenic activity (Farnsworth, Bingel, Cordell, Crane & Fong 1975) and these substances are commonly known as phyto-oestrogens. Phyto-oestrogens have 2-phenylnaphthalene-type chemical structures, which render their chemical structure similar to those of oestrogens (Zava & Duwe 1997; Tham, Gardner & Haskell 1998).

Trifolium spp. (red, white and subterranean clover), which are widely used to improve pastures, contain oestrogenic glycosides known as isoflavones and *Medicago* spp. (lucerne) contains coumestans (Kellerman, Coetzer, Naudé &

Botha 2005). Cattle and sheep are exposed to these oestrogenic toxic principles when grazing on pastures containing any of these plant species.

Isoflavones are contained by a variety of plants, including fruits and vegetables, but they are predominantly present in leguminous plants and are especially abundant in soya. These substances have a common diphenolic structure that resembles the structure of the potent synthetic oestrogen, diethylstilboestrol and hexestrol (Tham *et al.* 1998). Isoflavones can exert both oestrogenic and anti-oestrogenic effects on metabolism, depending on their own concentration and the concentration of endogenous oestrogens (Wood, Register, Franke, Anthony & Cline 2006).

The fungus *Fusarium graminearum* produces zearalenone, a mycotoxin with proven oestrogenic activity (Kellerman *et al.* 2005). Zearalenone is a polyketide (resorcyclic acid lactone) and is a natural contaminant commonly found in mouldy feeds and feed ingredients such as corn and grain sorghum, but has also been found as a contaminant in wheat, hay and silage (Welshons, Rottinghaus, Nonneman, Dolan-Timpe & Ross 1990).

2.3 ENVIRONMENTAL FATE OF EDCs

Most EDCs are synthetic organic chemicals being introduced to the environment by anthropogenic inputs (e.g. bisphenol A) and therefore are

ubiquitous in aquatic environments receiving wastewater effluent (Jiang, Yin, Zhou & Pearce 2005).

Endocrine disrupting compounds are markedly hydrophobic and tend to associate with organic or mineral matter (Wild & Jones. 1992) or are lipophilic and therefore sequestered in fat depots of exposed animals (Thomas & Colborn 1992; Nimrod *et al.* 1996). Some of these compounds also exhibit long half-lives in the environment, resulting in a continuing increase of the global environmental concentration (Brevini, Cillo, Antonini & Gandolfi 2005).

Unlike persistent organic pollutants (such as organochlorine pesticides), phthalates and their metabolites have a short half-life and don't accumulate in the environment. However, when adsorbed to soil and sediments, phthalates can persist in the environment for years (Chou & Wright 2006).

High concentrations of EDCs are detected in sewage sludge from agricultural, industrial and even domestic origin (Abbassy, Ibrahim & El-Amayem 1999). They are also present in river water in different concentrations reflecting the level of contamination (Abbassy 2000; Jiang *et al.* 2005). EDCs can also pollute agricultural soils and be transferred to crop plants and livestock (Wild *et al.* 1992). In fact, Boerjan *et al.* (2002) demonstrated that certain EDCs can accumulate in the fat and liver tissue of livestock grazing on pastures fertilized with sewage sludge. On the other hand, sound experimental data, indicate

negligible contamination of crop plants with toxic organic chemicals in sludge-amended soils (O'Connor 1996; Harrison, Oakes, Hysell & Hay 2006).

2.4 BIOACCUMULATION OF EDCs

Certain EDCs, being lipid soluble, are well absorbed, have a high bioavailability and accumulate in fatty tissues (Hooper & McDonald 2000). Polychlorinated biphenyls are chemically stable compounds and due to their lipophilic nature, they have been detected in fatty tissues of humans and animals (Sandvik 2002).

Organochlorines preferentially bioaccumulate in the adipose tissue, however, DDT and metabolites, γ-HCH, PCBs and PCDDs are also found in fluids of the female reproductive tract, such as in cervical fluid of women (Hanf, Behnisch, Körner, Sonntag, Tinneberg & Hagenmaier 1995).

Significant concentrations of some of these compounds are stored in the fat depots of animals which are mobilized from their storage sites during fasting or physiological conditions with high energy demands (Sweeney, Nicol, Roche & Brooks 2000). Bigsby, Caperell-Grant & Madhukar (1997) demonstrated that the release of pesticide residues from fat stores during fasting produces oestrogenic effects in ovariectomized mice.

Ahel, McEvoy and Giger (1993) evaluated the bioaccumulation potential of nonylphenol and lipophilic nonylphenol ethoxylates, such as nonylphenol monoethoxylate (NP1EO) and nonylphenol diethoxylate (NP2EO) in freshwater organisms under natural environmental conditions. The study was conducted at the Glatt Valley, a densely populated region in the northern part of Switzerland. They concluded that these compounds have a considerable potential to bioaccumulate in freshwater organisms, including algae, fish and wild ducks. In macrophytic algae, high concentrations of the compounds investigated were found (up to 38 mg/kg, 80 mg/kg, and 28 mg/kg of NP, NP1EO and NP2EO, respectively), the bioconcentration factors of NP reaching up to 10,000. The concentrations in fish were much lower (NP: < 0.03-1.6 mg/kg, NP1EO: 0.06-7.0 mg/kg, and NP2EO: <0.03-3.1 mg/kg suggesting that biomagnification did not take place. Similar concentrations to those in the fish were determined in a wild duck. The estimated bioconcentration factors in fish tissues ranged from 13 to 410 for NP, 3 to 300 for NP1EO and 3 to 330 for NP2EO.

Fossi, Casini, Marsili, Ancora, Mori, Neri, Romeo and Ausili (2004) performed an experiment in swordfish (*Xiphias gladius*) caught in the Strait of Messina, Sicily, Italy. Their results indicate a correlation between PCB concentrations in male fish livers and positive responses to various EDC screening tests, including the vitellogenin assay.

Even though phthalates can persist for years in soil and sediments, they don't bio-accumulate in living organisms and therefore high concentration of these compounds in body tissues indicate recent exposure (Chou *et al.* 2006).

2.5 EFFECTS OF EDCs

2.5.1 Introduction

Environmental chemicals are generally less potent than endogenous hormones, such as oestradiol, but it is clear that they act additively (Hyder, Kirkland, Loose-Mitchell, Makela & Stancel 1999). Therefore, very low levels of environmental contaminants with oestrogenic activity could have a significant impact when added to the existing circulating levels of endogenous steroidal hormones (Silva, Rajapakse & Kortenkamp 2002). In addition, chronic or long-term exposure to small concentrations in the animal's diet may eventually increase the body burden to elicit an oestrogenic response (Welshons *et al.* 1990).

The fact that animals are generally exposed to mixtures of EDCs has been taken into account by Rajapakse *et al.* (2002), who demonstrated that even though each component of a mixture of EDCs is present at concentrations that produce no observable biological effect, the mixture might exert significant effects.

2.5.2 Effects of EDCs in humans

Endocrine disruptors have been implicated in a wide range of undesirable biological phenomena. Over the past 60 years, numerous epidemiological studies have demonstrated negative effects of these environmental pollutants on human health (Auger, Kunstmann, Czyglik, & Jouannet 1995; Lamb 1996). These compounds affect the reproductive system of mammals, various fish, bird and other wildlife species (Colborn, Vom Saal & Soto 1993; Campbell & Hutchinson 1998) and also affect the immune system (Rier, Martin, Bowman & Becker 1995) and thyroid function (Zoeller 2005).

Epidemiological studies have indicated that during the past six decades the quality of human spermatozoa have decreased and the incidence of male genital tract defects and testicular, prostate and breast cancer has increased. Developmental, reproductive and endocrine effects have also been documented in wildlife during the same period of time (Maffini *et al.* 2006).

Carlsen, Giwercman, Keiding and Skakkebaek (1992) have described in their influential paper *"Evidence for decreasing quality of semen during past 50 years"*, that there has been a decline in semen quality during that period. As male fertility is correlated with spermatozoa count, these results may be a true reflection of a reduction in male fertility. Three years later, they demonstrated that there is a correlation between the decline of human sperm concentration

and the exposure to environmental EDCs (Carlsen, Giwercman, Keiding & Skakkebaek 1995).

Kumar, Gautam and Saiyed (2000) monitored workers at a pesticide plant and determined that exposure to various contaminants could cause abortion, stillbirth, neo-natal deaths, infertility and testicular dysfunction and abnormalities.

These and other studies, suggest that the observed worldwide decline in male reproductive health may be caused by environmental contamination. This statement generated significant public and scientific concerns about the possible role of endocrine disruptors (Irvine 2000).

2.5.3 Effects of EDCs in wildlife

Endocrine disruptive syndromes have also been described in a variety of wildlife species. Reproductive abnormalities such as reduced fertility, reduced egg hatchability, reduced viability of offspring, abnormalities in reproductive anatomy and impaired hormone secretion, have been reported (Guillette 2000(b)).

Guillette, Gross, Masson, Matter, Percival and Woodward (1994) examined the reproductive development of alligators at a contaminated lake - mainly DDT and other contaminants derived from extensive agricultural activities around the

lake and a sewage treatment facility. They determined that, female alligators from the contaminated lake had plasma 17ß-oestradiol concentrations almost two times greater than females from a control lake and they also showed abnormal ovarian morphology. Male juvenile alligators had decreased plasma testosterone concentrations compared to levels observed in the males from the control lake and they also showed abnormal testicular morphology and abnormally small phalli.

In recent studies, Guillette (2000(a)) demonstrated that reptiles living in contaminated environments exhibit population declines, developmental abnormalities of embryos and abnormalities of the endocrine system.

Bowerman, Best, Grubb, Sikarskie and Giesy (2000) conducted a study at the Great Lakes of North America. They collected blood plasma from nesting bald eagles and examined the influence of locally available toxicants on reproduction. They reported that concentrations of PCBs in blood plasma of nesting eagles were abnormally high and inversely correlated to the health of the nesting eagles across the Great Lakes basin. They also found that the breeding population which nest inside the Great Lakes food web exhibited reproductive impairments, while those eagles nesting at a remote locality and long distance from the Lakes showed a significantly higher and unimpaired reproduction.

Brower, Reijnders and Koeman (1989) noticed that common seals *(Phoca vitulina)* exposed to PCB contaminated fish, had abnormal low concentrations of Vitamin A and thyroid hormone.

It should be noted that the reported studies on wildlife populations are limited to a few animal species and studies have often focussed on 'hot-spots' of chemical pollution (Tyler, Jobling & Sumpter 1998).

2.5.4 Effects of EDCs in laboratory animals

Laboratory experiments confirmed that exposure of rodents to sex hormones pre-natally or during early post-natal periods can cause permanent and irreversible alterations of the endocrine and reproductive organs. These organs include the uterus, fallopian tube, ovary, cervix, vagina and mammary gland in females; and the testis, epididymus, prostate and seminal vesicle in males; as well as non-reproductive organs, including bones, muscle, immune and nervous systems of both sexes (Iguchi, Watanabe & Katsu 2001).

Wang, Bartolucci-Page, Fenton and You (2006) performed studies in male rats to evaluate mammary responses to the phyto-oestrogen genistein. After *in utero* and dietary exposure, the inguinal mammary gland of male rats, 90 days of age, had various morphological alterations (lobular enlargement and epithelial proliferation) compared to the control group.

The potential endocrine disrupting effects of dicyclohexyl phthalate (DCHP) in rats were evaluated by Hoshino, Iwai and Okazaki (2005). They exposed male and female rats to different concentrations of DCHP in the diet. Those animals exposed to doses of 1200 ppm and higher showed, amongst other abnormal changes, decreased body weight gain and food consumption and hypertrophy of hepatocytes and thyroidal follicular epithelial cells.

The reported studies support the well-documented role of environmental contaminants as potential endocrine disruptors (Saradha & Mathur 2006).

2.5.5 Potential effects of EDCs in livestock

Concerns have been expressed about the potential effects on reproductive health and immune status of farm animals following exposure to a range of natural and synthetic environmental compounds that disrupt normal hormonal homeostasis (Sweeney 2002; Brevini *et al.* 2005).

Even though there is a paucity of scientific literature on possible reproductive effects of EDCs in cattle, there is evidence of an overall decline in the fertility of high yielding dairy cows, which is supported by international data (Darshaw, Lamming & Woolliams 1999).

In the past few years, more evidence regarding the effects of exposure to multiple pollutants on animal production became available. Meijer, De Bree,

Wagenaar & Spoelstra (1999) conducted a study where dairy cattle were exposed to drinking water contaminated with sewage. Adverse effects on reproductive and production parameters, such as reduced milk production and increased age at first calving were recorded. Even though these effects were seen in adult animals, more subtle effects of exposure to EDCs may be on the development of oocytes, embryos or neonates, which are generally more sensitive to the effects of EDCs (Pocar, Perazzoli, Luciano & Gandolfi 2001; Gandolfi *et al.* 2002).

Red clover containing isoflavones and lucerne containing coumestans (Kellerman *et al.* 2005) have been reported to cause infertility in cattle. Although oestrogenic subterranean clover is a major cause of infertility in sheep, reports of infertility in cattle on subterranean clover are uncommon (Adams 1995). The observed clinical signs in cattle are those related to hyper-oestrogenism, such as swelling of the vulva, discharge of cervical mucus and enlargement of the uterus. Other manifestations including cystic ovaries, irregular oestrus cycles, nymphomania and anoestrus were also seen in cattle consuming these plants (Adler & Trainin 1960).

Hyper-oestrogenism in gilts has been related to zearalenone, a mycotoxin synthesized by the fungus *Fusarium graminearum*. Clinical signs such as hyperaemia and oedema of the vulva and enlargement of the mammary gland can be observed within 5 to 8 days after consumption of the contaminated feed (Kellerman *et al.* 2005).

Nicol, Roche and Brooks (2000) demonstrated that male foetuses born from ewes that were exposed to octylphenols during pregnancy showed an abnormally low number of Sertolli cells and decreased testicular size.
Bogh, Christensen, Dantzer, Groot, Thofner, Rasmussen, Schmidt and Greve (2001) found evidence suggesting that exposure of sows to octylphenol during pregnancy might interfere with foetal maturation and cause decreased litter sizes.

When bovine oocysts were exposed *in vitro* to different concentrations of various pesticides, DDT decreased the rate of normal oocyte maturation and caused degeneration in a dose-dependent manner (Alm, Torner, Tiemann & Kanitz 1998). Studies conducted on pre-pubertal female lambs demonstrated that certain EDCs can suppress gonadotrophin secretion (Evans, North, Dye & Sweeny 2004). Beard, Bartlewski, Chandolia, Honaramooz and Rawlings (1999), exposed rams to organochlorine pesticides from conception to 28 weeks of age. At necropsy, these animals showed more severe seminiferous tubule atrophy and reduced epididymal sperm density compared to untreated rams.

Currently there are not many controlled studies that had confirmed adverse effects of EDCs on cattle, although there is sufficient evidence that there are casual relationships between EDCs and production and reproductive functions of farm animals (Rhind 2005). Furthermore, it is also important to consider the age of the animals at the time of exposure to EDCs. The impact of EDCs might

be more apparent in the developing foetus and post-natal offspring as many of the normal homeostatic endocrine feedback mechanisms and the immune system are not yet fully developed and there is a greater potential for low dose exposures to EDCs to exert adverse effects on those systems (Crisp *et al.* 1998; Sweeny 2002).

2.6 SCREENING TEST FOR EDCs

2.6.1 Introduction

The issue of "endocrine disruption" originally centred on chemicals that mimic the action of the natural hormone oestrogen. However, the focus has now shifted to include effects on the entire endocrine and immune systems. In response to public awareness, a significant effort over the past few years has focused on the development of testing methods to detect the potential effects of endocrine disrupting chemicals. Regulatory agencies, such as the Environmental Protection Agency of the United States (US EPA), are formulating potential testing strategies and are in the process of validating tests to assess a chemical's endocrine-disruptive activity, quantitatively and qualitatively.

Screening tests to evaluate endocrine disrupting activity can be divided in *in vitro* and *in vivo* tests. *In vitro* screening systems are used to characterise the potency and mechanism of action of certain chemicals. However, *in vitro*

assays can not always predict the outcome of *in vivo* studies since they lack, for example, appropriate metabolic activation. Therefore, the results obtained might be of limited value in risk assessment, as *in vitro* assays are simple and don't mimic the complexicity of humans and animals. Thus, *in vivo* assays are more important for hazard identification and risk assessment (Gelbke, Kayser & Poole 2004).

2.6.2 *In vitro* screening tests

A large number of *in vitro* tests have been developed for the screening of potential endocrine disrupting compounds. They are sensitive, show a high specificity and are cheaper than *in vivo* test systems (Eertmans, Dhooge, Stuyvaert & Comhaire 2003). Limitations of *in vitro* assays are that metabolic activation or deactivation of the test compound (Clode 2006) and its bioavailability can not be determined (Mueller 2002). Another major disadvantage of *in vitro* assays is that due to their lack of metabolism "false negative" responses may be obtained (Gray *et al.* 2003). For example, several compounds, which are not oestrogenic *in vitro*, can be transformed to oestrogenic active metabolites *in vivo*. Therefore, *in vivo* bioassays are needed for further analyses of possible effects of EDCs (Eertmans *et al.* 2003).

2.6.2.1 Receptor binding assays

Receptor binding tests assess the ability of a compound to bind directly to the hormone receptor (Bolger, Wiese & Ervin 1998). Steroid hormones such as oestradiol act on their target cells by binding to specific, high-affinity receptors within the cell. The receptor binding assays can utilize tissue extracts from the uterus from various animal species, such as rodents or bovines or human cell lines that contain oestrogen receptors, like MCF-7 (Soto, Maffini, Schaeberle & Sonnenschein 2006).

In the same manner, various cell-free binding assays involving the androgen and progesterone receptors have also been developed (Gray, Kelce, Wiese, Tyl, Gaido, Cook, Klinefelter, Desaulniers, Wilson, Zacharewski, Waller, Foster, Laskey, Reel, Giesy, Laws, Mclachlan, Breslin, Cooper, Di Giulio, Johnson, Purdy, Mihaich, Safe & Colborn 1997). Receptor binding assays are fast, easy to perform and relatively cheap, hence they are a good choice for large-scale screening. Some limitations of receptor binding assays include their inability to distinguish between agonistic and antagonistic effects (Soto *et al.* 2006).

2.6.2.2 Cell proliferation assays

In these *in vitro* systems, the ability of a test substance to stimulate the growth of oestrogen-dependent cell lines is measured (Lippman & Huff 1976). Most commonly, the oestrogen-dependent human breast cancer cell lines, such as

MCF-7 (E-screen), are used (Welshons, Grady, Engler & Judy 1992). This test measures specifically a biological response, which is a measure of the direct interaction of agonists with the oestrogen receptor and equates this with oestrogenic potential (Soto, Sonnenschein, Chung, Fernandez, Olea & Serrano 1995). This assay is one of the most sensitive assays to analyse the oestrogenicity of compounds (Rasmussen & Nielsen 2002) and is highly suitable to distinguish between oestrogen agonists and antagonists (Wakeling & Bowler 1992; Eertmans *et al.* 2003).

2.6.2.3 Recombinant cell lines

The "yeast screen" utilizes yeast cells, transfected with the human oestrogen receptor (Gray *et al.* 1997). Yeast cells do not contain endogenous steroid receptors. However, mammalian steroid receptors introduced into yeast cells, function as they do in mammalian cells, as steroid-dependent transcriptional activators. Chemical interaction with that receptor can be determined by measuring the reporter gene product (Gaido, Leonard, Lovell, Gould, Babai, Portier & McDonell 1997). The amount of β-galactosidase produced in culture reflects the binding to the oestrogen receptor in the transformed yeast cells. The optical density (OD) indicates galactosidase activity.

The "yeast screen" has many advantages such as the ability to rapidly attain the stable transformant, the ability to process a large number of samples quickly and is also relatively inexpensive (Gaido *et al.* 1997). One of the

limitations of this test is that the impermeability of the cells to some substances may generate "false negative" results and some strain specific effects have also been reported (Gray *et al.* 1997).

2.6.2.4 Production of gamma-interferon (γ-IFN) as an indicator of immunosuppressive activity

This screening test evaluates the production of γ-IFN in response to mitogenic stimulation of bovine peripheral blood mononuclear cells. This assay measures immunosuppression by quantifying the production of γ-IFN using a BoviGam ELISA kit (Maue, Waters, Palmer, Whipple, Minion, Brown & Estes 2004).

2.6.2.5 Production of interleukin-6 (IL-6) as an indicator of immunosuppressive activity

The *in vivo* IL-6 synthesis can also be stimulated *in vitro*, using cell culture assays (Pool, Johaar, James, Petersen & Bouic 1998). Cytokines, such as IL-6, are important regulators and initiators of immune response. Hence, IL-6 could be a valid indirect marker of immune suppression (Pool, Robson, Smith, van Wyk & Myburgh 2002).

2.6.3 *In vivo* screening tests

Results, obtained with *in vitro* bioassays, need to be extended by conducting animal testing. Indeed, *in vitro* bioassays measure only one endpoint or one specific characteristic of a possible endocrine disrupting agent. *In vivo*, endocrine disruptors interact in a more complex manner with the regulation mechanism of the endocrine system (Eertmans *et al.* 2003). Some of these *in vivo* assays are suitable for screening large numbers of chemicals in contaminated matrices, including food and water. Thus they can be useful tools for prioritising chemicals for more extensive studies (Andersen, Andersson, Arnold, Autrup, Barfoed, Beresford, Bjerregaard, Christiansen, Gissel, Hummel, Bonefeld Jorgensen, Korsgaard, Le Guevel, Leffers, McLachlan, Moller, Nielsen, Olea, Oles-Karasko, Pakdel, Pedersen, Perez, Skakkebok, Sonnenschein, Soto, Sumpter, Thorpe & Grandjean 1999).

It is important to take into account that there are species and strain variations in sensitivity and data varies depending on the protocol used (Endocrine Disruptor Screening and Testing Advisory Committee - EDSTAC 1998).

2.6.3.1 Vitellogenin assay

Vitellogenin (Vtg) is a yolk protein synthesized by the liver of egg-laying animals and is under oestrogen control. The Vtg gene is normally silent in males, but if they are exposed to oestrogens, activation of this gene can occur

resulting in increased Vtg concentration in blood (Sumpter & Jobling 1995). Increases in the plasma Vtg concentration of egg-laying vertebrates, have been identified as a potentially useful biomarker of exposure to chemicals with oestrogen-like properties (Jones, De Coen, Tremblay & Giesy 2000; Hurter, Pool & van Wyk 2002). This assay is successfully performed in male African Clawed Frogs (platanna; *Xenopus laevis)* (van Wyk, Pool & Leslie 2003).

2.6.3.2 Rodent assays

Various studies on rodents have been developed to measure the endocrine activity of EDCs. The "rodent 3-day uterotrophic assay" screens for oestrogenic and anti-oestrogenic activities of chemicals by evaluating the binding of these compounds to the oestrogen receptor. The endpoint measured with this test is the uterine weight changes of immature ovariectomized rodents injected with a suspected oestrogenic compound (Eertmans *et al.* 2003; Kanno, Onyon, Peddada, Ashby, Jacob & Owens 2003). However, available evidence indicates that relatively large quantities of certain substances without oestrogenic activity, such as progesterone, can also stimulate an oestrogenic response and interfere with the results of this assay (Jones & Edgren 1973).

The "rodent 5-7 day Hershberger assay" measures the androgenic or anti-androgenic activities of suspected endocrine disruptors. In this assay, the ability of a compound to restore the weight of the prostate and seminal vesicles of castrated immature male rodents is determined (Gelbke *et al.* 2004).

The "20-day rodent pubertal female assay" screens for oestrogenic activity of suspected endocrine disruptors in immature female rats exposed during puberty. This test evaluates abnormalities in the development of female sex organs as well as secondary sex characteristics (Gray *et al.* 2003). This assay is also useful to detect compounds with potential thyroid activity (Marty, Crissman & Carney 1999).

2.6.3.3 Assays on aquatic vertebrates

Various screening tests to identify EDCs have been developed in aquatic vertebrates (Hutchinson, Brown, Brugger, Campbell, Holt, Länge, McCahon, Tattersfield & van Egmond 2000; Gray *et al.* 2003). One of them is the "fish reproduction assay". This test is used to screen compounds with potential oestrogenic and androgenic activities. After exposing fish to a suspected EDC, abnormalities associated with survival, reproductive behaviour, secondary sex characteristics and fecundity are examined (EDSTAC 1998; Hutchinson *et al.* 2000).

Another widely used screening test is the "frog metamorphosis assay". This assay is based on the assessment of tail resorption by *Xenopus laevis* tadpoles and evaluates effects under thyroid hormone control (Eertmans *et al.* 2003). Metamorphosis in tadpoles is a thyroid hormone-dependant process and exposure of tadpoles to a substance that exerts thyroid activity can cause deleterious effects in the metamorphosis process (Furlow & Brown 1999; Fort,

Rogers, Morgan, Miller, Clark, White, Paul & Stover 2000), thereby tadpoles provided an exceptional model for identifying thyroid-disrupting chemicals.

Thyroid hormone (TH) acts primarily via thyroid receptors (TRs) that initiate tissue-specific genetic programmes by the activation and repression of specific genes; thus, thyroid signaling is particularly amenable to analysis with a transcriptional assay (Turque, Palmier, Le Mévle, Alliot and Demeneix, 2005)

The thyroid hormone receptor β (TRβ) gene expression correlates with increased levels of endogenous TH during metamorphosis. The level of TRβ mRNA can be assessed by means of a reverse transcription-polymerase chain reaction (RT-PCR) (Manzon & Denver 2004).

This test assesses thyroid activity at the molecular level in a physiologically relevant situation. Moreover, translucent tadpoles are amenable to imaging with fluorescent reporter constructs that facilitate *in vivo* measurement of transcriptional activity (Turque, Palmier, Le Mévle, Alliot and Demeneix, 2005).

2.7 MOLASSES

Molasses is concentrated syrup consisting of sugar hemicelluloses and minerals and is usually obtained as a by-product of sugar refining operations. Molasses is the residue remaining after as much sugar as possible has been crystallized from the juices extracted from sugarcane (Morrison 1957). Apart

from sugarcane molasses, several other types are available, i.e. beet molasses and citrus molasses. In South Africa, sugarcane molasses is cheap and readily available as sugarcane is cultivated in large parts of the country.

Sugarcane (*Saccharum officinarum*) is a tropical or subtropical perennial grass, with a global production estimated at 1250 million tons a year; it contains 72–75 g liquid/100 g and the remaining 25–28 g/100 g is fibre (Sangnark & Noomhorn 2004).

The crop is produced for its sugar; the whole crop on a dry matter basis has a metabolisable energy (ME) value of about 9 MJ/kg and a low crude protein content of about 40 g/kg (McDonald, Edwards, Greenhalg & Morgan 1995). Molasses is an important component of stock feed in South Africa and the rest of the world, serving as a source of energy for growth and maintenance. Moreover, molasses is often included in a ration to improve its palatability, to improve rumen microbial activity, to reduce dustiness of the ration or as a binder for pelleting (Perry, Cullison & Lowrey 1999).

Feeding experiments demonstrated that the inclusion of 10-15 % levels of molasses in the diet of cattle, improves digestibility of dry matter, as well as nitrogen retention (Hatch & Beeson 1972). In South Africa molasses is usually included in cattle rations at an average of 6-8 % (G Harmse, University of Pretoria, personal communication, 2006).

Apart from its effect to deplete copper reserves, attributed to the high sulphur content of molasses (Beames 1959; Arthington & Pate 2002) and a condition known as "molasses toxicity" (McDonald *et al.* 1995), no other deleterious effect has been reported.

Although most cattle consuming high levels of sulphate successfully adapt (Gould, Cummings & Hamar 1997), a small number of them can develop signs of polioencephalomalacia (PEM) (Beke & Hirokana 1991). Dietary sulphur is relatively non-toxic *per se,* but is metabolised by the rumen micro-organisms to toxic sulphites and/or sulphides (Olkowski 1997). Cummings, Gould, Caldwell and Hamar (1995) conducted experiments where Holstein steers, fed a high sulphate diet, developed clinical signs and lesions of PEM. This condition is also known as cerebrocortical necrosis (CCN), a non-infectious neurodegenerative disease of ruminants, characterized amongst other clinical signs by episodic ataxia, opisthotonus, nystagmus, blindness and head pressing (Jensen, Griner & Adams 1956; Gould *et al.* 1997).

Niles, Morgan and Edwards (2000) evaluated calves from three different farms that exhibit signs of PEM where the diagnosis was confirmed by histopathology. One of the farms was using a liquid molasses-based feed as a supplement for calves grazing on a wheat pasture with decreased forage production due to a lack of rain. The sulphur content of the supplement was 8800 ppm.

A condition known as "molasses toxicity" has occurred when cattle with free access to molasses (with 3 % urea added) received severely restricted amounts of roughage. This condition is characterised by incoordination and blindness caused by deterioration of the brain similar to that seen in cerebrocortical necrosis (Rowe, Bobadilla, Fernandez, Encarnacion & Preston 1977). This condition is ascribed to unusual rumen fermentation of molasses, which gives rise to volatile fatty acid mixtures rich in butyrate and poor in propionate, leading to a glucose deficiency (Losada & Preston 1973). Initially it was suggested that the toxicity could be due to a thiamine deficiency, but administration of exogenous thiamine proved ineffective in preventing this condition (Losada, Dixon & Preston 1971).

CHAPTER 3

JUSTIFICATION

Despite the wide and apparent safe use of molasses in stock feed in South Africa, a relatively new syndrome was attributed to the product. The purported deleterious effect of molasses has occurred in both beef and dairy herds over the last decade (TW Naudé, University of Pretoria, unpublished observations, 2003).

Molasses is a vitally important component of stock feed in South Africa and the rest of the world and it was necessary to ascertain if there was indeed a problem. The allegations that molasses was implicated in the aetiology of the syndrome led to market resistance. In addition, sensational media reports were published and broadcasted on national television. Thus a scientifically controlled study to investigate the suspected toxicity of molasses was essential to improve our understanding of the syndrome.

Endocrine disrupting compounds are reported to be present as contaminants in South African river systems (E.C. Burger, Water Research Council (WRC), report in print). Contamination of molasses with these compounds could occur as a result of uptake of chemicals by growing sugarcane or by the use of contaminated river water during the extraction of sugar from chopped

sugarcane. Contaminants could become concentrated during the process of evaporation and crystallization of the sugar and re-utilization of water in successive batches of sugarcane and could end up in toxic concentrations in the highly concentrated molasses residue (TW Naudé, University of Pretoria, unpublished observations, 2003).

If contamination of molasses was the cause of the syndrome, it will most likely vary from one sugar-producing area to another. The two main sugarcane producing areas in South Africa are the southern region (the coastal area of KwaZulu-Natal) and the northern region (the Mpumalanga lowveld). The sugar mills and farms draw their water supply from different river systems. Moreover, sugarcane ripeners (herbicides) are used in the northern region, but not the southern region and they may also enter the sugar crop and become concentrated (Wild *et al.* 1992).

It was essential to investigate the safety of molasses, currently available as stock feed, to allay any fears created by farmers. The results of the project would have far-reaching implications for the sugar industry, the stock feeds manufacturers, cattle producers, the veterinary profession and environmental agencies. If it was confirmed that the syndrome was caused by molasses, the sugar and stock feed industries would be obliged to develop quality assurance methods for identifying toxic batches and if the toxicity of molasses was proved to originate from contaminated rivers, appropriate monitoring, surveillance and preventative measures will have to be implemented immediately by the

responsible government regulatory authority. On the other hand, if no adverse effects attributed to molasses were found, then the search for the cause(s) of this syndrome would have to be directed elsewhere.

The fact that some EDCs have been implicated in carcinogenesis (Colborn *et al.* 1993; Helleday, Tuominen, Bergman & Jenssen 1999) and the potential risk of exposure of humans to EDCs residues through the ingestion of edible products of animal origin have focused attention on EDCs. The possibility exists that chronic, low-level exposure of humans to oestrogenic chemicals in the diet can have effects comparable to those observed in animals exposed to EDCs (Colborn *et al.* 1993).

The extrapolation of data obtained from studies in laboratory rodents can always be challenged due to the physiological diversity of the animal kingdom (Magnusson 2005). However, laboratory animals are considered to be valuable indicators of potential effects in livestock.

The objectives of this project were threefold:

- To perform the initial screening of four batches of molasses to select potential harmful batches for inclusion in a calf feeding trial.
- To ascertain if molasses causes reduced growth and poor production in calves by comparing the weight gain over a 6-month study period.

- To evaluate the safety of molasses by evaluating clinical and clinico-pathological parameters, immune responses and endocrine effects between the groups.

CHAPTER 4

SELECTION OF THE BATCHES OF MOLASSES USED IN THE FEEDING TRIAL

4.1 INTRODUCTION

The clinical signs observed in cattle suffering from the aforementioned syndrome, were suggestive of an "endocrine disruptive syndrome". Endocrine disruptors can act via a wide range of mechanisms, including receptor dependent and independent processes and can also be influenced by metabolism. In addition, they can demonstrate species-, tissue- and cell-specific effects. No single *in vitro* test is able to detect all properties of hormonally active substances and a battery of tests (including *in vivo* tests) should be performed to evaluate results (Eertmans *et al.* 2003). Therefore, a screening test strategy, based on three principle elements, was decided upon, namely:

- oestrogenicity,
- immunotoxicity and
- thyroid activity.

In addition, chemical and nutritional analyses on the different batches of molasses were performed prior to selecting two batches of molasses to be used in the calf feeding trial.

4.2 MATERIALS AND METHODS

4.2.1 Collection of the batches of molasses

During 2004, molasses samples from four different sugar mills were collected. Two of the sugar mills, namely Umzimkulu (UK) and Noodsberg (NO), situated in the southern part of the country where sugarcane is harvested after two years. Samples were collected directly from the bulk tanks (Fig. 4.1 & 4.2). The other two sugar mills, namely Pongola (P) and Komatipoort (KP), situated in the northern part of the country where sugarcane is harvested after 12 months, were also visited. Samples were collected directly from the production line (Fig. 4.3 & 4.4). The total quantity of molasses estimated to be used during the feeding trial was collected from each sugar mill, using 200-liter metal drums, which have never been used or washed with any detergent before.

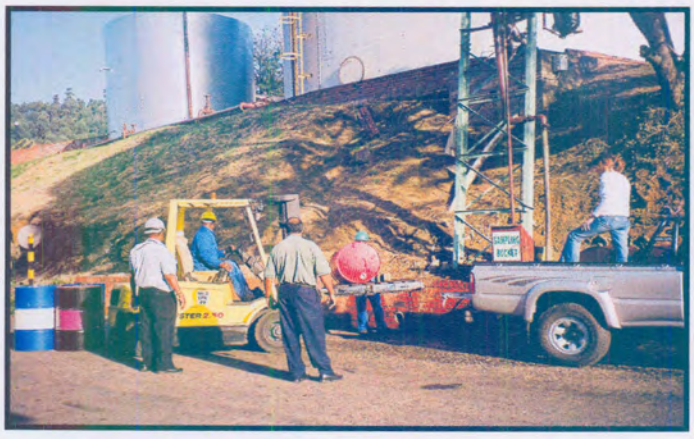

Fig. 4.1 and 4.2: Collection of molasses from the bulk tank at Noodsberg Sugar Mill

Fig. 4.3 and 4.4: Collection of molasses from the production line at Pongola Sugar Mill

4.2.2 Extraction of molasses

Chemical extraction of the four batches of molasses was performed at the Toxicology Laboratory, Onderstepoort Veterinary Institute (OVI) and the Phytomedicine Laboratory of the Faculty of Veterinary Science (Fig. 4.5), using analytical grade reagents (ethyl acetate and acetone) and EDC-free water kindly supplied by the Department of Andrology, Faculty of Health Sciences. The glassware used was washed every time with chromic acid and rinsed with EDC-free water and finally rinsed with methanol and allowed to dry. The extraction process was performed in triplicate and a blank was also obtained.

Composite samples of the three drums of each batch were made (*ca.* 30 g of each composite) and extracted. These *ca.* 30 g aliquots were each mixed with 40 ml of EDC-free water in 500 ml bottles and extracted three times by shaking at room temperature for 30 min in a mechanical shaker with 300 ml ethyl acetate. The *ca.* 900 ml slightly yellowish-brown extract was evaporated under reduced pressure at 80-90 °C in a Buchi R114 rotavapor to a small volume and quantitatively transferred to a 100 ml volumetric flask.

The final 100 ml ethyl acetate extract was then divided in exactly 25 ml aliquots with a volumetric pipette, evaporated to a small volume and, again with the aid of small volumes of acetone, quantitatively transferred to the final test tubes in *ca.* 5 ml of solvent. This was evaporated to dryness in a stream of nitrogen at 70 °C, capped with tin foil and stored in a refrigerator at 8 °C. Each tube,

therefore, represents the extract of *ca.* 7.5 g of molasses. For the blanks of each of the four batches of molasses, 40 ml of EDC-free water was similarly shaken three times with 300 ml ethyl acetate of the particular batch of extractant used for this batch of molasses and this "extract" also concentrated and divided into four 25 ml aliquots and similarly evaporated to dryness in the final test tubes.

Fig. 4.5: Extraction of molasses at the Phytomedicine Laboratory of the Faculty of Veterinary Science

4.2.3 Chemical and nutritional analyses of molasses

The four batches of molasses were analysed to determine their chemical and nutrient composition. The chemical analysis was performed by the Institute for Soil, Climate and Water (ISCW), Pretoria and the nutrient analyses by the Sugar Milling Research Institute (SMRI), University of KwaZulu-Natal, Durban.

4.2.4 Laboratory screening tests

Extracts of the four batches of molasses were sent to different laboratories for oestrogenicity and immune and thyroid toxicity screening tests.

4.2.4.1 Oestrogenicity assays

- A cell proliferation assay was performed using a human cancer cell line (MCF-7) to measure oestrogenic activity. This assay was performed by the Medical Faculty of the Texas A & M University, USA
- The yeast screen was performed by the Ecophysiology Laboratory, Department of Botany and Zoology, University of Stellenbosch. The assay was repeated three times and the average calculated.
- Frog vitellogenin assay. Male frog (*Xenopus laevis*) liver slices were exposed to molasses extracts and the Vtg synthesized by the liver slices was measured by means of radio-immunoassay, as an indication of oestrogenic activity. This assay was performed by the Ecophysiology Laboratory, Department of Botany and Zoology, University of Stellenbosch.

4.2.4.2 Immunotoxicity screening tests

- An γ-IFN production assay was performed to evaluate the production of γ-IFN in response to mitogenic stimulation of bovine

peripheral blood mononuclear cells as an indication of immunosuppressive activity. This assay was performed by the Laboratory at the Medical Faculty of the Texas A & M University, USA.

- Production of interleukin-6 (IL-6) as an indicator of immunosuppressive activity was measured by the Ecophysiology Laboratory, Department of Botany and Zoology, University of Stellenbosch. For this assay, after incubation of molasses samples with human blood, the supernatants were assayed for IL-6 concentrations. The difference in IL-6 response was then calculated and expressed as a percentage recovery using a control sample containing no extract as 100 % recovery. Samples giving recoveries of less than 90 % indicate inflammatory response suppression.

4.2.4.3 Thyroid hormone activity test

- Frog metamorphosis assay. In this study *Xenopus laevis* tadpoles were exposed to triiodothyronine (T_3) and thyroxine (T_4) as well as molasses and combinations of T_4 and molasses. Tail biopsies from metamorphic tadpoles were collected at 48 and/or 96 hours post-treatment. The level of thyroid hormone receptor β (TRβ) mRNA was evaluated by means of a reverse transcription-polymerase chain reaction (RT-PCR).

4.3 RESULTS

The results of the molasses analyses are depicted in Table 4.1.

Table 4. 1: Chemical and nutritional analyses of molasses

Nutritional data	UK	P	KP	NO	National average*
Dry solids (%)	77.05	81.45	79.03	74.89	
Fructose (%)	6.7	9.5	8.9	7.7	7.4
Glucose (%)	4.3	7.4	6.5	5.2	5.2
Sucrose (%)	27.7	28.1	27.6	27.6	31.3
Starch (ppm)	1342	922	542	1586	1900
Elements (mg/kg)					
Arsenic	0.0000	1.1029	2.3084	0.0000	
Beryllium	0.0166	0.0270	0.0220	0.0188	
Boron	8.5466	21.6150	9.8574	12.1501	
Cadmium	0.3039	0.3215	0.3240	0.3229	
Chromium	0.0000	0.1351	0.2778	0.0000	
Cobalt	0.6140	1.6929	1.7604	1.0301	
Copper	2.5636	3.1790	4.8766	2.6086	
Lead	1.1576	0.9294	1.2400	1.4927	
Lithium	0.1509	0.1587	0.1302	0.1034	
Mercury	1.1796	0.9263	1.3735	0.9608	
Molybdenum**	<0.01	-	0.1700	-	
Nickel	1.8036	4.2833	13.3637	3.1614	
Selenium	3.8844	3.6971	3.8244	3.6116	
Tin	0.0937	0.1771	0.0816	0.2538	
Uranium	0.0000	0.0000	0.0000	0.0000	
Vanadium	0.0000	0.0000	0.0000	0.0000	
Zinc	13.9484	14.3986	17.5980	16.4702	

*** Information supplied by the SMRI**

*** * Analysed by Central Analytical Laboratories, Pretoria**

The laboratory results of potential oestrogenicity, immunotoxicity and thyroid toxicity are presented in Table 4.2.

Table 4.2: Screening tests results

Test*	MCF-7	Yeast screen	Vtg	γ-IFN	IL-6	TH activity
Laboratory	Texas	Stellenbosch	Stellenbosch	Texas	Stellenbosch	Stellenbosch
P blank		0.6 OD	2 µg/ml			
P sample	1090 ppt	1.5 OD	40 µg/ml	1.3 OD	73 %	0.21 units
K blank		0.6 OD	2 µg/ml			
K sample	467 ppt	1.3 OD	5-25 µg/ml	1.35 OD	68 %	0.32 units
NO blank		0.5 OD	negative		>90 %	
NO sample	1090 ppt	0.6 OD	negative	1.1 OD	89 %	0.18 units
UK blank		0.5 OD	7 µg/ml			
UK sample	2729 ppt	3.0 OD	2-12 µg/ml	0.3 OD	92 %	0.23 units

* Maximum results highlighted in red

4.4 DISCUSSION AND CONCLUSIONS

The UK molasses showed the highest oestrogenic activity based on the MCF-7 assay as well as in the yeast screen and a marked immunosuppressive activity with the γ-IFN assay. The KP molasses showed the highest immunosuppressive effect with the IL-6 assay and a high thyroid hormone (TH) activity.

The selection of the molasses to be used in the calf feeding trial was based on the laboratory screening tests since no major differences were noticeable in the

chemical composition of the four batches. Based on the results obtained with the laboratory screening tests, the UK and KP samples were selected for the calf feeding trial.

CHAPTER 5

CALF FEEDING TRIAL

5.1 INTRODUCTION

A preliminary, inadequately controlled molasses feeding trial in Holstein veal calves performed by stockowners have yielded equivocal, but apparently disturbing results (TW Naudé, University of Pretoria, unpublished observations, 2003). In an attempt to clarify the role of molasses in the aetiology of the syndrome, the sugar industry agreed to support a scientifically designed, controlled feeding trial in calves.

5.2 MATERIALS AND METHODS

5.2.1 Experimental animals

Thirty-two, 4 to 6 week-old Holstein bull calves of approximately the same age and weight, supplied by the same rearing facility, were included in a single phase, three treatment, parallel design experiment. The calves were identified and numbered by means of a plastic ear tag. Before inclusion in the trial all the animals were clinically examined (normal general appearance and habitus, temperature, respiratory and heart rates, rumen movements, absence of

noticeable clinical signs of disease) and clinical pathology parameters (haematology, serum proteins, serum enzyme activities and serum nitrogenous compounds) were determined. The calves were weighed before commencement of the trial, ranked from the heaviest to the lightest and sorted into replicates of three animals each. Within each replicate the calves were randomly allocated, by means of a table of random numbers, to the treatment groups.

5.2.2 Treatment groups and feeding

The calves (n=32) were allocated to three groups and each group was fed one of the following rations at 3 % body weight:

- a ration containing 6 % Umzimkulu (UK) molasses (n=11)
- a ration containing 6 % Komatipoort (KP) molasses (n=10)
- a ration where no molasses was added, but where approximately 3 % fermentable sugar (mainly sucrose, but also glucose and fructose) and minerals (mainly potassium, magnesium and sulphate) equivalent to that of the national average for molasses (as supplied by the SMRI, University of KwaZulu-Natal, Durban) were added to compensate for the mineral and energy content of molasses (n=11).

Eragrostis hay was also available *ad libitum*.

The calves were weighed weekly and the amount of the ration to be fed was adjusted accordingly. The rations were balanced by an independent nutritionist (Prof. C.W. Cruywagen from the Department of Animal Sciences, University of Stellenbosch) to ensure that they were equivalent in all aspects. The three different rations were mixed separately in batches of one ton each on a farm near Bronkhorstspruit and transported to Onderstepoort. The mixer used for this procedure was cleaned of all loose material before mixing and the control ration without molasses was mixed first followed by the two molasses-containing rations.

5.2.3 Feed analyses

Samples of each batch of feed (eight in total) were collected and submitted to UP Nutrilab, Department of Animal and Wildlife Sciences, Faculty of Natural and Agricultural Sciences, University of Pretoria for complete feed analyses. The analyses were performed according to the laboratory's official standard operating procedures (Giron 1973; Association of Official Analytical Chemists 2000).

5.2.4 Housing and water

The calves were individually housed, in pens, at the University of Pretoria Biomedical Research Centre (UPBRC), Onderstepoort so that each calf only had access to its own feed (Fig. 5.1 and 5.2). Animals were allowed access to

an adjacent communal camp, for 6 hours a day, during weekdays for exercise and socialization (Fig. 5.3).

The calves had free access to potable municipal water. Water samples were collected at commencement of the trial and submitted to the Institute for Soil, Climate and Water, Pretoria for ICP analyses.

Fig. 5.1 and 5.2: Individual housing facilities for the calves at the UPBRC, Faculty of Veterinary Science

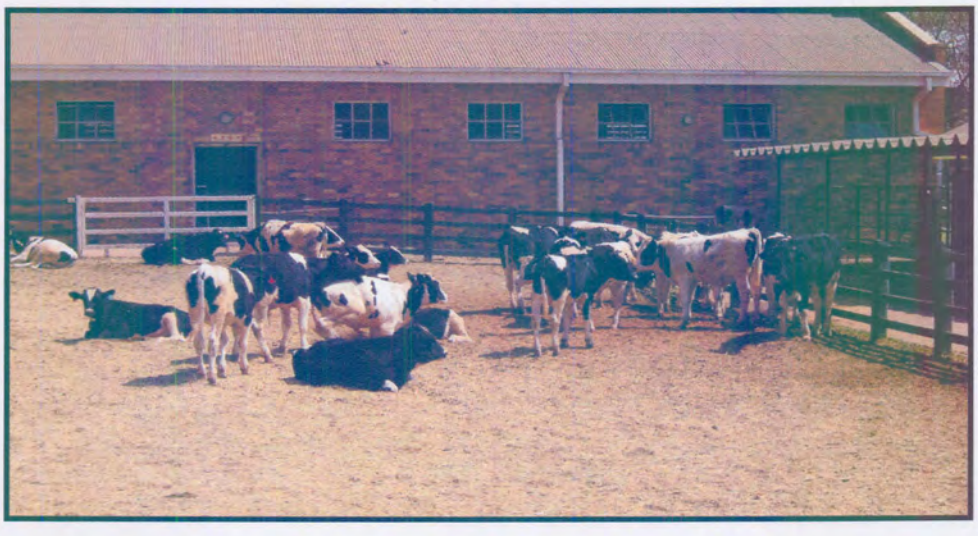

Fig. 5.3: Calves in the communal camp adjacent to the housing facility

5.2.5 Clinical examination

Animals were observed daily for any abnormalities. Diarrhoea as a clinical sign was included as part of the statistics when:

- calves exhibited mild diarrhoea on three or more days during the specific week
- calves exhibited moderate diarrhoea on two or more days during the specific week
- calves exhibited severe diarrhoea on one or more occasions during the specific week.

The calves were clinically examined every week. However, daily clinical examinations were performed whenever an animal's physical condition deteriorated.

Due to persistent diarrhoea in a number of calves, faecal samples were collected from six animals at Day 90 of the trial and submitted to the Department of Tropical Diseases, Faculty of Veterinary Science, University of Pretoria for bacterial culture, as well as to the Department of Anatomy and Physiology, Faculty of Veterinary Science, University of Pretoria for virus particle identification with electron microscopy (EM). The diarrhoeal fluid was also screened for coccidial oocysts and helminth eggs.

5.2.6 Body weight

Animals were weighed weekly on a digital built-in floor scale and the weekly weight gain was calculated. Accuracy of the scale was ascertained periodically by placing weights with a known mass on the scale.

5.2.7 Clinical pathology

Clinical pathology parameters were determined before the commencement of the trial and at days 120 and 148 after the feeding of molasses commenced. The following parameters were determined by the Section of Clinical Pathology, Faculty of Veterinary Science, University of Pretoria.

- Haematology (red cell count, white cell count [including differential count], thrombocyte count, haematocrit).
- Serum proteins (total serum proteins, albumin and globulins).
- Serum enzyme activities (Alkaline phosphatase [ALP], γ-glutamyltransferase [GGT], Lactate dehydrogenase [LDH] and Aspartate aminotransferase [AST]).
- Serum nitrogenous compounds (urea & creatinine).

The haematology tests were done using a CELL-DYN® 3700, according to the manufacturer's instructions. This system is a multi-parameter, automated haematology analyser designed for *in vitro* diagnostics.

For the determination of total protein, albumin and urea in serum, the NexCT™ Total Protein/ Albumin/Urea Reagents were used with the NexCT™ clinical chemistry system. The globulin fraction was calculated as the difference between total proteins and albumin. For the determination of creatinine, the ACE™ Creatinine Reagent Kit was used with the ACE™ clinical chemistry system. The ACE® Alkaline Phosphatase Reagent was used for the quantitative determination of ALP activity in serum using the ACE® and the NexCT™ clinical chemistry system. The Alfa Wassermann Lactate Dehydrogenase Activity Reagent was utilized for the quantitative determination of LDH activity in serum using the Alfa Wasserman clinical chemistry systems (ACE® and NexCT™). For the quantitative determination of Aspartate Aminotransferase/γ-Glutamyltransferase, the ACE™ AST/GGT Reagent was used with the ACE™ clinical chemistry system.

5.2.8 Thyroid hormone analyses

Animals were bled four times during the trial to measure triiodothyronine (T_3) and thyroxine (T_4) concentrations *viz.*, before commencement of the trial (Day 0), at 1 month (Day 29), at 3 months (Day 86) and at 5 months (Day 148). The analyses were performed by the staff of the Endocrinology Laboratory, Faculty of Veterinary Science, using a Count-A-Count Total T_3/T_4 method. This is a solid-phase ^{125}I radioimmunoassay designed for the quantitative measurement of the total circulating T_3 and T_4 in serum or plasma. It is intended for *in vitro* diagnostic use as an aid in the assessment of thyroid status.

5.2.9 Testosterone analysis

Serum of the 32 animals, bled on Day 154 of the feeding trial, was submitted to the Reproduction Laboratory, Faculty of Veterinary Science, University of Pretoria for determination of total testosterone levels. The analysis was performed by means of a Count-A-Count Total Testosterone radioimmunoassay. This test is designed for the quantitative measurement of the total circulating testosterone in heparinized plasma or unextracted serum.

5.2.10 Infectious Bovine Rhinotracheitis (IBR) - Indirect fluorescent antibody test

All calves where vaccinated against IBR at the rearing facility. To asses the immunocompetency, animals were bled on Day 120 of the feeding trial and serum samples submitted to the Department of Tropical Diseases, Faculty of Veterinary Science, University of Pretoria for an indirect fluorescent antibody (FA) assay.

5.2.11 Seroconversion with *Brucella abortus* Strain 19 vaccine

Animals were bled 4 months (Day 120) after commencement of molasses feeding. The serum samples were sent to the Onderstepoort Veterinary Institute (ARC-OVI) to determine *Brucella* antibody titers using the complement fixation test (CFT). Following blood collection, the calves were immunized (on

Day 120) with *Brucella abortus* Strain 19 vaccine®, Onderstepoort Biological Products (a freeze-dried suspension of live *Brucella abortus* Strain 19 bacteria). Four weeks later blood was again collected and the CFT repeated to measure antibody titers in all the experimental animals.

5.2.12 *Brucella* protein allergen skin test

On Day 170 of the trial, an area of the skin of the neck was selected in each animal and its thickness was measured using a calliper. Animals were then injected intradermally with Brucellergene OCB®, Synbiotics Corporation, a *Brucella* protein allergen. The skin thickness was again measured 72 hours later (Day 173) to evaluate the inflammatory reaction against the allergen (Fig. 5.4 and 5.5).

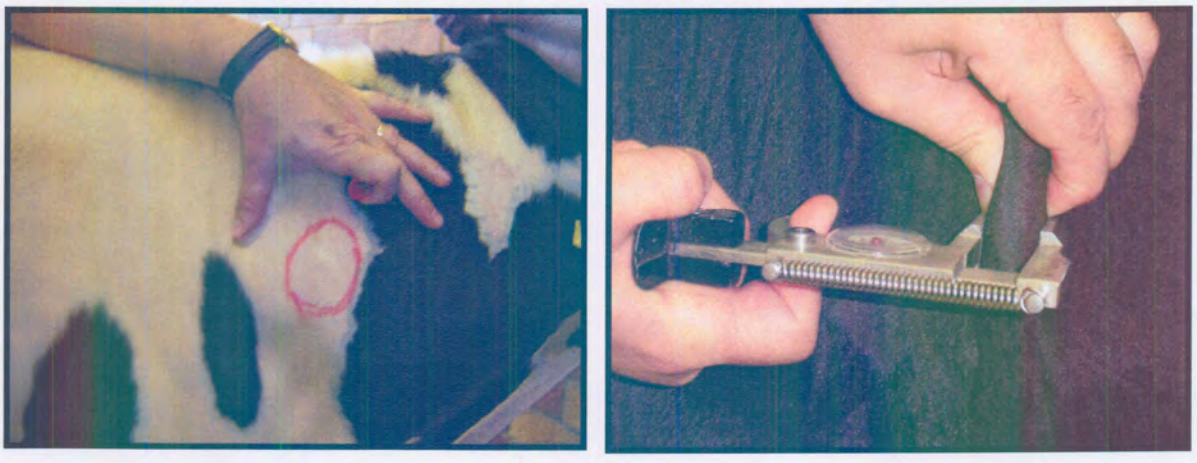

Fig. 5.4 and 5.5: *Brucella* protein allergen skin test

5.2.13 Lymphocyte transformation test

This test was done before the feeding trial and at 4 months of molasses feeding (Day 120 of the trial). To ascertain immune competency of the animals, the lymphocytes were challenged with the mitogen - Concanavilin A (Con-A) (Van Kleef, Macmillan, Gunter, Allsop, Shkap & Brown 2000), an unstimulated animal was used as control. Results are presented as a stimulation index (SI), where SI = mean counts per minute of test sample/mean counts per minute of unstimulated control. A SI higher than 2 was considered to be an indication of Con-A induced proliferation. This test was performed by the Division of Molecular Biology, Onderstepoort Veterinary Institute, under the supervision of Dr. M. Van Kleef.

5.2.14 Immunoglobulin (IgG and IgM) titers

Immunoglobulin G and Immunoglobulin M were first measured at the beginning of the trial to determine a basal level. Animals were bled again at 4 months (Day 120) and at 5 months (Day 154), before and after the immunization with *Brucella abortus* Strain 19 vaccine, to determine their immunoglobulin titers and therefore their immunocompetency. A radial immunodiffusion for IgM and IgG was performed using "SRID Kit"® (VMRD, Inc), which contains monospecific antisera for the protein to be measured in buffered agarose. The titers were determined by the Section of Clinical Pathology, Faculty of Veterinary Science, University of Pretoria.

5.2.15 Carcass weight

The carcass weight of each animal was recorded at slaughter at the abattoir.

5.2.16 Pathology and histopathology

Animals were slaughtered during week 26 of the trial at an approved abattoir. Samples of various organs or tissues were collected at necropsy (Fig. 5.6). These included: cerebrum, cerebellum, midbrain, brainstem, heart, lung, rumen wall, small and large intestine, several lymph nodes, spleen, liver, kidney, testis, thyroid gland, pancreas, thymus, adrenal gland. Samples for histopathology (10 mm thickness) were collected and fixed in 10 % buffered formalin.

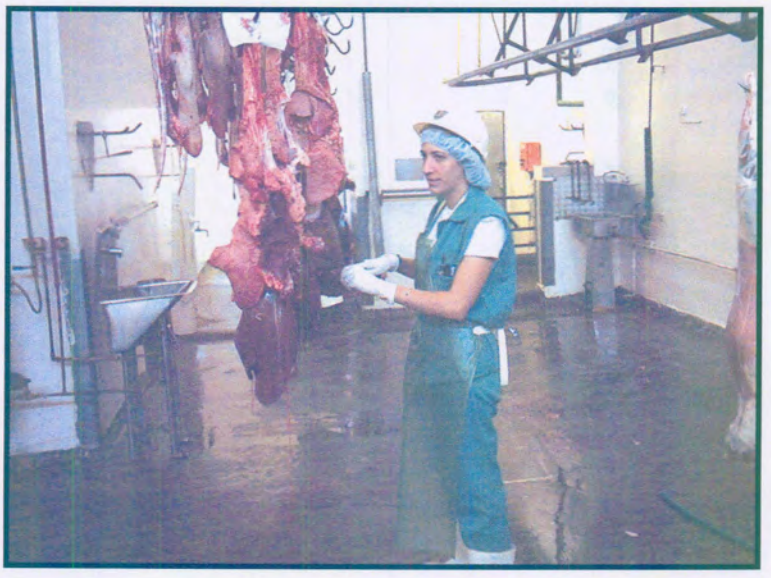

Fig. 5.6: Collection of samples at the abattoir

The tissues were routinely processed, embedded in paraffin wax, cut and mounted on glass slides and stained with haematoxylin and eosin at the Section of Pathology, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria. The organs were evaluated macroscopically and the histopathology slides were viewed under the light microscope by two independent pathologists (Fig. 5.7).

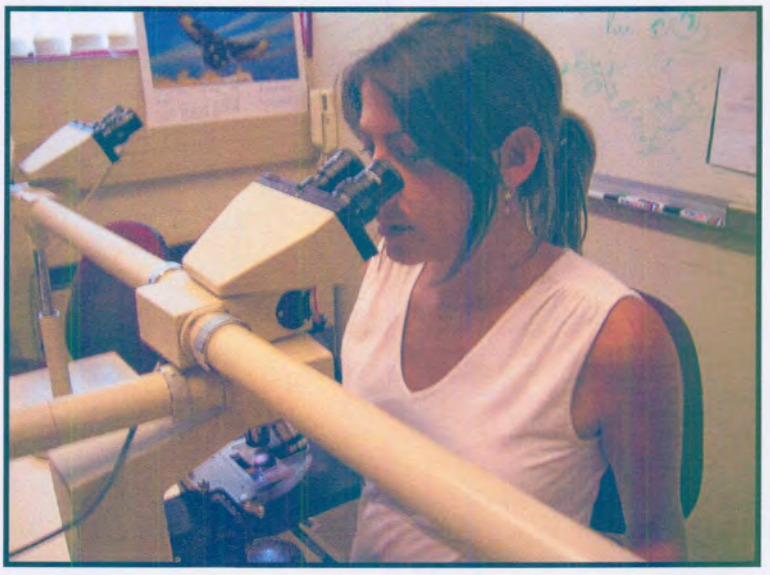

Fig. 5.7: Evaluation of histopathology samples under the light microscope

5.2.17 *Brucella abortus* culture on animal tissues

Tissue samples were collected at the abattoir and submitted to the Bacteriology Division, OVI, for *Brucella* culture. The following tissues were collected: retro-pharyngeal-, superficial inguinal- and pre-scapular lymph nodes, tonsils, spleen, testis and epididymus. Bacterial culture was done using standard operating procedures (Alton, Jones, Angus & Verger 1988).

5.2.18 Mineral analyses

Liver samples (100-200 g) were collected and submitted to the UP Nutrilab, Department of Animal and Wildlife Sciences, Faculty of Natural and Agricultural Sciences for copper (Cu), iron (Fe), manganese (Mn) and zinc (Zn) determination by means of atomic absorption spectrophotometry (Giron 1973).

5.2.19 Statistical analyses

The data was captured and arranged in animals per replicate in spreadsheets. Various statistical analyses were performed by Prof. H.S. Steyn (Jr), Statistical Consultation Service, Potchefstroom Campus of the North-West University. All statistical calculations were performed using a statistical programme (StatSoft. Inc. 2005). The significance level was set at $p < 0.05$. Clinical significance was based on the partial eta-square. An eta-square value of 0.14 can be viewed as a large effect (Cohen 1988).

One-way analyses of variance (ANOVA) on experimental groups were utilized to test for differences in: carcass weights, the *Brucella* complement fixation test and testosterone concentration.

Repeated measures analyses were done on weekly weight gains, T_3, T_4, IgG and IgM levels. These were followed by two-way analyses of variance (ANOVA) on groups within block-replicates. Tukey multiple comparisons were

performed to compare groups or Bonferroni multiple comparisons to compare different times over all groups.

A two-way analysis of variance (ANOVA) on groups within block-replicates was used for: monthly weight gain, the *Brucella* skin test and lymphocyte transformation test. Significance was set at $p < 0.05$.

5.3 RESULTS AND DISCUSSION

5.3.1 Feed analyses

The mean values of the feed analyses of the eight samples collected during the feeding trial were calculated for each group. The results are presented in Tables 5.1 and 5.2. There were no major differences in the composition of the rations fed to the three groups.

Table 5.1: Feed analyses "as is" basis

	KP	UK	Control
	Mean ± SD	Mean ± SD	Mean ± SD
DM (g/100g)	87.1 ± 0.44	87.2 ± 0.40	88.0 ± 0.43
Moisture (g/100g)	12.8 ± 0.44	12.8 ±0.40	11.9 ± 0.43
Ash (g/100g)	5.5 ± 0.42	5.5 ± 0.32	6.6 ± 0.52
CP (g/100g)	12.8 ± 0.70	12.7 ± 0.54	12.5 ± 0.56
C Fat (g/100g)	3.8 ± 1.23	3.8 ± 1.37	3.7 ± 1.26
ME (MJ/kg)	15.9 ± 0.26	15.9 ± 0.34	15.7 ± 0.40

SD= Standard deviation

Table 5.2: Feed mineral analyses "as is" basis

	KP	UK	Control
	Mean ± SD	Mean ± SD	Mean ± SD
Ca (g/100g)	0.78 ± 0.08	0.77 ± 0.05	0.82 ± 0.07
P (g/100g)	0.37 ± 0.06	0.38 ± 0.06	0.46 ± 0.06
Mg (g/100g)	0.19 ± 0.03	0.19 ± 0.03	0.22 ± 0.03
Cu (mg/kg)	30.76 ± 4.34	31.01 ± 6.09	34.86 ± 9.58
Fe (mg/kg)	357.37 ± 93.47	356.11 ± 99.08	340.86 ± 62.27
Zn (mg/kg)	125.11 ± 9.81	121.90 ± 8.55	140.72 ± 22.01
Mn (mg/kg)	62.92 ± 13.11	71.29 ± 14.98	113.78 ± 59.52
K (g/100g)	1.04 ± 0.06	0.99 ± 0.11	1.32 ± 0.18
Na (g/100g)	0.22 ± 0.07	0.17 ± 0.05	0.19 ± 0.07

SD = Standard deviation

5.3.2 Water analyses

The results of the water analyses are shown in Tables 5.3 and 5.4. All values were within normal ranges.

Table 5.3: Water analyses

Water sample	Northern Stables	Western Stables	RML*	TWQR**
pH	6,9	7,57		5-9
ANIONS	mg/l	mg/l	mg/l	mg/l
Fluoride	0,24	0,11	<1.2-2.0	0-2
Nitrites	0,00	0,00	_	0-40
Nitrates	0,82	2,13	<100	0-400
Chloride	10,79	10,30	<1000	0-3000
Sulphate	21,79	19,47	<500	0-1000
Phosphate	1,27	0,21	<0.7	_
Carbonate *(a)*	0,00	0,00	_	_
Bicarbonate	94,55	88,45	<1000	_
CATIONS	mg/l	mg/l	mg/l	mg/l
Sodium	8,80	8,35	<150-800	0-2000
Potassium	2,68	2,54	<20	0-100
Calcium	28,44	26,10	<1000	0-1000
Magnesium	4,22	3,67	<90-250	0-500
	mg/l	mg/l	mg/l	mg/l
Sodium Carbonate *(a)*	0,00	0,00	_	_
Sodium Bicarbonate *(a)*	0,00	0,00	_	_
Alkalinity	77,50	72,50	<2000	_
TDS	125,73	116,78	_	0-2000

*RML: Recommended maximum levels for livestock (Puls, 1994)

** Target Water Quality Range (Department of Water Affairs and Forestry, 1996)

(a): No guideline values available

Table 5.4: Water analyses: Microelements

Water sample	Northern Stables	Western Stables	RML*	TWQR**
Element*	**µg/l**	**µg/l**	**mg/l**	**mg/l**
Antimony	1,538	1,310	<0.0002	<0,006
Arsenic	2,063	1,573	<0.05-0.2	0-1
Barium	31,726	23,806	<1.0	_
Beryllium	0,017	0,026	<1.0	<0,04
Bismuth *(a)*	0,000	0,000	_	_
Bromine	369,879	323,705	_	<0,01
Cadmium	0,288	0,033	<0.05	0-0,01
Caesium	0,015	0,001	_	_
Chromium	4,469	3,694	<0.1	0-1
Cobalt	0,461	0,417	<1.0	0-1
Copper	3,281	14,588	<0.5	0-1
Iodine	22,960	26,646	<10	_
Lanthanum *(a)*	0,026	0,010	_	_
Lead	8,281	7,796	<0.05-0.1	0-0.1
Lithium *(a)*	1,169	0,642	_	_
Manganese	9,846	11,887	<0.05	0-10
Mercury	0,454	1,861	<0.003-0.01	0-0.001
Molybdenum	0,838	0,499	<0.06	0-0,01
Nickel	4,595	4,234	<1.0	<0,02
Platinum *(a)*	0,000	0,000	_	_
Rubidium *(a)*	0,991	0,941	_	_
Selenium	9,144	5,997	<0.01-0.05	0-0,02
Strontium	72,444	61,732	<10	<0,1
Tellurium *(a)*	0,000	0,000	_	_
Thallium	0,000	0,042	_	<0,002
Titanium	10,980	9,260	_	<0,2
Tungsten *(a)*	0,000	0,033	_	_
Uranium	0,000	0,000	<0.2	0-0,02
Vanadium	6,557	5,201	<0.1	0-1
Zinc	151,076	336,124	<5.0-25	0-20

*RML: Recommended maximum levels for livestock (Puls, 1994)

** Target Water Quality Range (Department of Water Affairs and Forestry, 1996)

(a): No guideline values available

5.3.3 Clinical examination

Four months after the commencement of the molasses feeding trial, one of the calves from the control group (calf number 5) developed an abomasal displacement and the condition was corrected surgically. When the displacement re-occurred on Day 162 of the trial, the calf was slaughtered at the abattoir. Another calf (calf number 8) from the UK group was also

hospitalised on Day 93 of the trial due to severe persistent diarrhoea; the calf recovered uneventfully and was returned to the group. Another animal from the UK group (calf number 28) developed an upper respiratory tract infection on Day 157 of the trial. This calf was treated with an antibacterial agent (Danofloxacin, Advocin®, Pfizer Laboratories (Pty) Ltd.) and a non-steroidal anti-inflammatory drug (Flunixin meglumine, Cronyxin®, Kyron Laboratories (Pty) Ltd.) and subsequently recovered.

Bouts of diarrhoea were observed in all the calves before and during the first half of the feeding experiment (Fig. 5.8). However, the KP group had a higher number of animals with diarrhoea, which did not seem to affect their weight gain. No viral particles were noticed with the EM scan and no bacterial pathogens were cultured in any of the samples. The diarrhoeal fluid was also negative for coccidial oocysts and helminth eggs.

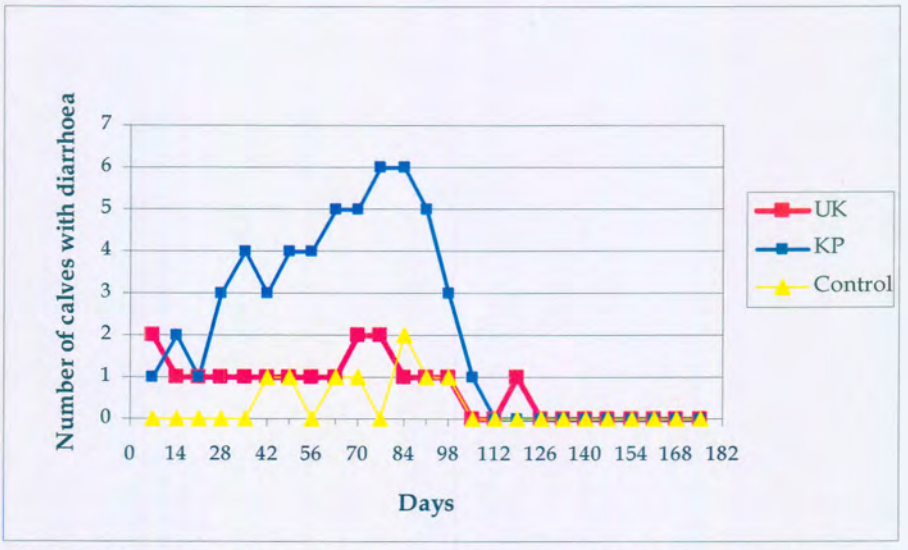

Fig. 5.8: Diarrhoea in calves from the three experimental groups

5.3.4 Body weight

Mean live body weight of the three groups are depicted in Fig. 5.9. No significant difference between the groups was noticed through-out the trial.

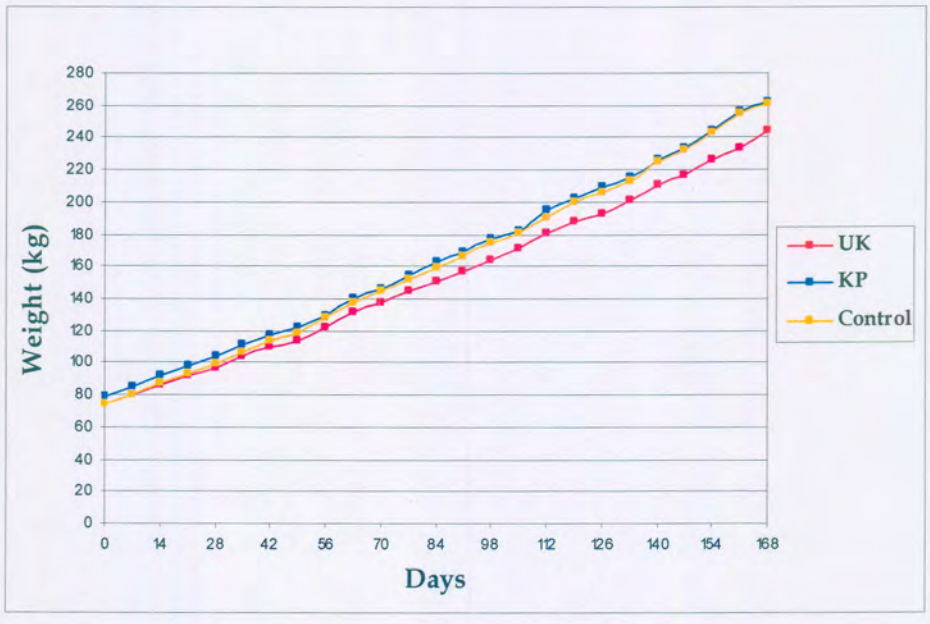

Fig. 5.9: Mean live body weight of the three experimental groups through-out the trial

The weekly weight gain of each animal was calculated against its initial weight at the beginning of the trial (Fig. 5.10). Average weekly weight gain of the two molasses fed groups was compared with the control group (Fig. 5.11). A notable lower mean weight gain, although not statistically significant, was found in the UK group compared with the KP and control groups. However, when the experimental error was corrected (due to the large differences in body weight between different replicates) the monthly weight gain was only statistically significant ($p = 0.025$) between the three experimental groups at 4 months following molasses feeding and at no other time.

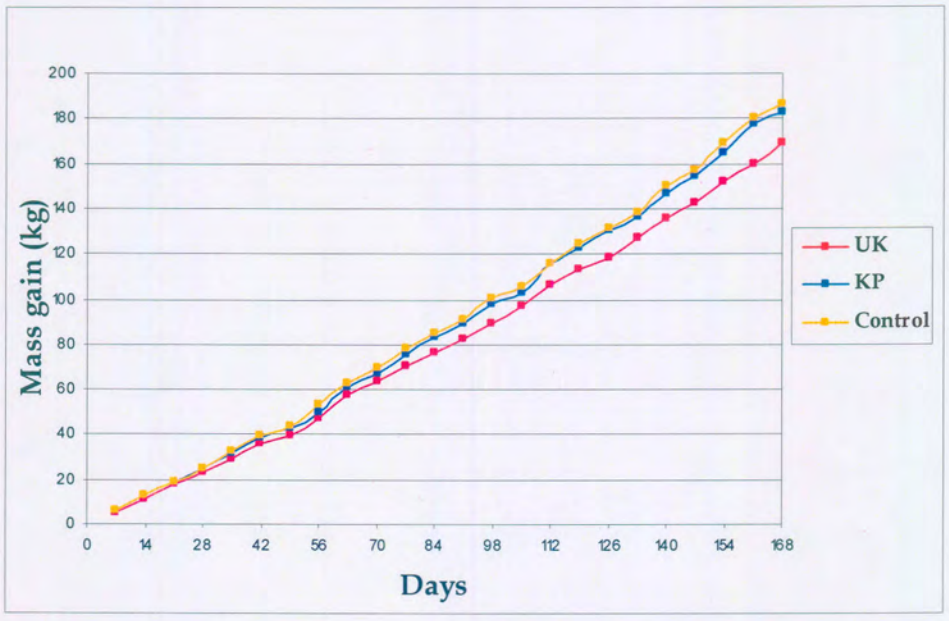

Fig. 5.10: Mean weekly weight gain of the three experimental groups

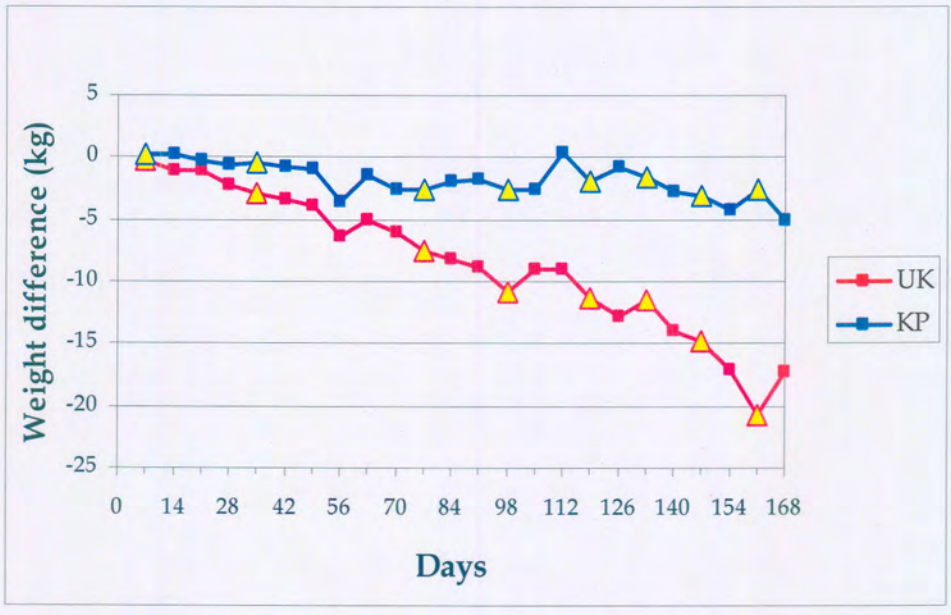

Fig. 5.11: Average weekly weight gain of the two molasses groups compared to the control group (yellow triangle indicates new batch of feed).

5.3.5 Clinical pathology

The mean of the clinical pathology parameters was calculated for each group (Table 5.5: Haematology and Table 5.6: Serology). All the parameters fluctuated within or near normal ranges.

Table 5.5: Haematology parameters

		Before Trial			Day 120			Day 154		
		UK	KP	Control	UK	KP	Control	UK	KP	Control
	Normal range	Mean ± SD	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
Hb (g/dl)		100 ± 14.51	94.1 ± 9.81	88.36 ± 11.34	110 ± 4.96	114 ± 8.6	110.1 ± 6.41	114 ± 6.95	117.4 ± 7.89	113 ± 6.27
RCC ($x10^{12}$/l)	5.0-9.0	8.6 ± 1.29	8.0 ± 0.67	7.75 ± 0.86	8.44 ± 0.53	8.55 ± 0.34	8.38 ± 0.37	8.82 ± 0.72	8.83 ± 0.43	8.59 ± 0.48
Ht (l/l)	0.24-4	0.30 ± 0.04	0.28 ± 0.03	0.27 ± 0.03	0.32 ± 0.01	0.33 ± 0.02	0.32 ± 0.02	0.33 ± 0.02	0.34 ± 0.02	0.33 ± 0.02
MCV (fl)	40-60	34.8 ± 1.95	35.33 ± 1.1	34.35 ± 1.69	37.78 ± 2.07	38.52 ± 2.12	38.05 ± 0.02	38.04 ± 2.1	38.8 ± 2.18	38.43 ± 1.58
MCHC (g/dl cells)	30-36	33.25 ± 0.56	33.23 ± 0.63	33.15 ± 0.65	34.54 ± 0.21	34.45 ± 0.55	34.56 ± 1.77	34.07 ± 0.43	34.21 ± 0.57	34.20 ± 0.31
RDW (%)		25.12 ± 2.03	26.36 ± 2.34	25.92 ± 3.26	24.49 ± 1.48	24.60 ± 1.25	25.06 ± 0.64	23.93 ± 1.71	24.64 ± 1.37	24.69 ± 1.74
WCC ($x10^9$/l)	4-10.0	8.68 ± 3.42	8.66 ± 1.47	7.94 ± 3.12	10.30 ± 2.33	10.47 ± 1.33	10.81 ± 1.89	9.53 ± 2.24	10.81 ± 1.77	10.28 ± 2.53
AbNmat ($x10^9$/l)	0.6-4.0	2.98 ± 1.62	3.36 ± 1.3	2.58 ± 1.23	4.03 ± 1.16	3.85 ± 0.79	4.24 ± 2.21	3.67 ± 1.52	3.98 ± 1.21	3.87 ± 1.41
AbNimm ($x10^9$/l)	0.0-.012	0.01 ± 0.05	0.08 ± 0.14	0.01 ± 0.04	0.00 ± 0	0.00 ± 0	0.02 ± 0.93	0.00 ± 0	0.00 ± 0	0.00 ± 0
AbLymp ($x10^9$/l)	2.5-7.5	4.28 ± 1.79	4.50 ± 2.01	4.48 ± 2.39	5.59 ± 1.4	5.73 ± 1.26	5.74 ± 0.07	4.96 ± 1.57	5.65 ± 1.61	5.46 ± 1.54
AbMono ($x10^9$/l)	0.03-0.84	0.86 ± 0.58	0.64 ± 0.44	0.76 ± 0.39	0.58 ± 0.34	0.81 ± 0.36	0.75 ± 2.18	0.81 ± 0.34	0.84 ± 0.36	0.89 ± 0.44
AbEos ($x10^9$/l)	0.0-2.4	0.05 ± 0.11	0.02 ± 0.05	0.04 ± 0.09	0.07 ± 0.1	0.03 ± 0.04	0.06 ± 0.31	0.04 ± 0.05	0.25 ± 0.39	0.04 ± 0.10
AbBaso ($x10^9$/l)	0.0-0.2	0.03 ± 0.07	0.07 ± 0.09	0.073 ± 0.11	0.04 ± 0.06	0.06 ± 0.09	0.00 ± 0	0.03 ± 0.03	0.09 ± 0.12	0.03 ± 0.04
Thr C ($x10^9$/l)	200-600	1022 ± 427.3	802 ± 285.9	950 ± 240.5	638.6 ± 78.2	546.5 ± 82.96	640 ± 117.76	683 ± 99.74	604.4 ± 11.86	656 ±140.36

SD = Standard deviation (±)

Table 5.6: Serum proteins, nitrogenous compounds and enzyme activities

		Before Trial			Day 120			Day 154		
		UK	KP	Control	UK	KP	Control	UK	KP	Control
Parameter	Normal Range	Mean ±SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ±SD	Mean ± SD	Mean ± SD	Mean ± SD
TSP (g/l)	65-78	62.1 ± 4.07	62.73 ± 3.82	62.1 ± 5.09	64.7 ± 3.02	64.86 ± 3.78	64.7 ± 4.18	66.9 ± 3.99	68.85 ± 2.23	66.6 ± 4.94
ALB (g/l)	28-37	33.3 ± 0.92	33.21 ± 2.15	32.5 ± 2.25	33.0 ± 0.9	33.06 ± 1.68	33.7 ± 1.71	32.8 ± 1.49	33.24 ± 2.85	34.0 ± 1.99
GLOB (g/l)	28-42	28.7 ± 4.24	29.52 ± 4.85	29.5 ± 5.05	31.7 ± 2.68	31.8 ± 4.07	31.0 ± 4.02	34.2 ± 4.42	35.61 ± 3.68	32.7 ± 4.99
A/G	0.9-1.4	1.2 ± 0.19	1.2 ± 0.23	1.1 ± 020	1.1 ± 0.08	1.1 ± 0.16	1.1 ± 0.15	1.0 ± 0.16	0.94 ± 0.16	1.1 ± 0.17
ALP (U/l)	33-328	252.8 ± 70.2	296.8 ± 102.14	297.4 ± 154.6	277.6 ± 103.8	263.3 ± 66.7	278.7 ± 78.1	256.3 ± 66.4	239.3 ± 112.4	268.0 ± 87.79
AST (U/l)	21-167	68.9 ± 25.51	51.0 ± 6.27	57.5 ± 19.75	80.7 ± 16.28	71.0 ± 12.9	68.5 ± 8.20	68.7 ± 14.00	69.3 ± 7.73	69.5 ± 9.05
GGT (U/l)	0-45	20.2 ± 6.23	19.1 ± 3.98	23.1 ± 5.20	16.5 ± 3.75	16.9 ± 3.78	16.4 ± 2.50	23.9 ± 5.63	21.9 ± 4.72	21.5 ± 4.32
LD (U/l)	38-2885	1857 ± 486	1701.5 ± 237.4	1813 ± 399	1883.7 ± 243.7	1790.2 ± 177.5	1899 ± 231	1909 ± 284	1916.4 ± 198.7	1859.2 ± 242.2
Urea (mmol/l)	3.6-10.7	8.6 ± 1.2	7.4 ± 0.78	7.9 ± 1.51	3.9 ± 1.07	2.6 ± 0.92	3.6 ± 1.33	3.1 ± 0.63	3.04 ± 1.3	3.4 ± 1.08
Creat (µmol/l)	10-133	68.5 ± 9.88	65.6 ± 7.24	70.4 ± 1.51	93.4 ± 9.39	91.6 ± 4.81	92.0 ± 9.84	84.9 ± 8.00	85.8 ± 6.09	82.7 ± 10.14

SD = Standard deviation (±)

5.3.6 Thyroid hormone analyses

The results of the T_3 and T_4 analyses were plotted (Figures 5.12 & 5.13). The mean T_3 concentrations of the three groups showed similar curves. The UK group showed a slightly lower mean T_4 concentration compared to the other two groups at 3 months after commencement of molasses feeding. However, no statistical significant difference could be demonstrated. In the control group an appreciable decrease in mean T_4 concentrations occurred from the initial bleeding before molasses feeding commenced, up to 3 months of molasses feeding.

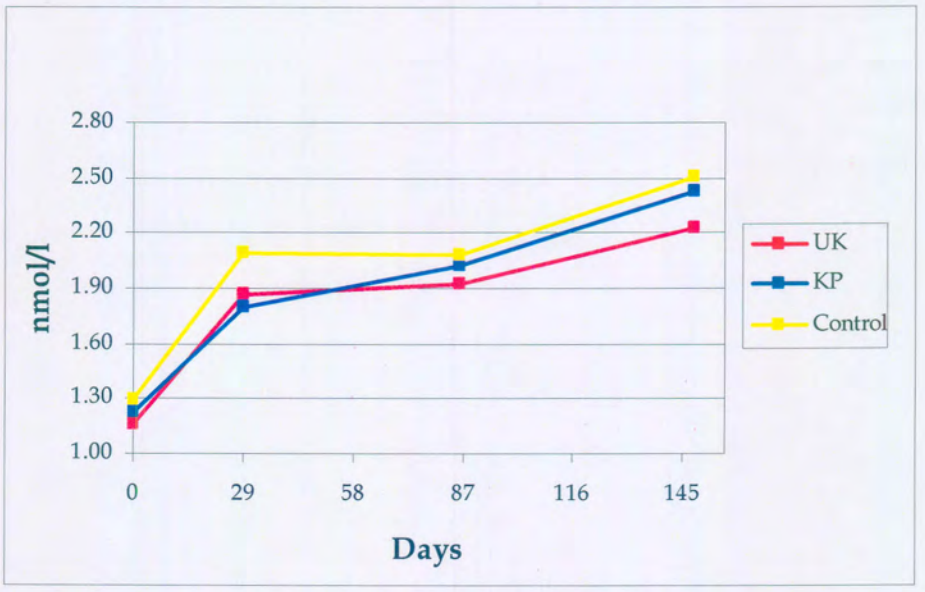

Fig. 5.12: Mean triiodothyronine (T_3) concentrations of the three experimental groups at D0, D29, D86 and D148 of the feeding trial

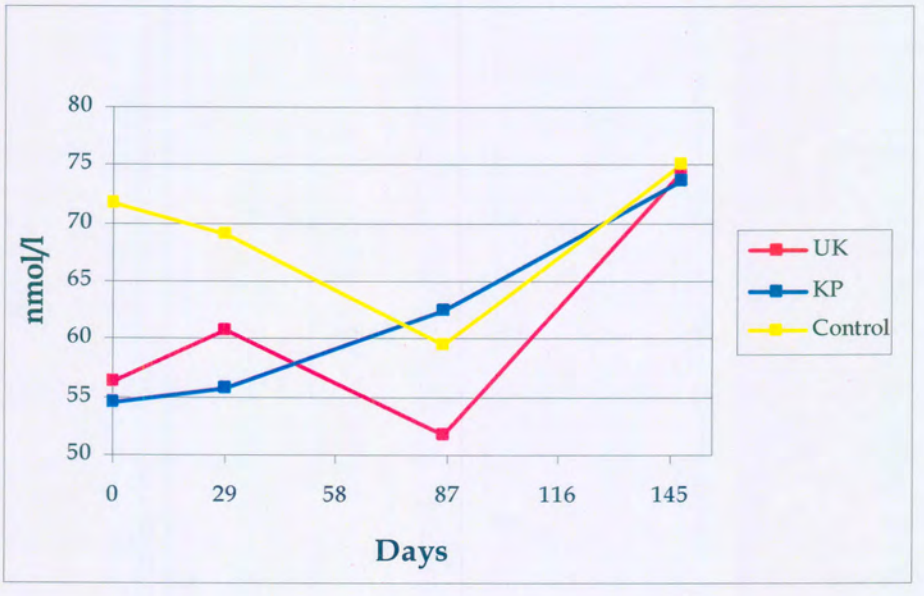

Fig. 5.13: Mean thyroxine (T_4) concentrations of the three experimental groups at D0, D29, D86 and D148 of the feeding trial

5.3.7 Testosterone analysis

There was not a significant difference amongst the groups with regard to their mean testosterone concentrations (Fig. 5.14).

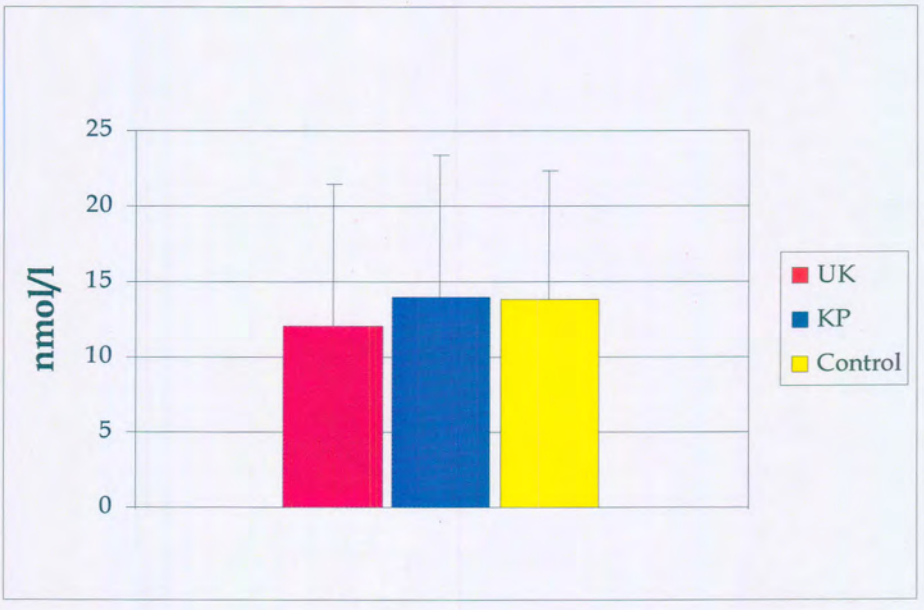

Fig. 5.14: Mean testosterone concentration of the three experimental groups at D154 of the trial

5.3.8 Infectious Bovine Rhinotracheitis (IBR) - Indirect fluorescent antibody test

Using a serum screening dilution of 1:20, all animals in the three groups showed to be positive for antibodies against IBR.

5.3.9 Seroconversion with *Brucella abortus* Strain 19 vaccine

The complement fixation test is very specific and is regarded throughout the world as the definitive test for serological detection of *Brucella* infected/vaccinated animals (Coetzer, Thomson & Tustin 1994). Before the immunization all the animals tested negative for *Brucella abortus* antibodies. After vaccination all the calves had a positive reaction to the vaccine. In South Africa, titers of 30 IU/ml or higher are considered positive in those animals that have been vaccinated between 4 and 8 months of age (Coetzer *et al.* 1994). The antibody titers against *Brucella abortus* were similar in the three different groups 1 month after the immunization of the calves with *Brucella abortus* Strain 19 vaccine (OBP) (Fig. 5.15). No statistical significant difference was found between the three experimental groups.

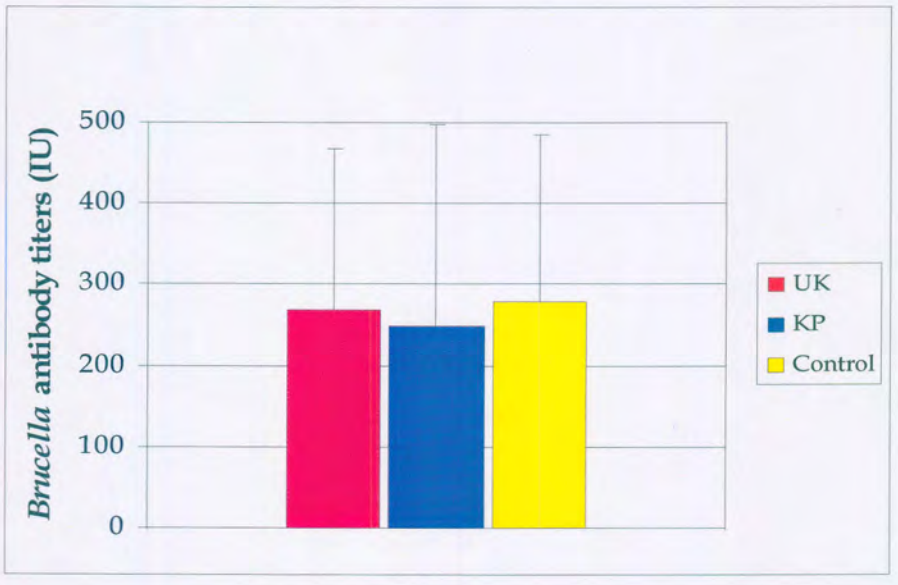

Fig. 5.15: Mean *Brucella abortus* antibody titers of the three experimental groups after immunization

5.3.10 *Brucella* protein allergen skin test

No statistical significant difference in skin thickness was measured in any of the groups 72 hours after the intradermal administration of the *Brucella* antigen (Fig. 5.16).

Brucellergene OCB® was successfully used in the past as an allergen to define the intrinsic parameters of a skin test and to compare its properties with serology for the diagnosis of bovine brucellosis. The skin test was also evaluated for its capacity to solve problems associated with "false positive" reactions in serological tests (Saegerman, Vo, De Waele, Gilson, Bastin, Dubray, Flanagan, Limet, Letesson & Godfroid 1999). Although the sensitivity of this test, compared to CFT, can vary between 52 and 62 %, its specificity exceeds 99 % (Coetzer *et al.* 1994).

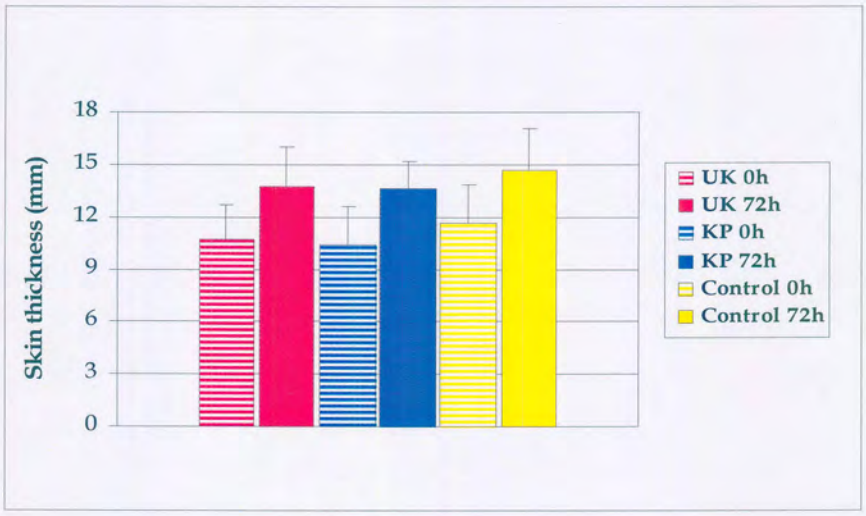

Fig. 5.16: Mean skin thickness of the three experimental groups before and after intradermal injection with Brucellergene OCB®

5.3.11 Lymphocyte transformation test

The control group showed more transformation after the challenge with the mitogen Concanavilin A, but due to a very large standard deviation the difference was not statistically significant (Fig. 5.17).

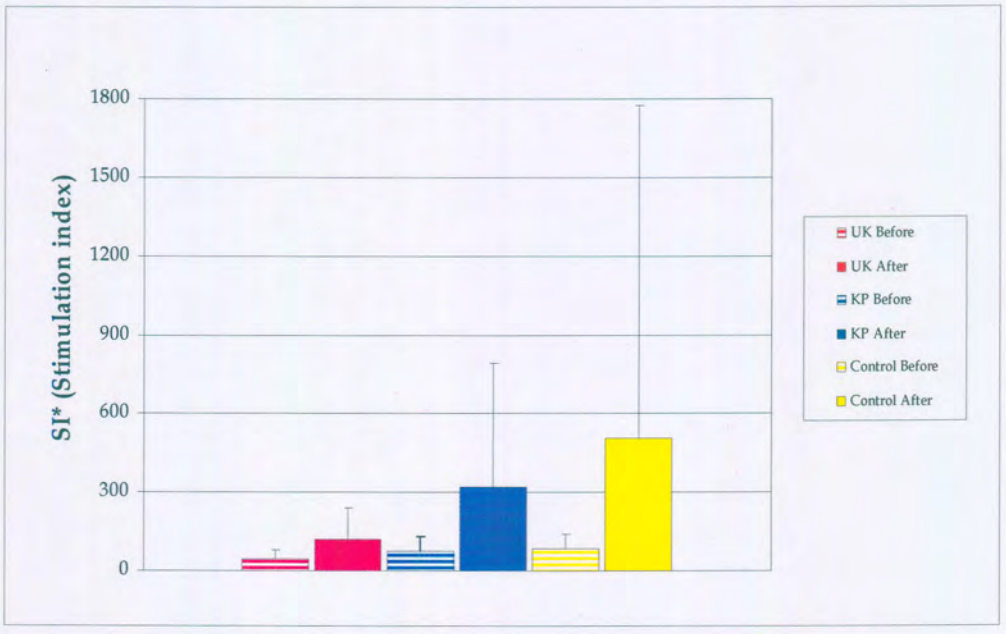

Fig. 5.17: Results of the lymphocyte transformation test of the three experimental groups, before and after 4 months of molasses feeding [*SI= mean counts per minute (cpm) of tested sample/mean cpm of unstimulated control]

5.3.12 Immunoglobulin (IgG and IgM) titers

Mean IgG titers in all three groups increased consistently during the molasses feeding trial (Fig. 5.18), most probably due to natural exposure to different pathogens. Mean IgM concentrations increased after the start of molasses feeding at 4 months, but decreased slightly when determined again at 5

months into the trial (Fig. 5.19). Only slight variations occurred between the groups. No statistical significant difference was found between the three experimental groups.

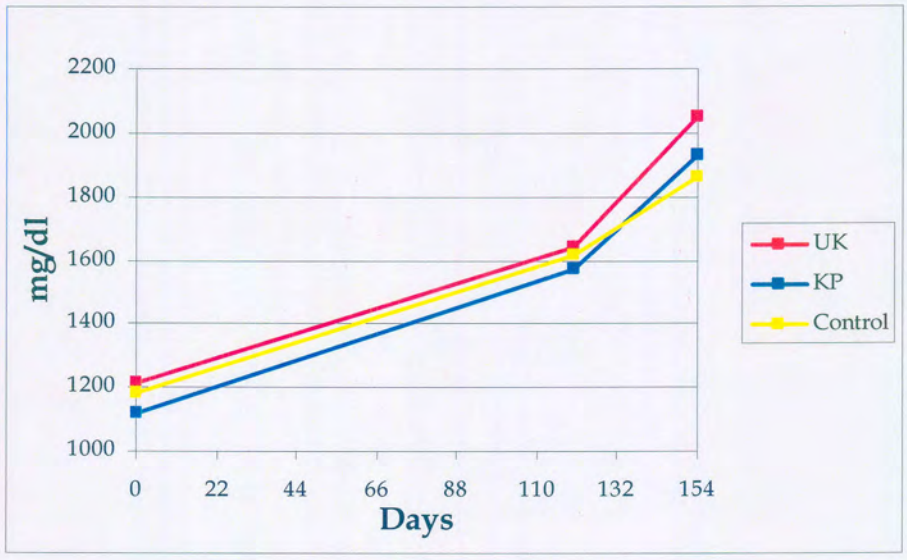

Fig. 5.18: IgG levels of the three experimental groups at D0, D120 and D154 of the trial [normal range of IgG: 1700-2700 mg/dl (Coetzer *et al.* 1994)]

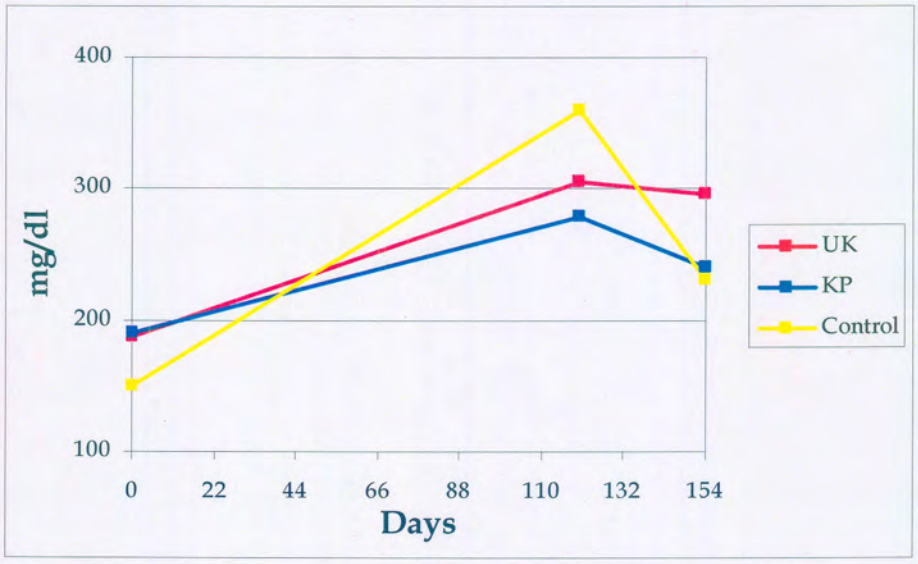

Fig. 5.19: IgM levels of the three experimental groups at D0, D120 and D154 of the trial [normal range of IgM: 250-400 mg/dl (Coetzer *et al.* 1994)]

5.3.13 Carcass weight

Mean carcass weight of each group is shown in Figure 5.20. No significant differences were found between groups.

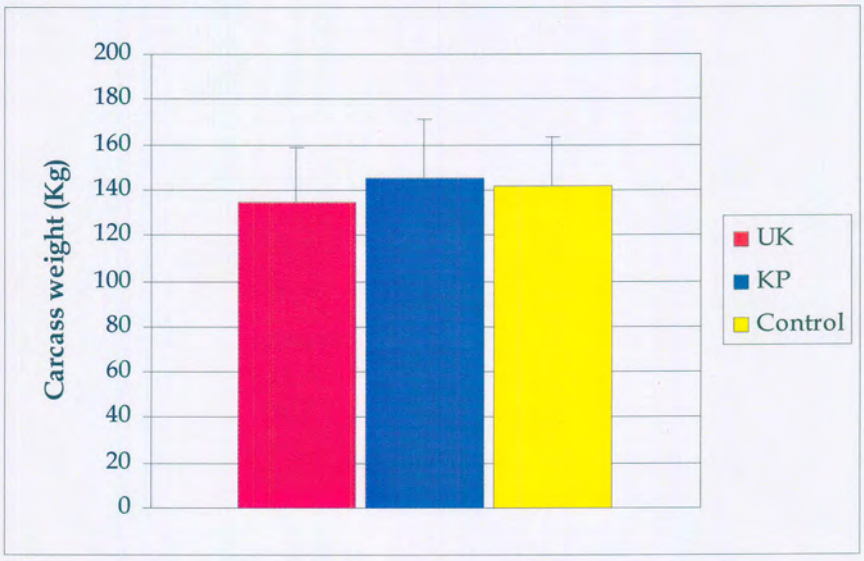

Fig. 5.20: Average carcass weights of the three experimental groups

5.3.14 Pathology and histopathology

No noteworthy macroscopic lesions were detected in the organs/tissues examined at necropsy and no histopathological lesions were seen in the tissues collected.

5.3.15 *Brucella abortus* culture on animal tissues

All calves in the control group tested negative for *Brucella abortus* organisms at culture. *Brucella abortus* was cultured in the left pre-scapular lymph node of one animal in the KP group and two animals in the UK group. However, these results do not represent any difference amongst the experimental groups.

5.3.16 Mineral analyses

The mean liver concentration of Cu, Fe, Zn and Mn for each group was calculated (Table 5.7). Even though mean liver Cu concentrations in the three groups were higher than the normal range given for cattle, this concentration did not reach toxic levels (250-800 mg/kg) (Puls 1994). On the other hand, the mean liver Fe concentrations in the three experimental groups were slightly lower than the normal range, but did not reach deficient levels (<30 mg/kg) (Puls 1994). No relevant differences were found in the levels of these trace elements between the three groups.

Table 5.7: Mean concentrations of Cu, Fe, Zn and Mn in liver samples

	UK Mean (±SD)	KP Mean (±SD)	Control Mean (±SD)	Normal range in cattle*
Cu (mg/kg WM)	223.3 (±57.1)	218.2 (±39.3)	189.6 (±40.3)	25-100
Fe (mg/kg WM)	40.4 (±9.4)	40.8 (±3.8)	40.5 (±9.8)	45-300
Zn (mg/kg WM)	31.9 (±8.0)	33.8 (±8.1)	35.8 (±11.5)	25-100
Mn (mg/kg WM)	2.9 (±0.3)	2.9 (±0.5)	2.8 (±0.4)	2.5-6

* Mineral levels in animal health (Puls 1994)

WM = Wet mass

SD = Standard deviation

5.4 CONCLUSIONS

Calves from the three experimental groups were kept in similar facilities, under the same conditions and fed diets with comparable energy, protein and minerals composition (Tables 5.1 and 5.2). On the majority of the days all the feed was consumed by the calves and only rarely small amounts remained (left over occurred in all three groups), which were not regarded as having an influence on weight gain.

All clinical pathology, endocrine and immunocompetency parameters measured, showed no significant differences between the UK, KP and control groups.

Even though it would seem that the UK group had a lower mean weight gain (compared to the other two experimental groups) (Fig. 5.11), only one significant difference was found at 4 months of molasses feeding, when monthly weight gains were statistically analysed. Considering that this was the only significant difference during the whole feeding trial, it is unlikely that this could be attributed to molasses in the feed. Although the calves in the KP group exhibited a higher incidence of diarrhoea when compared with the other two groups (Fig. 5.8), it did not appear to affect their weight gain over the whole feeding period.

The immunity of the calves was not compromised. Animals in the three experimental groups tested positive for antibodies against IBR following vaccination at a very young age at the rearing facility. All the calves seroconverted when challenged with *Brucella abortus* Strain 19 vaccine and all the animals in the three experimental groups showed similar antibody titers against *Brucella abortus* a month after the immunization (Fig. 5.15). These results confirm and verify that the calves' immune responses were not affected.

No relevant differences between the three groups were detected in the mean liver concentrations of Cu, Fe, Zn and Mn (Table 5.7).

The mean dietary Cu levels supplied to the three experimental groups ranged from 30.76 - 34.86 mg/kg, which were higher than the requirement for cattle (10 - 25 mg/kg). However, this was far below the maximum safe dietary level for calves of 50 mg/kg (Puls 1994). The higher Cu inclusion level in the ration is most probably the reason why the mean liver Cu concentrations in the three groups were higher than the normal range for cattle.

Although Fe inclusion levels in the diet of the three experimental groups were adequate, the liver Fe concentrations in all the calves were slightly lower than the normal range. However, no abnormalities were detected in the haematology parameters such as Ht, RCC, Hb, MCHC and MCV, throughout the feeding trial that could indicate an Fe deficiency (Puls 1994).

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

Molasses is an important feed supplement for livestock in South Africa and the rest of the world and it was necessary to ascertain if there was indeed a problem. The allegations that molasses was implicated in the aetiology of a suspected "endocrine disruptive syndrome" led to market resistance and also raised concerns about the consumption of edible products, obtained from molasses-fed animals, by humans. If no adverse effects of molasses were found, then the search for the cause(s) of this syndrome would have to be directed elsewhere.

Preliminary feeding trials of cattle with molasses carried out by stockowners lead to equivocal and conflicting results. In an attempt to clarify the role of molasses in the aetiology of the syndrome, an investigation of molasses and a scientifically designed, controlled feeding trial in calves was conducted.

Four batches of molasses from four different sugar mills (KP, P, NO & UK) were screened to select two potential harmful batches for inclusion in a calf feeding trial. Since no major differences were found in the chemical composition of the four batches of molasses (Table 4.1), the selection of the batches to be used in the calf feeding trial was based on the laboratory

screening tests (Table 4.2). The two batches chosen were:

- KP molasses, with the highest immunosuppressive effect and a high TH activity and
- UK molasses, with the highest oestrogenic activity and a marked immunosuppressive activity.

Even though endocrine disruptive activity was detected in laboratory tests, these could not be reproduced in calves under experimental conditions. However, it should be realized that *in vitro* potency comparisons could be misleading.

In vitro assays do not always reliably predict the *in vivo* outcome, due to differences in metabolic capabilities of the test systems (and lack of *in vivo* pharmacokinetics and pharmacodynamics) and the diverse range of mechanisms by which endocrine disrupting chemicals may act (Clode 2006). For example, screening tests that measure only the binding to oestrogen receptors do not take into account the differences between endogenous hormones and exogenous EDCs in their ability to activate subsequent events after receptor binding that ultimately lead to changes in gene expression (Amaral Mendes 2002). Similarly, they do not take into account the rates of absorption and metabolism of exogenous EDCs (Gray *et al.* 2003). Because oestrogenic chemicals compete with endogenous oestrogens for binding to oestrogen receptors, the concentration of endogenous oestrogen may influence the oestrogenic effects of the chemicals and a chemical that on its

own shows weak oestrogenicity might therefore potentially act as an anti-oestrogen *in vivo* (Andersen *et al.* 1999).

Indeed, *in vitro* bioassays measure only one endpoint or one specific characteristic of a possible endocrine disrupting agent. *In vivo*, endocrine disruptors interact in a more complex manner with the homeostasis of the endocrine system.

In vitro assays can therefore lead to "false positive" results (active *in vitro,* but not *in vivo*). Reasons for *in vivo* inefficacy could be attributed to a compound that is not absorbed or distributed to the target tissue; a compound that is rapidly biotransformed to an inactive metabolite and excreted or when some other form of toxicity of that compound predominates *in vivo* (Gray *et al.* 1997).

Despite widespread use, it has been reported that the MCF-7 cells proliferate in response to a range of non-oestrogenic substances, including progesterone, dihydrotestosterone, insulin-like growth factors, lithium chloride and ethanol (Jones, Baker, Irwin & Earl 1998), thus generating "false positive" data.

On the other hand, *in vitro* assays can also result in "false negative" responses (positive *in vivo*, but negative *in vitro*), since they do not account for absorption, distribution, metabolic activation and excretion of potential EDCs. For example, purified methoxychlor is inactive or weakly active *in vitro*, whereas *in vivo* it is

almost as potent as 17ß-oestradiol when both are administered orally (Gray *et al.* 2003).

Dose and age of exposure should also be considered. When xeno-oestrogens identified through *in vitro* assays were tested using the *in vivo* uterotrophic assay, their low potency relative to that of oestradiol was confirmed. However, significant effects were observed when these compounds were administered peri-natally at doses that were of a lower magnitude than those producing an uterotrophic effect. This lack of correlation, between the potency and the developmental effects, suggests that the relative potency revealed by the conventional assays should not be used to infer whether a chemical has or has not deleterious effects at current exposure levels (Soto *et al.* 1995).

It is therefore obvious that the complex nature of the endocrine system in the living organism and the relationship of endocrine toxicity to other systemic effects cannot be simulated *in vitro.* Therefore, future research needs to be conducted on the relationship between screening tests for suspected endocrine disruptors and their *in vivo* effects in mammals.

Farmers reported that this suspected "endocrine disruptive syndrome" caused, amongst other problems, seriously decreased production (weaning weights, milk yield and loss in condition particularly of dairy cows). During the feeding trial, calves from the three experimental groups were kept in similar facilities, under the same conditions and fed diets with comparable composition

regarding energy, protein and minerals (Tables 5.1 and 5.2). Drinking water parameters were also within normal limits (Tables 5.3 and 5.4).

Even though mean monthly weight gains of the three experimental groups were analysed statistically and some of the results tended towards significance, a statistical significant difference between the groups was only seen at 4 months after the commencement of molasses feeding. However, it is unlikely that this could be attributed to molasses feeding.

The mean weights of the three experimental groups at Week 24 of the trial (when the calves were between 29 and 33 weeks of age) ranged from 243 – 261 kg (Fig. 5.9). Although these calves were weaned of milk within days of birth, their weights were comparable with the national average weaning weights of Bonsmara calves, a beef breed known for high weaning weights (220 kg at 205 days of age) (ARC, Livestock Business Unit 2006).

The farmers also reported increased reproductive disorders with poor conception, resorptions and abortions, which were attributed to an "endocrine disruptive syndrome". Since the animals used in the feeding trial were bull calves, only certain parameters could be evaluated to detect reproductive disorders, *viz.*, testosterone levels and the morphology and histopathology of male reproductive organs. No significant differences were detected between the three groups with respect to testosterone concentrations (Fig. 5.14) and

reproductive organs of the bull calves from the three groups showed no visible abnormalities or microscopical lesions.

The farmers also highlighted apparent immune incompetency/breakdown in their herds resulting in calf diarrhoea, verminosis, relapses of protozoal diseases in previously immune competent animals and pneumonia caused by various pathogens.

In the current study, calves from the three experimental groups were considered to be immunocompetent. When tested for IBR antibodies at 4 months into the feeding trial, after being vaccinated at a very young age at the rearing facility, all the animals showed positive titers. When challenged with *Brucella abortus* vaccine, all the animals seroconverted and the three experimental groups showed similar antibody titers against *Brucella abortus* one month after the immunization (Fig. 5.15). While all calves in the control group tested negative for *Brucella abortus* organisms at culture, only one animal from the KP group and two animals from the UK group tested positive. However, these results were not regarded as indicative of immune incompetency.

In addition, immunoglobulin levels in the three groups showed no significant differences. While IgG titers increased consistently during the molasses feeding trial (Fig. 5.18), most probably due to natural exposure to different pathogens in the environment, mean IgM concentrations increased at 4 months

months (Fig. 5.19). The lymphocyte transformation test performed at 4 months also corroborated the immunocompetency of the animals in the three experimental groups. Even though the control group showed a higher degree of lymphocyte transformation after challenge with the mitogen Concanavilin A, this was not statistically significant due to a very large standard deviation (Fig. 5.17).

Only three calves became sick during the trial, one calf from the control group developed a left sided abomasal displacement, which is not an infectious disease. One calf, from the UK group, was hospitalised due to a severe persistent diarrhoea, but recovered uneventfully. Even though the calves from the KP molasses fed group showed a higher incidence of diarrhoea when compared with the other two groups (Fig. 5.8), it did not appear to affect their weight gain over the whole feeding period. Faecal samples were sent for bacterial culture as well as for virus particle identification and were also screened for coccidial oocysts and helminth eggs. These results were all negative and the diarrhoea was thus considered to be of metabolic origin. Another animal from the UK group developed an upper respiratory tract infection, but recovered uneventfully after appropriate treatment.

All the parameters measured, indicate that the calves' immune response was not affected. Immunosuppression could, therefore, not be confirmed in the molasses fed calves.

Various mineral imbalances observed in the field were also blamed on the "endocrine disruptive syndrome". Although mean liver Cu concentrations in the three groups were higher than the normal range for cattle (Table 5.7), but still within acceptable limits, this could probably be attributed to the high dietary Cu levels fed during the trial (Table 5.2). These results contradict molasses feeding experiments conducted in the past. Beames (1959) fed different rations to three groups of Hereford heifers and concluded that the two groups of animals receiving molasses as a supplement showed significantly depleted liver Cu concentrations when compared with the group of heifers receiving no molasses supplement. He concluded that the reduction of Cu reserves in the molasses-fed groups could be explained on the basis of a copper-molybdenum-inorganic sulphate interaction. On the other hand, Arthington and Pate (2002), performed experiments on Brahman heifers and compared liver Cu concentrations between animals given corn vs. molasses supplements. Their results indicated that components in molasses-based supplements decreased the accumulation of Cu in the liver of beef heifers and suggested that selenium and molybdenum components in molasses could be in part responsible for this decrease.

Although dietary levels of Fe were considered adequate (100-500 mg/kg), liver Fe concentrations in the three experimental groups were slightly lower than the normal range (Table 5.7), but did not reach deficient levels for cattle (<30 mg/kg). Iron deficiency is associated with anaemia, reduced growth, poor immune function and weakness (Graham 1991; Puls 1994). During the trial, no

abnormalities in the haematology parameters were noticed and growth rate and immune function were also as expected.

Despite goitre being reported by the farmers manifesting as enlarged and/or cystic thyroids, no abnormalities were detected during macroscopical and histopathological examination of the thyroid glands of the animals from the three experimental groups. An assessment of thyroid hormone status (T_3 and T_4) was also performed in the calves at four different times during the feeding trial (Fig. 5.12 & 5.13). The mean T_3 concentrations of the three groups showed similar curves and even though the UK group showed a slightly lower mean T_4 concentration compared to the other two groups at 3 months after commencement of molasses feeding, no statistical significant difference could be demonstrated. Although not statistically significant, an appreciable decrease in mean T_4 concentrations of the control group occurred from the initial bleeding before molasses feeding commenced, up to 3 months of molasses feeding.

Although a lower weight gain was observed in the UK group, this effect was not statistically significant, but tended towards significance. With larger sample sizes a significant difference may become evident.

Under the conditions of the present study, these two batches of molasses induced no endocrine disruptive or immunosuppressive effects in Holstein bull

calves. It can therefore be concluded that these two batches of molasses *per se* were not deleterious when fed to calves.

CHAPTER 7

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