University of Glasgow Veterinary School

Report

Effect of urea supplementation and plasma cholesterol levels on *T. congolense* infection in Scottish Blackface sheep

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Appendix I

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EEC DG XII Project: The interaction between nutrition and genetic resistance to trypanosomiasis in trypanotolerant livestock.
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REPORT

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Introduction

In previous studies plasma cholesterol levels were found to be reduced after *T.congolense* infections in ruminants (Roberts, 1974; Katunguka-Rwakishaya, 1992). Cholesterol is a necessary component of all cell surface and intracellular membranes, and is a precursor for bile acids and numerous steroid hormones (Swenson, 1984). Since trypanosomes are able to take up cholesterol from the blood plasma (Vandeweerd and Black, 1989) the growth of trypanosomes may be partly related to the plasma cholesterol levels (Wassink *et al.*, unpublished data). The cholesterol is taken by *T.brucei* via rapid receptor-mediated endocytotic uptake of low density lypoproteins followed by lysosomal digestion and the release of cholesterol into the cytosol (Coppens *et al.*, 1988). Blood stream forms of *T.brucei* has been found to use almost exclusively host sterols, but other livestages may synthesize endogenous sterol (Coppens *et al.*, 1995).

Cholesterol levels are partly heritable in cattle (Arave *et al.*, 1974) and may play an important role in trypanotolerance. Traore-Leroux *et al.* (1987) found significantly higher HDL-cholesterol levels in trypanosensitive zebu cattle than in trypanotolerant Baoule cattle.

There is also evidence that plasma cholesterol levels in sheep are also related to the diet (Nestel *et al.*, 1978; Wassink *et al.*, unpublished data). In a recent study it was found that high protein diets can ameliorate the effects of *T.congolense* infections (Katunguka-Rwakishaya *et al.*, 1993). Similar results have been demonstrated in ovine fascioliasis (Berry and Dargie, 1976) and haemonchosis (Abbott *et al.*, 1986).

To investigate the effects of plasma cholesterol levels, urea supplementation and possible interaction between the two, an experiment was conducted using twin pairs of Scottish Blackface lambs.

Materials and methods

Experimental design

Twelve pairs of male, twin Scottish Blackface lambs were selected and divided into two groups of six pairs (N and U) each group with a high range in plasma cholesterol concentrations. All animals received 428 grams grass hay dry matter (DM) per day. One group of six pairs (N) were offered an additional amount of 395 grams DM of barley grain and the other group (U) 400 grams DM of barley grain plus urea. The diets resulted in estimated crude protein levels of 86 and 123 grams per day for normal and urea supplemented diet respectively. Energy levels in both diets were very similar. The composition of the diets is shown in table I. Two weeks after the start of the experiment one animal of each pair was infected (I) with *T.congolense* and the other animal used as a control (C).

Infection

Two weeks after the experiment started the group I lambs were infected with *T.congolense* 1180 (GRVPS 57/6) (Nantulya *et al..*, 1984). The trypanosomes were obtained from irradiated mice during the first rising parasitaemia. Each lamb were inoculated intravenously with 5×10^5 trypanosomes in 3 to 4 ml phosphate buffered saline (PBS) (containing 1.5% glucose).

Measurements

General measurements

Clinical observations were made daily for any abnormal behaviour. Body weight was measured weekly.

Blood haematology

On Mondays and Thursdays 5 ml of blood was collected into tubes containing ethylene tetra acetic acid (EDTA) for the determination of a range of haematological indices (packed cell volume (PCV), red blood cell count (RBC), white blood cell count (WBC), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular haemoglobin (MCH), platelet count (PLT), mean corpuscular volumes (MCV), and haemoglobin concentration (Hb)). Blood in which trypanosomes could be detected in the buffy coat (Table II) was used to make Giemsa stained thick blood smears. The ratio of white blood cells to trypanosomes was determined using a phase contrast microscope. The number of parasites per ml of blood could then be estimated by using the equation:

$$\text{WBC/ml} \times \text{Trypanosomes/WBC} = \text{Trypanosomes/ml}$$

The samples were collected from the jugular vein using 20 gauge needles between 7:30 and 8:30 a.m., just before the morning feeding.

Two microhaematocrit tubes were filled with blood from each sample and the mean PCV determined by spinning the tubes in a microhaematocrit centrifuge for 5 minutes.

Blood biochemistry

On Mondays and Thursdays 5 ml of blood was also collected into tubes containing lithium heparin for plasma cholesterol pre- and post-infection, and urea and albumin post-infection. The samples were also collected from the jugular vein using 20 gauge needles between 7:30 and 8:30 a.m., just before the morning feeding.

Statistical analysis

All parameters were subjected to statistical analysis using a randomised block design with each block consisting of a pair of lambs (one I, one C). Mean effect over time was calculated and subjected to split plot analysis of variance with consideration of variation between treatments, between animals within treatments and interaction between treatments. Growth rates of the sheep were determined using linear regression analysis.

Results

Parasitaemia

No differences in parasitaemia could be detected between the normal and urea supplemented diet in either the buffy coat nor the thick blood smear method (Table III) A high correlation was found between the results of both methods (Kruskal Wallis, $H=102.9$, $p<.0001$) although at higher parasitaemias the buffy coat method appears to underestimate the number of trypanosomes in the bloodstream whereas at very low parasitaemias the ratio trypanosomes to white blood cells in the thick blood smear is difficult to assess accurately. The level at which there are not enough trypanosomes in the thick blood smear and the ratio trypanosomes to white blood cells cannot be estimated accurately is estimated to be 100,000 or less trypanosomes per ml of blood ($^{10}\log 5$).

The first peak parasitaemia appeared to be slightly higher in the urea fed group as determined with the thick blood smear method. After the initial peak the parasitaemia started to fluctuate in both dietary groups (Figure 1).

Body weight gain

The average body weight of the control lambs on the urea supplemented diet was higher from the start of the experiment than that of the other three groups (Figure 2). However, the body weight gains were very similar in all four groups and no significant effects were found of either diet or infection. However, a small interaction effect ($p<.05$) was found between diet

and infection. The infected animals on the normal diet had slightly higher body weight gains than their control counterparts, whereas the infected animals on the urea supplemented diet showed lower body weight gains than their controls (Table IV). However, the differences in body weight gains are so small that they are not apparent in figure 2.

Blood haematology

Packed cell volume followed the normal pattern during a trypanosome infection and fell sharply after infection (Figure 3). Approximately 20 days after infection the packed cell volume started recovering slightly. Average packed cell volume was significantly ($p<.01$) lower in the infected than in the control animals (Table V). No diet effect could be observed and the decrease in packed cell volume after infection was very similar between the two diet groups.

The changes in red blood cell counts (Figure 4) and the haemoglobin concentration (Figure 5) during the T.congolense infection developed in a similar manner as the changes in packed cell volume.

The mean corpuscular volume increased significantly during infection ($p<.01$) and appeared to be slightly higher in the infected group fed the normal diet (Figure 6; Table VI). However, no significant diet effect was found. The mean corpuscular volume in the control lambs also increased but not as much as in the infected groups. The mean corpuscular volume appeared to stabilise at around day 35 after infection in all four groups.

The mean corpuscular haemoglobin (Figure 7) increased slightly due to infection ($p<.05$). The mean corpuscular haemoglobin concentration (Figure 8) decreased after infection in the infected group fed the normal diet, but not in the infected lambs fed the urea supplemented diet ($p<.01$) (Table VI) which could be partly explained by the slightly higher mean corpuscular volume in the infected, normal diet fed group.

Platelet counts decreased rapidly after infection (Figure 9), but tended to recover after about 14 days. The variance in platelet counts between animals was high and no significant differences were found between the groups (Table VI).

The variance in white blood cell counts between animals within treatment groups was very high, especially in the infected groups, and none of the differences were significant (Table VI, Figure 10).

Blood biochemistry

The addition of urea to the diet did not result in changes in plasma cholesterol concentrations.and there were no significant differences in plasma cholesterol levels between groups. The variation in plasma cholesterol levels between animals within groups was also low (Table VII) and no significant relationship between cholesterol and parasite levels was observed. Plasma cholesterol concentration of the urea supplemented control animals were

lower pre-infection, though not significantly, than in the other three groups. The plasma cholesterol concentration decreased rapidly after infection in both groups ($p<0.01$) (Figure 11).

The plasma urea concentration was significantly higher in the urea supplemented groups ($p<.01$). Infection decreased plasma urea concentration in the normal-fed lambs but not in the urea supplemented lambs ($p<0.01$) (Table VIII; Figure 12).

There is no explanation for the decrease in plasma albumin concentration in the control groups immediately after infection (Figure 13). Plasma albumin concentration decreased to a similar extent in both dietary groups due to infection ($p<0.01$) (Table VIII).

Discussion

In this experiment the influence of urea supplementation and cholesterol levels was studied in sheep infected with *Trypanosoma congolense*. The cholesterol concentration of the blood plasma was not affected by the urea supplementation. The plasma cholesterol concentration was not different between the two infected groups and no effect could be detected on parasite growth. The urea supplementation did not have a beneficial effect on the pathogenesis of the trypanosome infection.

In this experiment two methods for the determination of parasitaemia were used. Although, the results of both methods correlated strongly, both methods showed shortcomings. The buffy coat method appears to underestimate the number of parasites at higher parasitaemias while low parasitaemias are difficult to estimate with the thick blood smear method.

Body weight gains were not affected by the *T.congolense* infection, indicating that either the particular strain of *T.congolense* used was not very virulent or the animals were relatively resistant to the disease or both.

The additional urea did not result in higher body weight gains indicating that the amount of energy available to the rumen microbes for 'catching' the ammonia produced was a limiting factor. No deleterious subclinical effects, such as a decrease in feed intake, were observed due to an increase in rumen ammonia load. The neutral molecule NH_3 can pass readily across cell membranes including the rumen wall (Smith, 1989). Ammonia concentrations in the portal blood have been found to parallel rumen concentrations (Bartley *et al.*, 1981). The liver can tolerate substantial amounts of ammonia before allowing appreciable amounts to enter the peripheral circulation (Symonds *et al.*, 1981). The higher plasma urea concentration in the sheep fed urea may be explained by the transformation of ammonia to urea in the liver for excretion.

The blood haematology parameters were all significantly affected by the *T.congolense* infection, except for the white blood cell count, and followed similar patterns as in previous experiments infections (Katunguka-Rwakishaya *et al.*, 1993; Wassink *et al.*, submitted). However, no obvious beneficial effects of the additional urea were found on the affected

haematology parameters, except for mean corpuscular haemoglobin concentration which decreased less in the urea fed infected sheep.

No effect of the additional urea on plasma cholesterol levels were found possibly due to the inability of the animals to use the urea. Unlike the previous experiment (Wassink *et al.*, submitted), the variance in plasma cholesterol levels between animals within groups was very low. As a consequence, the relationship between plasma cholesterol levels and parasitaemia found in the previous experiment (Wassink *et al.*, submitted) could not be verified in this experiment.

The lower plasma urea concentration in the infected animals fed the normal diets compared with their controls is difficult to explain. Since the animals were growing it is unlikely that body protein reserves were catabolised during infection as found in *T.congolense* infected straw fed sheep (Wassink *et al.*, submitted). It is more likely that the infected animals on the normal diet were preserving their body protein stores, hence the lower urea concentration.

The results presented here are in contrast to previous experiments in which protein supplemented diets were found to be beneficial to *T.congolense* infected (Katunguka-Rwakishaya *et al.*, 1993), *Fasciola hepatica* infected (Berry and Dargie, 1976) and *Haemonchus contortus* infected sheep (Abbott *et al.*, 1986). The crude protein levels in the low-protein groups of those experiments were much lower than the group fed a normal diet in the present experiment. It may be that the protein level of the normal-fed group was already adequate. Another explanation, already mentioned before, is that the energy levels in the diet of this experiment were not high enough for the rumen microbes to use the ammonia formed from the urea. The protein supplements used in the previous experiments was at least partly protected from rumen digestion, although no figures were presented on rumen protein digestion, and therefore more useful to the animals than the urea supplementation in this experiment. This result emphasises the importance of the energy to protein ratio in the diet both in healthy and sick animals.

References

Abbott, E.M., Parkins, J.J. and Holmes, P.H. (1986):

The effect of dietary protein on the pathogenesis of acute ovine haemonchosis.

Veterinary Parasitology 20, 275-289.

Arave, C.W., Miller, R.H., Lamb, R.C. (1974):

Genetic and environmental effects on serum cholesterol of dairy cattle of various ages.

Journal of Dairy Science, 58, 3, 423-427.

Bartley, E.E., Avery, T.B., Nagaraja, T.G., Watt, B.R., Davidovich, A., Galitzer, S. and Lassman, B. (1981):

Ammonia toxicity in cattle. 5. Ammonia concentration of lymph and portal, carotid and jugular blood after the ingestion of urea.

Journal of Animal Science 53, 494-498.

Berry, C.I. and Dargie, J.D. (1976):

The role of host nutrition in the pathogenesis of ovine fascioliasis.

Veterinary Parasitology 2, 317-332.

Coppens, I, Baudhuin, P., Opperdoes, F.R. and Courtoy, P.J. (1988):

Receptor for the low-density lipoproteins on the hemoflagellate *Trypanosoma brucei.* Purification and involvement in growth of the parasite.

Proceedings of the National Academy of Sciences, USA 85, 6753-6757.

Coppens, I, Bastin, P., Levade, T. and Courtoy, P.J. (1995):

Activity, pharmacologicalinhibition and biological regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase in *Trypanosoma brucei.*

Molecular and Biochemical Parasitology 69, 29-40.

Katunguka-Rwakisjaya, E.(1992):

The pathophysiology of ovine trypanosomiasis caused by *Trypanosoma congolense.*

PhD Thesis. pp 192-194.

Katunguka-Rwakishaya, E., Parkins, J.J., Fishwick, G., Murray, M. and Holmes, P.H. (1993):

The pathophysiology of *Trypanosoma congolense* infection in Scottish Blackface sheep.

Influence of dietary protein.

Veterinary Parasitology 47, 189-204.

Nantulya, V.M., Musoke, A.J., Rurangirwa, F.R. and Moloo, S.K. (1984):

Resistance of cattle to tsetse-transmitted challenge with *Trypanosoma brucei* or *Trypanosoma congolense* after spontaneous recovery from seringe-passaged infections.

Infection and Immunology 43, 735-738.

Nestel, P.J., Poyser, A., Hood, R.L., Mills, S.C., Willis, M.R., Cook, L.J., Scott, T.W. (1978):

The effects of dietary fat supplements on cholesterol metabolism in ruminants.

Journal of Lipid Research 19, 899-909.

Roberts, C.J. (1974):

Blood lipids of ruminants infected with trypanosomes.

Trans. Roy. Soc. Trop. Med. Hyg. 68, 155.

Smith, R.H. (1989):

Nitrogen metabolism in the ruminant stomach.

In: Protein metabolism in farm animals. Evaluation, digestion, absorption and metabolism.

Eds. Bock, H.D, Eggum, O., Low, A.G., Simon, O. and Zebrowska, T.

Oxford Science Publications. VEB Deutscher Landwirtschaftsverlag Berlin.

Swenson, M.J. (1984):

Dukes' Physiology of Domestic Animals. 10th edition.

Comstock Publishing Associates, Cornell University Press, Ithaca and London.

Symonds, H.W., Mather, D.L. and Collis, K.A. (1981):

The maximum capacity of the liver of the adult dairy cow to metabolize ammonia.

Brittish Journal of Nutrition 46, 481-486.

Traore-Leroux, T., Fumoux, F., Pinder, M. (1987):

High density lipoprotein levels in the serum of trypanosensitive and trypanoresistent cattle.

Changes during *Trypanosoma congolense* infection.

Acta Tropica 44, 315-323.

Vandeweerd, V. and Black, S.J. (1989):

Serum lipoprotein and *T.brucei brucei* interaction *in vitro*.

Molecular and Biochemical Parasitology 37, 201-212.

Table I Dry matter (DM), organic matter (OM), neutral detergent fibre (NDF), acid detergent fibre (ADF), ether extract (EE), gross energy (GE) and crude protein (CP) of the diet components

Diet Composition	Low Protein Concentrate	High Protein Concentrate	Grass Hay
DM (g/kg)	849.6	832.5	856.0
OM (g/kg DM)	959.6	955.1	939.0
NDF (g/kg DM)	309.8	319.3	654.4
ADF (g/kg DM)	55.6	61.6	320.9
EE (g/kg DM)	7.4	8.7	5.2
GE (MJ/Kg DM)	17.65	17.63	17.83
CP (g/kg DM)	114.8	204.1	96.0

Table II Parasitaemia scoring

Score	Trypanosomes per field	Estimated parasitaemia (trypanosomes per ml)
1	.-3 per film	10^2 - 10^3
2	4-10 per film	10^3 - 10^4
3	1 per field	5×10^3 - 5×10^4
4	2-10 per field	10^4 - 5×10^5
5	10-100 per field	$> 5 \times 10^5$
6	more than 100 per field	$> 6 \times 10^6$

* magnification = x 400

Table III Intensity of parasitaemia using the buffy coat and thick blood smear methods of *T.congolense* infected (I) sheep (n=6) fed either a normal (N) or a urea supplemented (U) diet

Group	Buffy Coat (Score)	Thick Blood Smear (^{10}log tryps/ml)
NI	2.4	5.35
SE	.3	.075
UI	2.6	5.40
SE	.2	.087
Significance		ns

ns : No Significant Difference

Table IV Body weight gain (g) of *T.congolense* infected (I) sheep and their respective controls (C) fed a normal (N) or a urea supplemented (U) diet

Group	Growth (g)
	102.7
NC	93.1
Pooled SE	4.5
UI	86.2
	102.8
Pooled SE	4.8
Diet effect	ns
Inf. effect	ns
Interaction	*

* · There is a significant difference between means ($p<.05$)
ns : No significant difference between means

Table V Packed cell volume (%) of *T.congolense* infected (I) sheep and their respective controls (C) fed a normal (N) or ureasupplemented (U) diet during pre- (day -9 - 0) and post-infection (day 14 - 49)

	Packed Cell Volume (%)	
	Period	
Group	pre	post
NI		27.8
NC	37.0	37.4
Pooled SE	1.0	1.6
UI	35.6	30.1
UC	36.9	37.4
Pooled SE	.7	1.3
Diet effect	ns	ns
Infection effect	ns	**
Interaction	ns	ns

** : There is a significant difference between means ($p<.01$)
ns : No significant difference between means

Table VI Haematocrit (HT (%)), Red blood cell count (RBC (x 10^{12} l^{-1})), haemoglobin concentration (Hb (g dl^{-1}), mean corpuscular volume (MCV (fl)), mean corpuscular haemoglobin (MCH (pg)), mean corpuscular haemoglobin concentration (MCHC (%)), platelet count (PLT (x 10^9 l^{-1})) and white blood cell count (WBC (x 10^9 l^{-1})) of *T.congolense* infected (I) sheep and their respective controls (C) fed a normal (N) or a urea supplemented (U) diet during post-infection (day 14 - 49)

Group	RBC	Hb	MCV	MCH	MCHC	PLT	WBC
NI	8.42	9.1	32.2	10.7	33.5	138	11.5
NC	11.68	12.2	29.6	10.4	35.3	117	11.7
Pooled SE	.54	.5	.6	1	.3	16	.7
UI	9.34	10.0	31.2	10.7	34.4	79	10.0
UC	12.07	12.2	29.1	10.1	35.0	155	11.4
Pooled SE	.48	.4	.4	.1	.2	20	.9
Diet effect	ns	ns	ns	ns	ns	ns	ns
Infection effect	**	**	**	*	**	ns	ns
Interaction	ns	ns	ns	ns	**	ns	ns

* : There is a significant difference between means ($p<.05$)
** : There is a significant difference between means ($p<.01$)
ns : No significant difference between means

Table VII Plasma cholesterol (mmol l^{-1}) during pre- (day -9 - 0) and post- (day 14 - 49) infection of *T.congolense* infected (I) sheep and their respective controls (C) fed a normal (N) or a urea supplemented (U) diet

	Cholesterol (mmol l^{-1})	
	Period	
Group	Pre	Post
NI	1.36	0.87
NC	1.36	1.47
Pooled SE	.04	.10
UI	1.35	0.92
UC	1.10	1.38
Pooled SE	.07	.09
Diet effect	ns	ns
Infection effect	ns	**
Interaction	ns	ns

** : There is a significant difference between means ($p<.01$)
ns : No significant difference between means

Table VIII Plasma urea (mmol l^{-1}) and albumin (g l^{-1}) concentration post-infection (day 14 - 49) of *T.congolense* infected (I) sheep and their respective controls (C) fed a normal (N) or a urea supplemented (U) diet

Group	Urea (mmol l^{-1})	Albumin (g l^{-1})
NI	3.9	27.7
NC	4.8	29.7
Pooled SE	.2	.5
UI	6.1	27.6
UC	5.9	29.4
Pooled SE	.2	.4
Diet effect	**	ns
Infection effect	*	**
Interaction	**	ns

* : There is a significant difference between means ($p<.05$)
** There is a significant difference between means ($p<.01$)
ns : No significant difference between means

Figure 1: Parasitaemia (Tryps/ml) of T.congolense infected sheep fed a normal (NI) or a urea supplemented (UI) diet

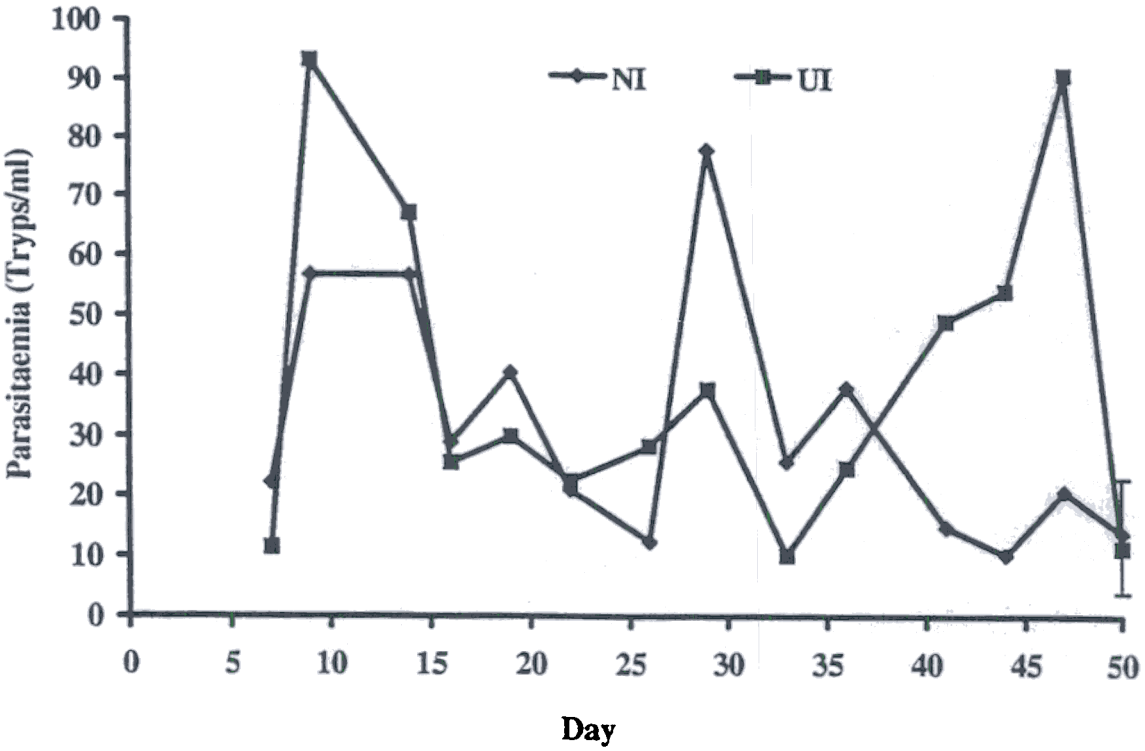

Figure 2: Body weight (kg) of *T.congolense* infected sheep fed a normal (NI) or a urea supplemented (UI) diet and their respective controls NC and UC

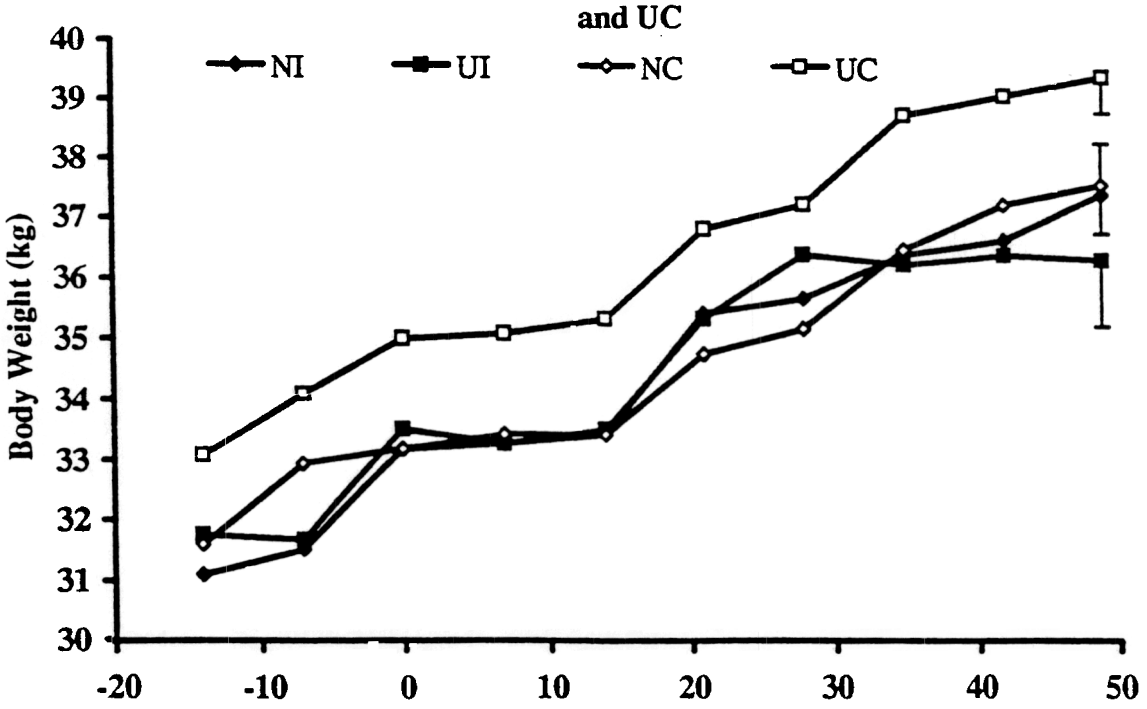

Figure 3: Packed cell volume (PCV (%)) of *T.congolense* infected sheep fed a normal (NI) or a urea supplemented (UI) diet and their respective controls NC and UC

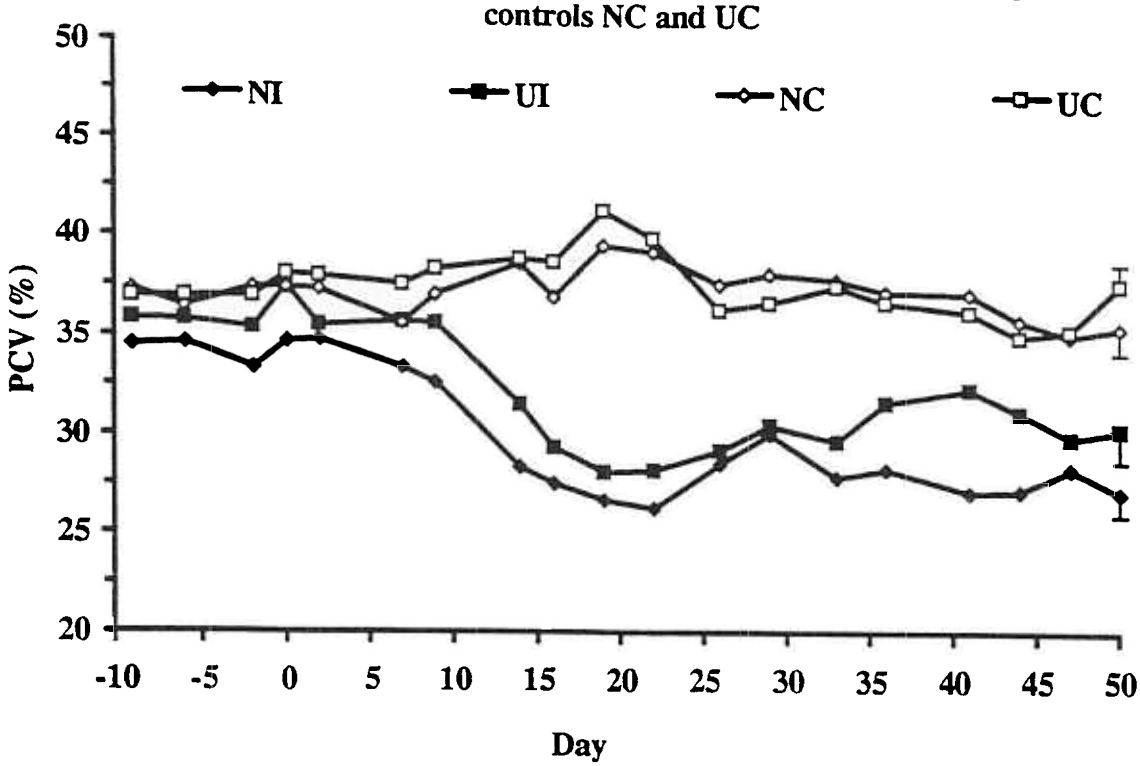

Figure 4: Red blood cells (RBC (x 10^{12} l^{-1})) of *T.congolense* infected sheep fed a normal (NI) or a urea supplemented (UI) diet and their respective controls NC and UC

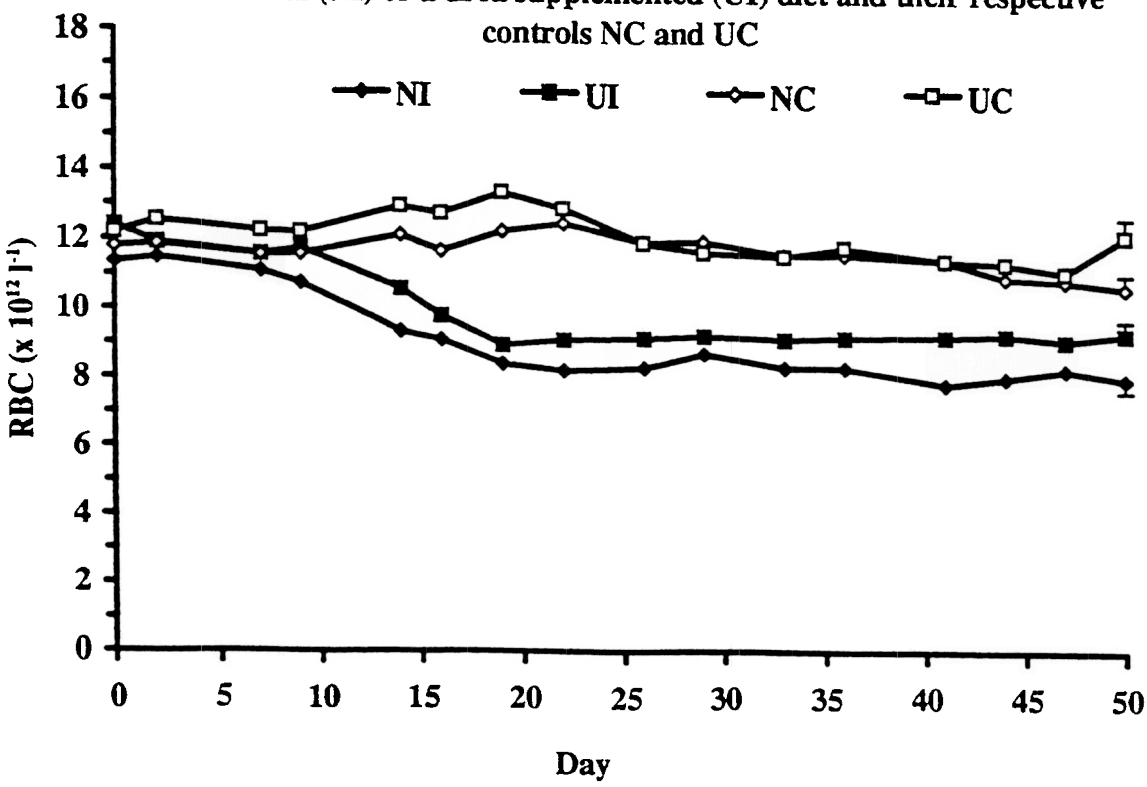

Figure 5: Haemoglobin (g dl^{-1}) of *T.congolense* infected sheep fed diet a normal (NI) or a urea supplemented (UI) diet and their respective controls NC and UC

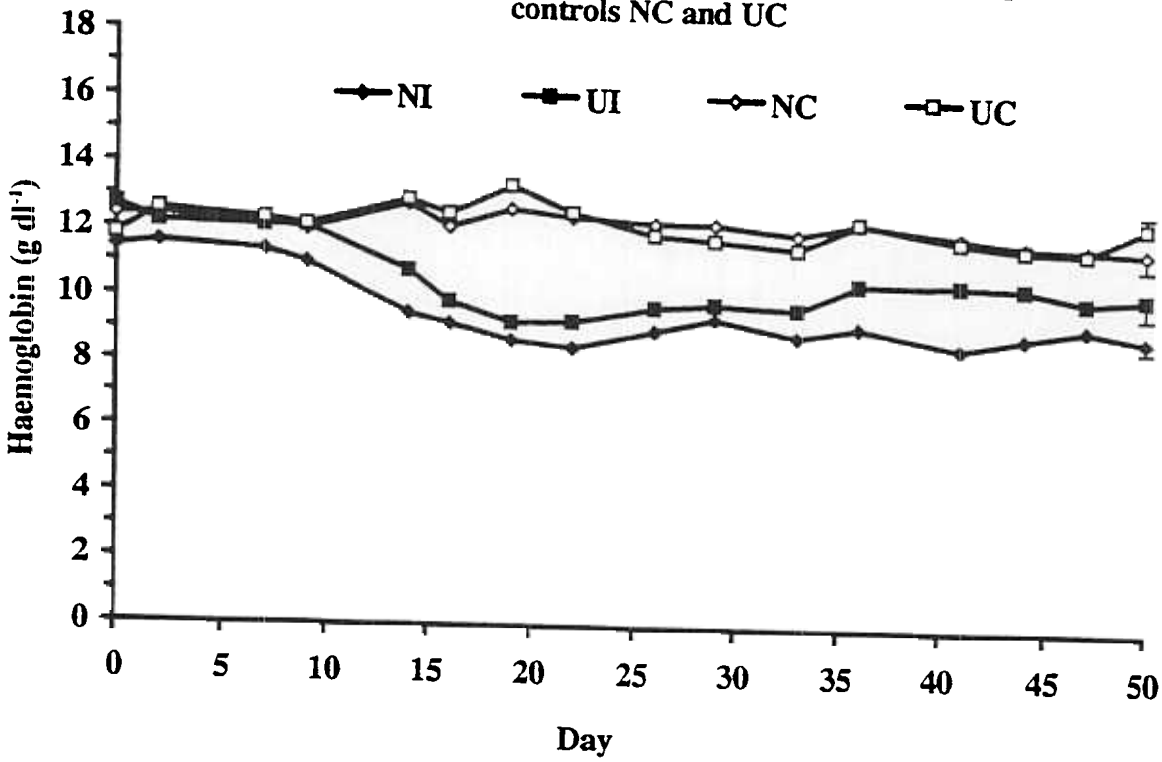

Figure 6: Mean corpuscular volume (MCV (fl)) of *T.congolense* infected sheep fed a normal (NI) or a urea supplemented (UI) diet and their respective controls NC and UC

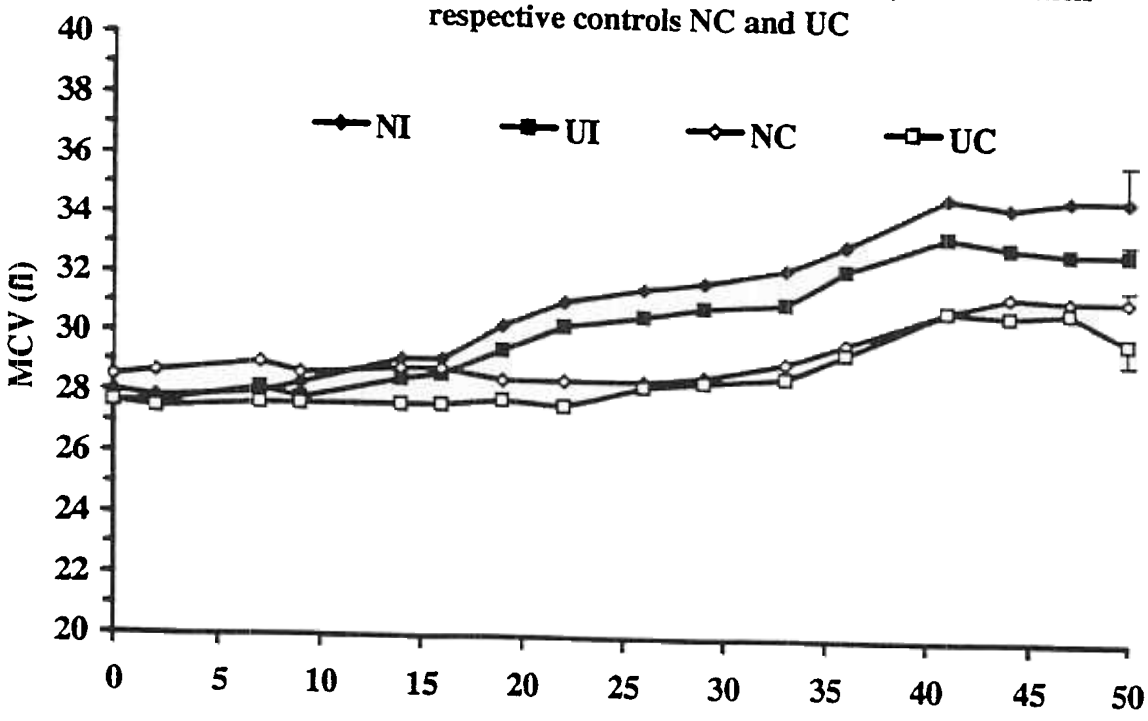

Figure 7: Mean corpuscular haemoglobin (MCH (pg)) of *T.congolense* infected sheep fed a normal (NI) or a urea supplemented (UI) diet and their respective controls NC and UC

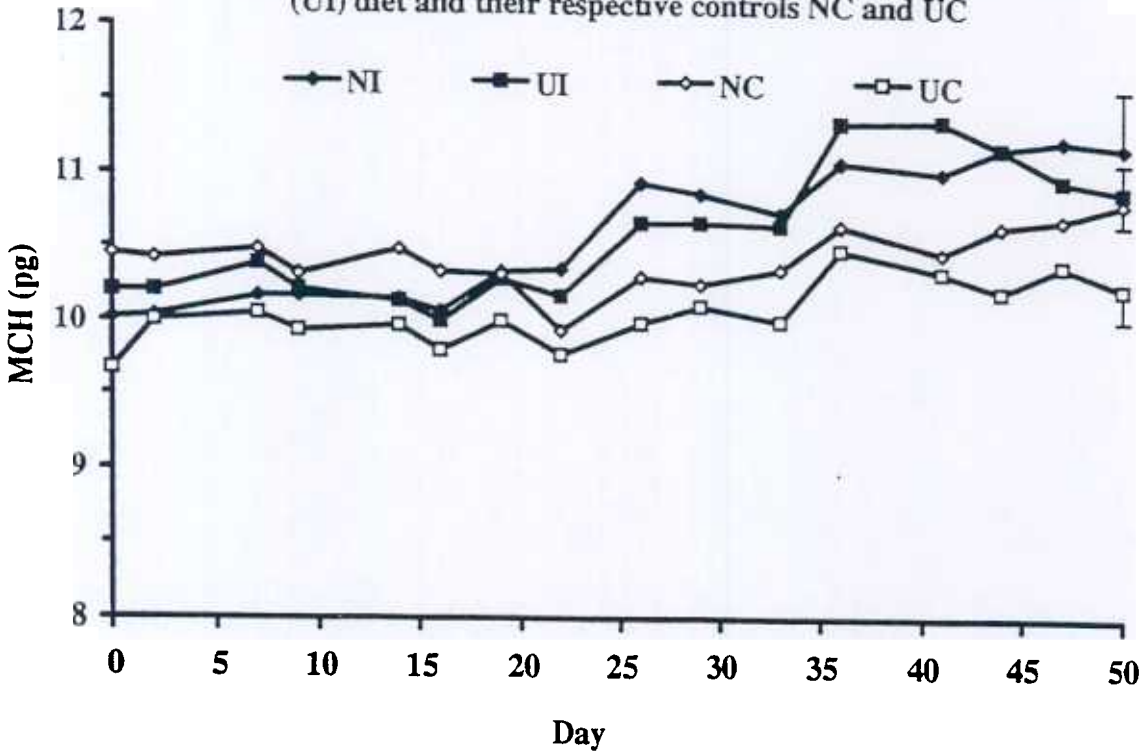

Figure 8: Mean corpuscular haemoglobin concentration (MCHC (%)) of *T.congolense* infected sheep fed a normal (NI) or a urea supplemented (UI) diet and their respective controls NC and UC

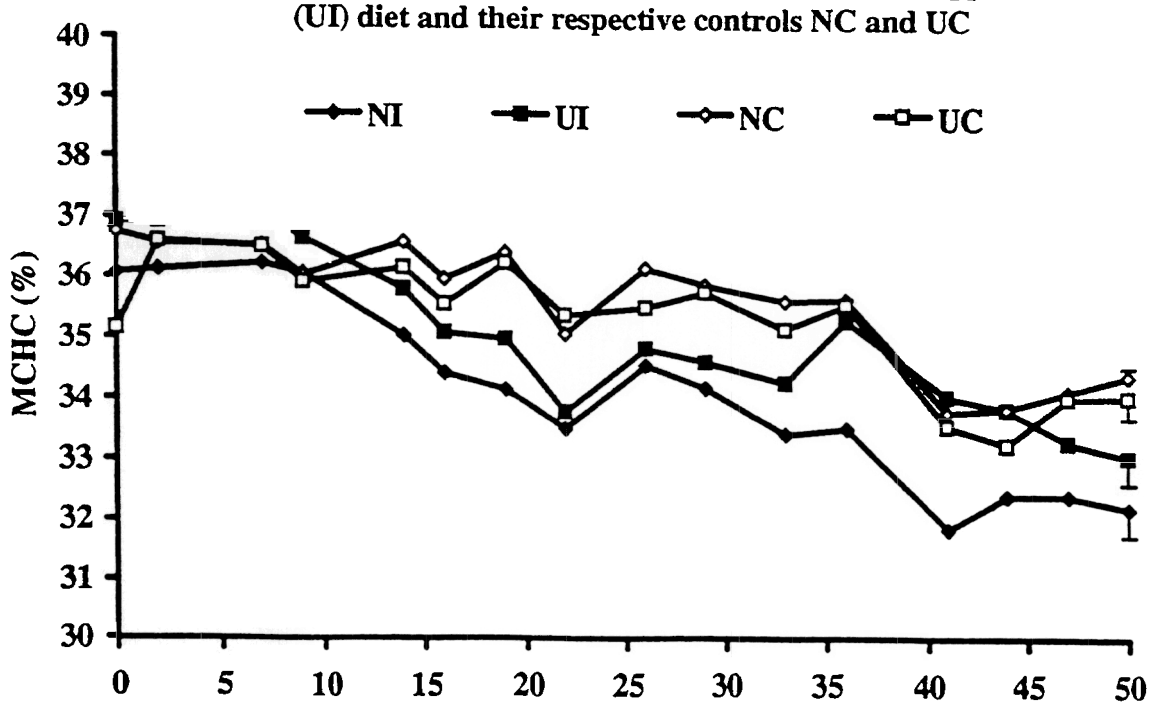

Figure 9: Platelets (x 10^9 l^{-1})) of *T.congolense* infected sheep fed a normal (NI) or a urea supplemented (UI) diet and their respective controls NC and UC

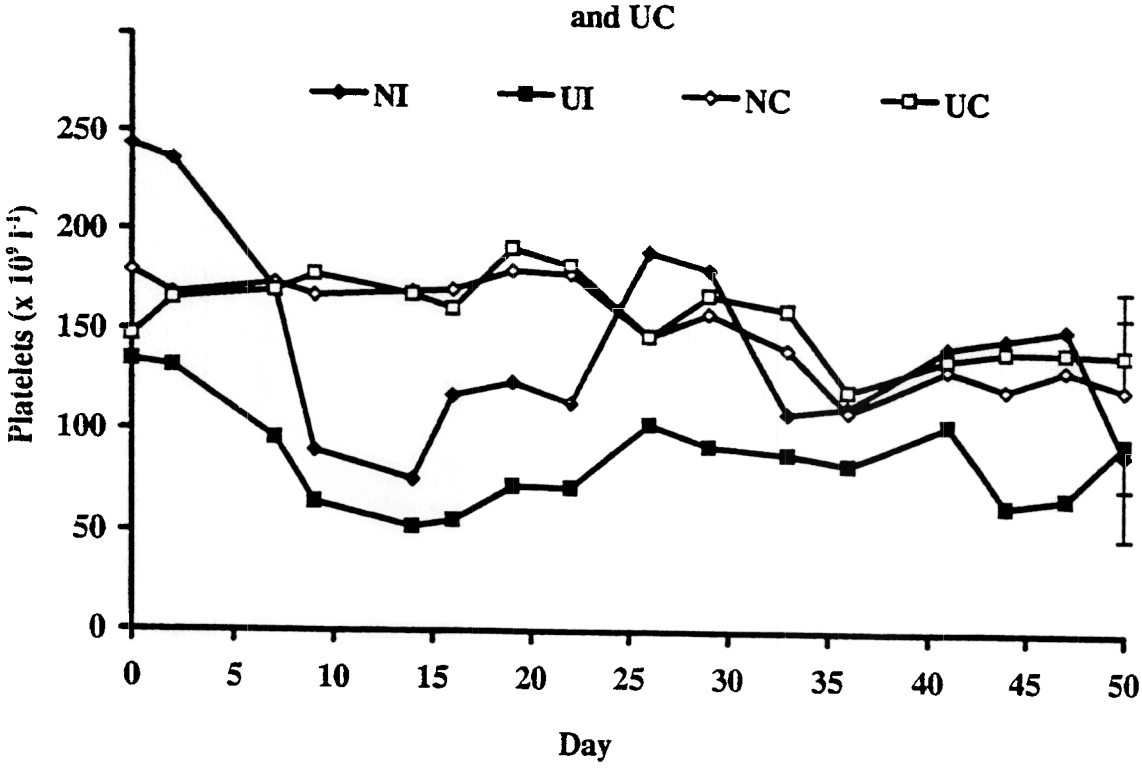

Figure 10: White blood cells (WBC (x 10^9 l^{-1})) of *T.congolense* infected sheep fed a normal (NI) or a urea supplemented (UI) diet and their respective controls NC and UC

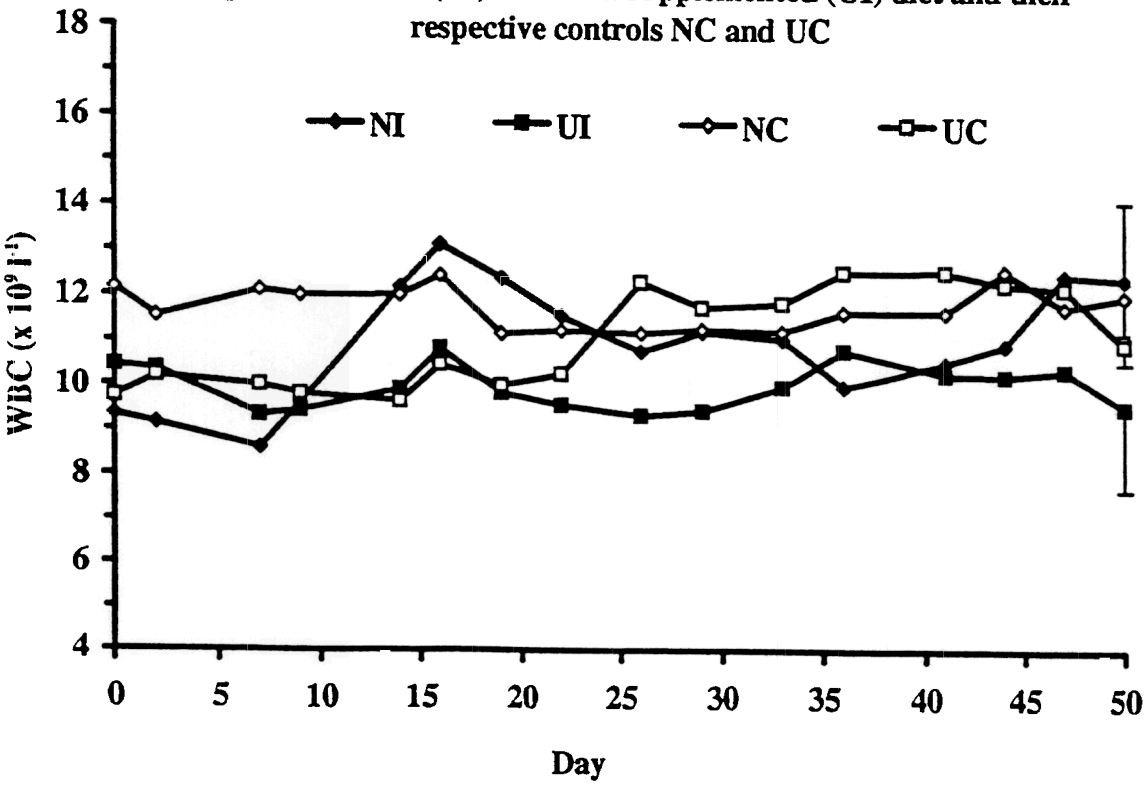

Figure 11: Plasma cholesterol (mmoll^{-1}) of *T.congolense* infected sheep fed a normal (NI) or a urea supplemented (UI) diet and their respective controls NC and UC

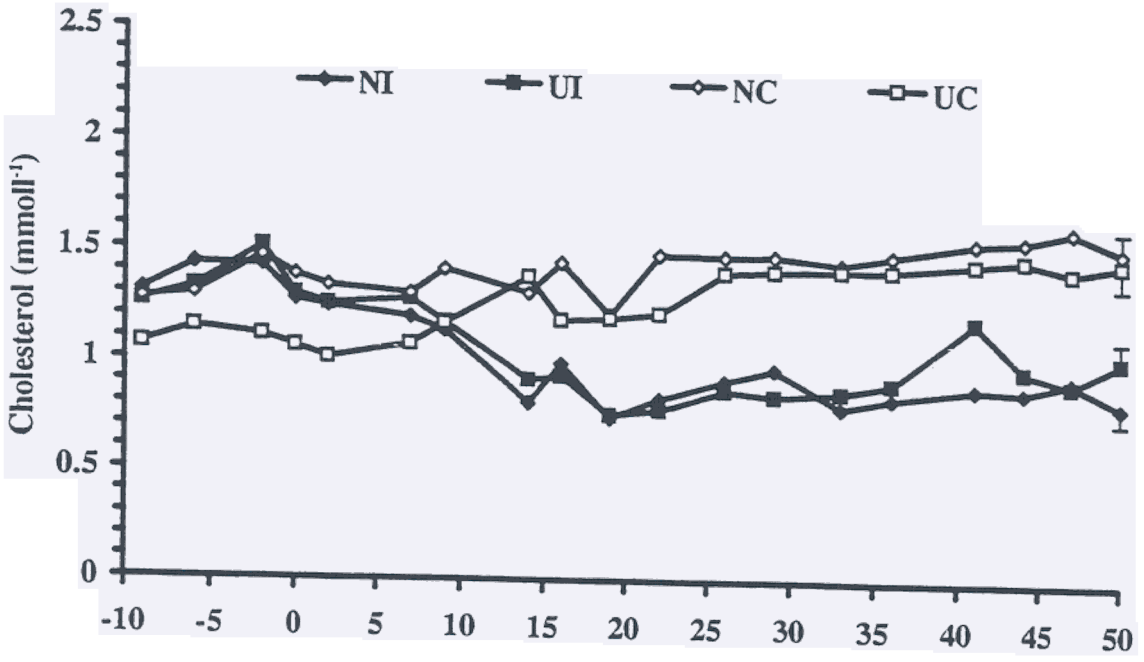

Figure 12: Plasma urea (mmoll^{-1}) of *T.congolense* infected sheep fed a normal (NI) or a urea supplemented (UI) diet and their respective controls NC and UC

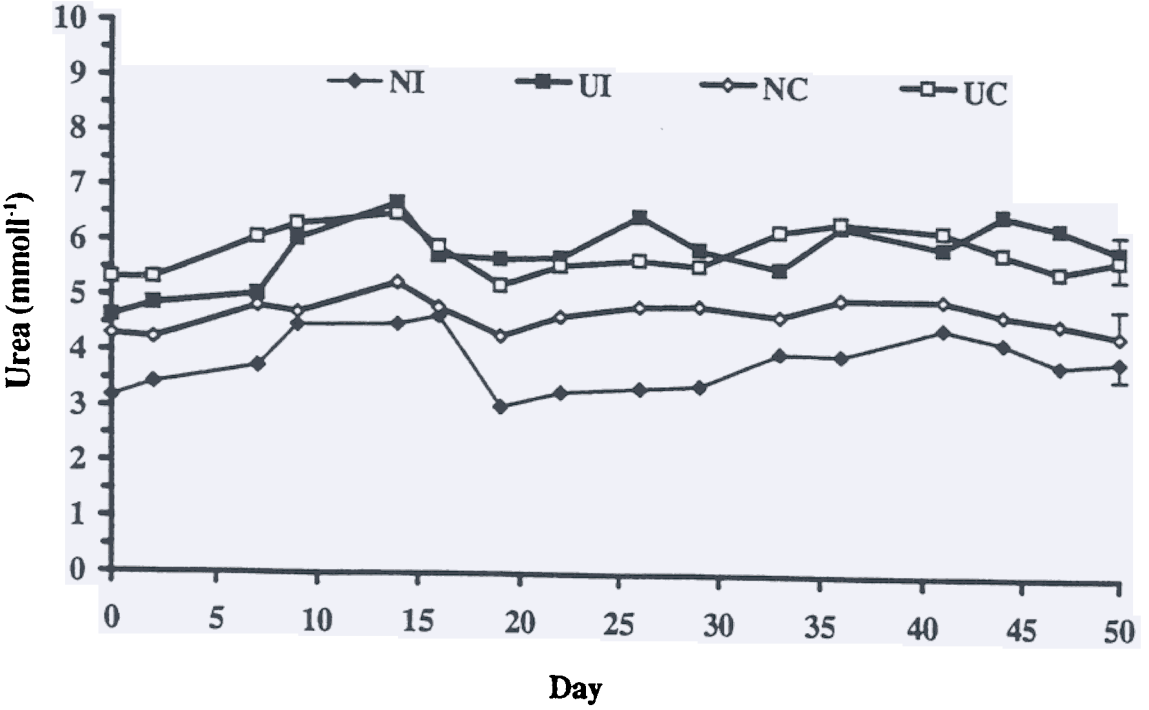

Figure 13: Plasma albumin (gl^{-1}) of *T.congolense* infected sheep fed a normal (NI) or a urea supplemented (UI) diet and their respective controls NC and UC