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Original Article

Urea Output by L_3 *Teladorsagia circumcincta* and some Properties of Two Urea Producing Enzymes

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ABSTRACT

Background: Like several other parasites, *Teladorsagia circumcincta* secretes or excretes urea, but neither the rate of efflux nor the possible metabolic sources of the urea has been considered.
Methods: Parasites were maintained by passage through sheep. Urea efflux was measured using phenol/hypochlorite after treatment with urea aminohydrolase. The kinetics of creatine amidinohydrolase and arginine amidinohydrolase were characterised by coupling the reactions with urea aminohydrolase and glutamate dehydrogenase.
Results: Infective L_3 *T. circumcincta* secreted or excreted urea at 25% of the rate of NH_3/NH_4^+. The rate of urea efflux was about 84 pmol h^{-1} $(10^3$ larvae$)^{-1}$ over 4 hours, corresponding to about 11 nmol h^{-1} mg^{-1} protein. We could not detect urea aminohydrolase activity, but urea production by both creatine amidinohydrolase and arginine amidinohydrolase could be detected. The apparent K_m and V_{max} of creatine amidinohydrolase were 1.1 mM and 48 nmol h^{-1} mg^{-1} protein, respectively, and the activity was greatest at pH 8. The apparent K_m and V_{max} of arginine amidinohydrolase were 0.7 mM and 62 nmol h^{-1} mg^{-1} protein, respectively, and the activity was greatest at pH 7.9.
Conclusion: The activity of creatine amidinohydrolase and arginine amidinohydrolase was sufficient to account for the rate of urea secretion or excretion.

Keywords: Kinetics, *Teladorsagia circumcincta*, Urea

Introduction

A number of nitrogenous small molecules are known to be secreted or excreted by nematodes. Of these, NH_3/NH_4^+ and urea are probably the most commonly observed. Urea secretion or excretion has been reported in *Ascaris lumbricoides*, *Ascaridia galli*, *Nematodirus* spp. and *Panagrellus redivivus*, but was not observed in *Trichinella spiralis*, *Nippostrongylus brasiliensis*, *Ditylenchus triformis*, or, to any significant extent, in *Caenorhabditis briggsae* (1). In the case of *A. lumbricoides* the rate of urea secretion or excretion is slower than that of NH_3/NH_4^+ (2-4). We have shown previously that L_3 *Teladorsagia circumcincta* secretes or excretes NH_3/NH_4^+ at 0.18-0.6 pmol h^{-1} $larva^{-1}$ (5, 6) and, while urea secretion or excretion has also been reported for L_3 *T. circumcincta*, no supporting data were shown and no indication of the rate was given (7).

The source of the urea secreted or excreted by *T. circumcincta* has not yet been investigated in any detail. Of the enzyme reactions known to involve urea, Rogers (2) reported activity of urea aminohydrolase in *A. lumbricoides* and *Nematodirus* spp. and a much lower activity in *Haemonchus contortus*. He also reported arginine amidinohydrolase activity in *A. lumbricoides*, *Nematodirus* spp. and *A. galli* (2) and it has been reported in *T. circumcincta* although no data were given (8, 9), but not in *Toxoplasma gondii* (10, 11). Paltridge and Janssens (4) confirmed that arginine amidinohydrolase activity could be observed in *A. lumbricoides*, but of the remaining urea cycle enzymes, only ornithine carbamoyltransferase (E. C. 2.1.3.3) was detectable.

Here we report on the initial kinetics of secretion or excretion of urea by L_3 *T. circumcincta*. We also attempted to assay urea aminohydrolase (E. C. 3.5.1.5)

$$\text{urea} + H_2O \rightleftharpoons CO_2 + 2NH_3 \qquad 1)$$

to determine whether the apparent rate of urea secretion or excretion might have been influenced by the activity of this enzyme. We also quantified some of the properties of two urea producing enzymes: arginine amidinohydrolase (E. C. 3.5.3.1)

$$\text{arginine} + H_2O \rightleftharpoons \text{ornithine} + \text{urea} \qquad 2)$$

and creatine amidinohydrolase (E. C. 3.5.3.3)

$$\text{creatine} + H_2O \rightleftharpoons \text{sarcosine} + \text{urea}. \qquad 3)$$

We show that the activity of either of these amidinohydrolases (eqns 2-3) would be sufficient in the absence of any detectable activity of urea aminohydrolase (eqn 1) to account for the observed rate of secretion or excretion of urea.

Materials and Methods

Parasite culture and homogenate preparation

Pure strains of *T. circumcincta* were maintained by regular passage through sheep to provide L_3. Larvae were concentrated by centrifugation at approximately 1000 × *g*, washed and then resuspended in 100 mM KH_2PO_4-KOH pH 7.5. Where necessary adult *T. circumcincta* were obtained from the abomasal contents of donor sheep as described previously (12). It was not practicable to culture sufficient L_3 or obtain enough adults to purify the enzymes and so all the work described here was carried out using nematode homogenates. In order to homogenise the nematodes, the suspension was frozen at -20°C and then ground manually at 4°C as described previously (13).

Ammonia and urea determination

The concentration of NH_3/NH_4^+ in was determined spectrophotometrically at 635 nm after reaction with hypochlorite and phenol (14). Concentrations were determined by reference to an NH_4Cl standard.

Ammonia and urea excretion or secretion

The excretion or secretion of NH_3/NH_4^+ and urea was monitored at 37°C in capped Eppendorf tubes. The tubes containing 50000 L_3 mL^{-1} in 1 mL 0.8 mM NaH_2PO_4-NaOH pH 7.0. Before the concentration of NH_3/NH_4^+

was determined, the incubation tubes were centrifuged briefly to pellet the larvae and the supernatant was adjusted to pH 7.

To determine the efflux of urea, 1 U urea aminohydrolase was added to 0.5 mL of the supernatant which was incubated at 37°C for 30 min, after which total NH_3/NH_4^+ was determined. The urea produced was estimated as half of the difference between the total NH_3/NH_4^+ measured after urea hydrolysis and the NH_3/NH_4^+ determined without enzymatic treatment.

Enzyme activities

By using urea aminohydrolase to hydolyse the urea produced by creatine or arginine amidinohydrolase and then employing the reductive amination reaction of glutamate dehydrogenase to incorporate the NH_4^+ into glutamate it was possible to monitor the oxidation of NADH at 340 nm. Using S to denote the substrate (arginine or creatine) and P to indicate the corresponding product (ornithine or sarcosine) of the amidinohydrolase (eqns 2-3), these reactions can be written

$$S + H_2O \rightleftharpoons P + \text{urea}$$

$$\text{urea} + H_2O \rightleftharpoons CO_2 + 2NH_3$$

$$2\ \alpha\text{-ketoglutarate} + 2\ NH_3 + 2\ NADH \rightleftharpoons 2\ \text{glutamate} + 2\ NAD^+ + 2\ H_2O,$$

so the observed rate of NADH oxidation was assumed to be twice the rate of the amidinohydrolase reaction. Details of the assays are provided in the relevant figure legends

Two approaches were employed to attempt to measure the activity of endogenous urea aminohydrolase. The first coupled the production of NH_4^+ to the reductive amination reaction of glutamate dehydrogenase in which the oxidation of NADH was monitored at 340 nm. The second approach involved detecting the NH_3/NH_4^+ directly using the chemical assay described in section 2.2.

The protein concentration of homogenates was determined using the Bradford method (15) and bovine serum albumin as the standard. No detergents were employed to solubilise the nematode homogenate as they can interfere with protein assays (16).

Analysis

While it is likely that the kinetics of L_3 *T. circumcincta* creatine amidinohydrolase and arginine amidinohydrolase can be modelled using the same mechanism as that of other species (17-19), we have not attempted to employ these models in the analysis presented here. Instead, apparent K_ms and V_{max}s were obtained by fitting the standard Michaelis-Menten expression to the activity data for each substrate. This approach is consistent with that adopted previously (20, 21). Estimates of apparent V_{max} depend on the enzyme concentration, so comparisons between L_3 and adult should be made with caution, but such comparisons can be made more reliably for estimates involving the same lifecycle stage. Estimates of apparent K_m are independent of enzyme concentration, so comparisons between values for L_3 and adult preparations can be made with more confidence.

All nonlinear regression and the other analyses described below were carried out using R (22).

Results

Accumulation of NH_3/NH_4^+ and urea

As we have previously reported (5, 7) NH_3/NH_4^+ accumulated in a medium containing L_3 *T. circumcincta*. However, treatment of the medium with urea aminohydrolase increased the total concentration of NH_3/NH_4^+ (Fig. 1), from which we infer that urea was also secreted or excreted by the larvae.

Within 5 min of setting up the suspensions of larvae, the concentration of NH_3/NH_4^+ was 8 ± 3 μM (P = 0.03), which was increased to 24 ± 7 μM (P = 0.005) by treatment with urea aminohydrolase (Fig. 1). From this we estimated that the corresponding concentration of urea was about 8 μM. Thereafter, the rate of NH_3/NH_4^+ accumulation was 0.336 pmol h^{-1} $larva^{-1}$ and this was increased by about 0.17 pmol h^{-1} $larva^{-1}$ by urea aminohydrolase treatment, both of which were approximately con-

stant for the first four hours of incubation. From the difference we infer that there was a constant rate of urea accumulation of 0.084 pmol h^{-1} $larva^{-1}$ over this time (Fig. 1).

Enzymatic reactions involving urea

Sustained attempts to measure endogenous urea aminohydrolase activity were unsuccessful. Neither the enzymatic assay, in which NH_3/NH_4^+ production was monitored using glutamate dehydrogenase, nor the chemical method, involving the measurement of NH_3/NH_4^+ production using hypochlorite and phenol (14), yielded any evidence of the activity of this enzyme. Of course, this was important in our efforts to monitor urea secretion or excretion and the activity of both arginine and creatine amidinohydrolase.

The activity of creatine amidinohydrolase was small (V_{max} = 48 ± 2 nmol min^{-1} mg^{-1} protein) and the apparent K_m was 1.1 ± 0.1 mM (Figure 2A).

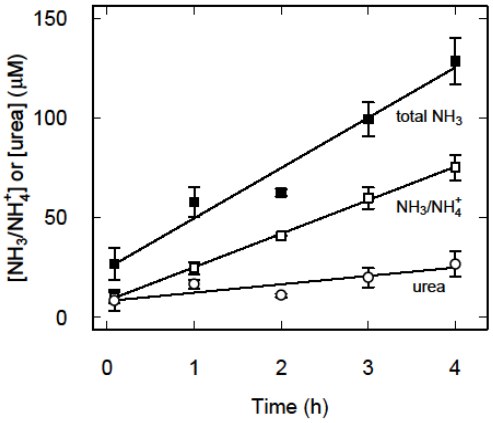

Fig. 1: Initial accumulation of total NH_3 (■), NH_3/NH_4^+ (□) and urea (○) in suspensions of 50000 larvae mL^{-1} in 0.8 mM KH_2PO_4-KOH pH 7 incubated in sealed Eppendorf tubes at 37ºC. Total NH_3 is the concentration of NH_3/NH_4^+ measured in the medium after treatment with urea aminohydrolase. Half of the difference between total NH_3 and the concentration of NH_3/NH_4^+ observed without enzyme treatment was used to estimate the urea concentration. The solid lines are the least squares fit to all of the appropriate data. The error bars represent ± SEM (n = 3)

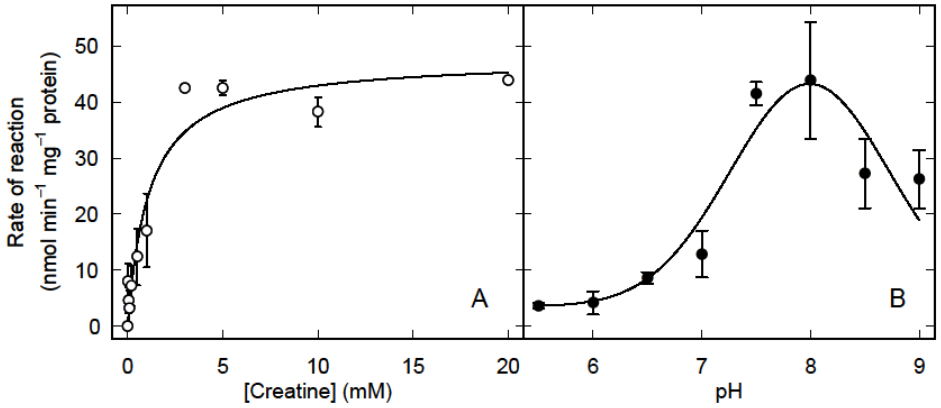

Fig. 2: Rate of creatine hydrolysis in homogenates of L_3 *T. circumcincta* as a function of creatine concentration (A) and pH (B). In (A) the smooth curve is the least squares fits of the Michaelis-Menten equation to all the data. The error bars represent ± SEM for n = 4 (A) or n = 2-5 (B). The reactions were carried out at 30ºC using 50 µM α-ketoglutarate, 0.2 mM NADH, 1 U urea aminohydrolase, 1 U glutamate dehydrogenase and 50 µg of homogenate protein in in 100 mM phosphate buffer. In (B) 5 mM creatine was used

The activity was greatest at pH 8 and declined at more acidic and alkaline pHs with apparent pK_as of 7.1 and 8.9 (Figure 2B). The activity of creatine amidinohydrolase was not affected by Fe(II), but was strongly inhibited by Fe(III), Mg(II), and Cu(II) (Table 1). The activity was slightly stimulated by 2 mM EDTA, but neither ATP nor ADP had any significant effect (Table 1).

Table 1: Effect of ions and adenine nucleotides on the activity of creatine amidinohydrolase in homogenates of L_3 *T. circumcincta*

Conditions	Rate of creatine hydrolysis (nmol min^{-1} mg^{-1} protein) (%)	
Control	42	(100)
+ 0.5 mM $FeSO_4$	40	(95)
+ 0.5 mM $FeCl_3$	7	(17)
+ 0.5 mM $CuSO_4$	5	(12)
+ 0.5 mM $MgCl_2$	12	(28)
+ 2 mM EDTA	52	(124)
+ 0.5 mM ATP	40	(95)
+ 0.5 mM ADP	43	(102)

In homogenates of adult nematodes (n = 2), creatine amidinohydrolase appeared to have kinetic properties that were very similar to those of the L_3 enzyme shown in Figure 2A. For the adult enzyme, the apparent V_{max} was 45 ± 3 nmol min^{-1} mg^{-1} protein and the apparent K_m was 0.6 ± 0.1 mM (data not shown).

In L_3 *T. circumcincta* the activity of arginine amidinohydrolase was also small (V_{max} = 62 ± 4 nmol min^{-1} mg^{-1} protein) and the apparent K_m was 0.7 ± 0.2 mM (Figure 3A), although for adults the V_{max} was 126 ± 6 nmol min^{-1} mg^{-1} protein and the apparent K_m was 1.4 ± 0.2 mM (n = 2). The activity in homogenates of L_3 nematodes was greatest at pH 7.9 and declined at more acidic and alkaline pHs with apparent pK_as of 7.4 and 8.7 (Figure 3B). The rate of arginine hydrolysis was halved by 0.1 mM Fe(II) and Mn(II) stimulated the activity slightly (Table 2), consistent with there being no significant loss from the enzyme of the Mn(II) required for activity (23). Low concentrations of Cu(II) or EDTA stimulated the activity of the enzyme, but slightly higher concentrations were inhibitory (Table 2).

Table 2: Effect of ions on the activity of arginine hydrolysis by L_3 arginine amidinohydrolase in homogenates of L_3 *T. circumcincta*

Conditions	Rate of arginine hydrolysis (nmol min^{-1} mg^{-1} protein) (%)	
Control	41	(100)
+ 0.1 mM $FeSO_4$	19	(46)
+ 1 mM $CuSO_4$	30	(73)
+ 0.1 mM $CuSO_4$	48	(117)
+ 0.1 mM $MnCl_2$	43	(105)
+ 1 mM $MnCl_2$	46	(112)
+ 0.1 mM EDTA	45	(124)
+ 1 mM EDTA	32	(78)

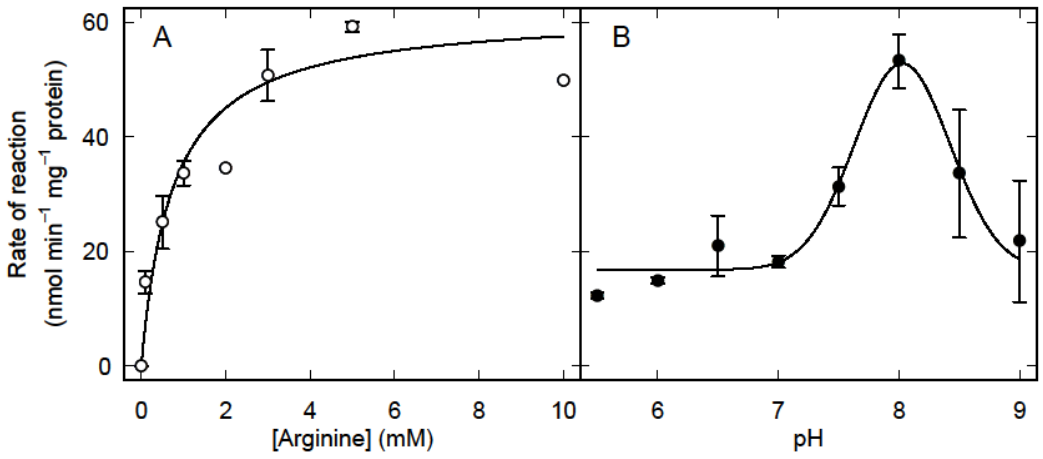

Fig. 3: Rate of arginine hydrolysis in homogenates of L_3 *T. circumcincta* as a function of arginine concentration (A) or pH (B). The smooth curve in (A) is the least squares fit of the Michaelis-Menten equation to all the data. The error bars represent ± SEM for n = 3 (A) or n = 2-5. The reactions were carried out at 30ºC using 100 µM α-ketoglutarate, 0.2 mM NADH, 1 U urea aminohydrolase, 1 U glutamate dehydrogenase and 50 µg of homogenate protein in 100 mM phosphate buffer. In (B) 5 mM arginine was used

Discussion

We have shown that the rate of urea secretion or excretion by L_3 *T. circumcincta* was similar to those reported by Rogers (2) for *Nematodirus* spp., but higher than those he reported for *A. lumbricoides* and *A. galli* (Table 3). The rate of urea secretion or excretion by *T. circumcincta* was about 25% of that of NH_3/NH_4^+, which is similar to previous reports (2-4) for *A. lumbricoides*, *Nematodirus* spp. and *A. galli* (Table 3). We have also shown that L_3 *T. circumcincta* have both arginine and creatine amidinohydrolase activity, but we were unable to detect any urea aminohydrolase activity.

Table 3: Rates of secretion or excretion of urea and NH_3/NH_4^+ reported for various parasites

Species	Rate of secretion or excretion (nmol h^{-1} g^{-1} WW)		Relative rate	Reference
	urea	NH_3/NH_4^+	(urea: NH_3/NH_4^+)	
A. lumbricoides	–	200	–	(29)
A. lumbricoides	17	80	0.21	(3)
A. lumbricoides	52	524	0.10	(4)
Nematodirus spp.	312	–	–	(2)
A. galli	89	–	–	(2)
T. circumcincta	252	1008	0.25	this work

The presence of arginine amidinohydrolase activity is consistent with the observations of activity in *A. lumbricoides*, *Nematodirus* spp. and *A. galli* (2, 4). It is possible that arginine deiminase (E. C. 3.5.3.6), which hydrolyses arginine to citrulline and NH_3/NH_4^+, might also be active in L_3 *T. circumcincta* homogenates. However, we think this is unlikely for two reasons. First, control reactions in which the urea aminohydrolase was omitted yielded very little activity, perhaps consistent with the low rates of chemical hydrolysis previously reported (24). Second, while there is also an orthologue of the human arginine amidinohydrolase gene in the *Caenorhabditis elegans* genome (T24F4.1 or NP_508948), we were unable to identify an arginine deiminase homologue in the *C. elegans* genome.

Our observation of creatine amidinohydrolase activity may be novel. We have been unable to identify either a previous report of this activity in a nematode or a creatine amidinohydrolase homologue in the *C. elegans* genome or among the sequences currently available for other nematodes. The maximum activity of creatine amidinohydrolase was observed at the same pH as that of the enzyme from *Pseudomonas putida* (25) and *Alcaligenes* sp. (20). However, the apparent K_m for creatine is much more similar to that of the *P. putida* enzyme (K_m = 1.33 mM, (26)) than that of the *Alcaligenes* sp. enzyme (K_m = 17.2 mM, (20)), although Schumann *et al.* (27) gives an estimate of 14.3 mM for the *P. putida* enzyme. The effects of Fe(II), Cu(II) and EDTA were similar to those reported for the enzyme from *Alcaligenes* sp. and *Arthrobacter ureafaciens* (20, 28), although Mg(II) had no effect on the *Alcaligenes* sp. enzyme (20). The crystal structure of the *P. putida* enzyme shows that the creatine is stabilised at the carboxyl end by a pair of arginines and at the guanidino nitrogen end by a pair of glutamate residues, and the substrate is sandwiched between a phenylalanine residue and a catalytically significant histidine (18). So, the lower pK_a in the pH dependence (Fig. 2B) might reflect changes in the protonation of either the glutamates or the histidine and the upper pK_a probably reflects pH-induced changes in the polarity of the guanidinium group.

Our inability to detect urea aminohydrolase activity may simply reflect a failure to identify the right assay conditions or test the parasite at the appropriate stage in its life cycle. Urea

aminohydrolase activity has been identified in *A. lumbricoides*, *H. contortus* and *Nematodirus* spp. (2) and nucleotide sequence similar to that encoding the β subunit of the enzyme from *Pseudomonas fluorescens* has been identified in the *Caenorhabditis elegans* genome (hypothetical protein F40G9.5, GenBank accession code: ACO15784.1). However, Rogers (2) reported that the urea aminohydrolase activity was much lower in *H. contortus* than in *A. lumbricoides* and *Nematodirus* spp. and concluded that it was unlikely to protect the nematode from urea in the host. If urea aminohydrolase is present, we conclude that the activity is unlikely to have significant impact on the rate at which urea is secreted or excreted by L_3 *T. circumcincta*.

The rate of urea secretion or excretion was about 0.084 pmol h^{-1} $larva^{-1}$ which corresponds to about 11 nmol h^{-1} mg^{-1} protein (5). The apparent V_{max}s for creatine and arginine amidinohydrolase are 48 and 62 nmol h^{-1} mg^{-1} protein, respectively, so either enzyme could account for the observed urea output. Moreover, the enzymes have similar apparent K_ms, so the most significant factor in determining their relative contribution to urea efflux is the intracellular concentration of each substrate.

Conclusion

The activity of creatine amidinohydrolase and arginine amidinohydrolase was sufficient to account for the rate of urea secretion or excretion.

Acknowledgements

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References

1. Wright DJ, Newall DR. Nitrogen excretion, osmotic and ionic regulation in nematodes. In: Croll NA, ed. The Organization of Nematodes. London: Academic Press Inc., London, Ltd, 1976:163-210.
2. Rogers WP. Nitrogen catabolism in nematode parasites. Aust J Biol Res. 1952; B5: 210-222.
3. Cavier RM, Savel J. Le métabolism protéique de l'Ascaris du porc, *Ascaris lumbricoides* Linné, 1758, est-il ammoniotélique ou uréotélique? C R Acad Sci. 1954; 238: 2448-2450.
4. Paltridge RW, Janssens PA. A reinvestigation of the status of the ornithine-urea cycle in adult *Ascaris lumbricoides*. Comp Biochem Physiol. 1971; 40B: 503-513.
5. Muhamad N, Pedley KC, Simpson HV, Brown S. Ammonium excretion/secretion by L_3 *Ostertagia circumcincta* and its connection with nitrogen metabolism. N Z J Zool. 2004; 31: 377.
6. Muhamad N, Walker LR, Pedley KC, Simcock DC, Brown S. The kinetics of NH_3/NH_4^+ efflux from L_3 *Teladorsagia circumcincta*. Parasitol Int. 2012; 61: 487-492.
7. Simpson HV, Muhamad N, Walker LR, Simcock DC, Brown S, Pedley KC. Nitrogen excretion by the sheep abomasal parasite *Teladorsagia circumcincta*. Exptl Parasitol. 2009; 123: 17-23.
8. Muhamad N, Brown S, Pedley KC, Simpson HV. Some aspects of nitrogen metabolism in *Ostertagia circumcincta*. N Z J Zool. 2005; 32: 278-279.
9. Umair S, Patchett ML, Bland RJ, Simpson HV. Arginine metabolism in the sheep abomasal nematode parasites *Haemonchus contortus* and *Teladorsagia circumcincta*. Exptl Parasitol. 2011; 127: 506-514.
10. Fox BA, Gigley JP, Bzik DJ. *Toxoplasma gondii* lacks the enzymes required for de novo arginine biosynthesis and arginine starvation triggers cyst formation. Int J Parasitol. 2004; 34: 323-331.
11. Cook T, Roos D, Morada M, Zhu G, Keithly JS, Feagin JE, Wu G, Yarlett N. Divergent polyamine metabolism in the apicomplexa. Microbiology. 2007; 153: 1123-1130.
12. Simcock DC, Brown S, Neale JD, Przemeck SMC, Simpson HV. L_3 and adult *Ostertagia* (*Teladorsagia*) *circumcincta* exhibit cyanide sen-

sitive oxygen uptake. Exptl Parasitol. 2006; 112: 1-7.
13. Walker LR, Simcock DC, Neale JD, Simpson HV, Brown S. Thermotolerance of L_3 *Ostertagia* (*Teladorsagia*) *circumcincta* and some of its enzymes. Vet Parasitol. 2007; 146: 77-82.
14. Bolleter WT, Bushman CJ, Tidwell PW. Spectrophotometric determination of ammonia as indophenol. Anal Chem. 1961; 33: 592-594.
15. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72: 248-254.
16. Sapan CV, Lundblad RL, Price NC. Colorimetric protein assay techniques. Biotechnol Appl Biochem. 1999; 29: 99-108.
17. Mora J, Tarrab R, Martuscelli J, Soberón G. Characteristics of arginases from ureotelic and non-ureotelic animals. Biochem J. 1965; 96: 588-594.
18. Coll M, Knof SH, Ohga Y, Messerschmidt A, Huber R, Moellering H, Rüssmann L, Schumacher G. Enzymatic mechanism of creatine amidinohydrolase as deduced from crystal structures. J Mol Biol. 1990; 214: 597-610.
19. Reczkowski RS, Ash DE. Rat liver arginase: kinetic mechanism, alternate substrates, and inhibitors. Arch Biochem Biophys. 1994; 312: 31-37.
20. Matsuda Y, Wakamatsu N, Inouye Y, Uede S, Hashimoto Y, Asano K, Nakamura S. Purification and characerization of creatine amidinohydrolase of *Alcaligenes* origin. Chem Pharm Bull. 1986; 34: 2155-2160.
21. Muhamad N, Simcock DC, Pedley KC, Simpson HV, Brown S. The kinetics of glutamate dehydrogenase of *Teladorsagia circumcincta* and the lifestyle of the parasite. Comp Biochem Physiol. 2011; 159B: 71-77.
22. Ihaka R, Gentleman R. R: a language for data analysis and graphics. J Comput Graphical Stat. 1996; 5: 299-314.
23. Christianson DW. Arginase: structure, mechanism, and physiological role in male and female sexual arousal. Acc Chem Res. 2005; 38: 191-201.
24. Warner RC. The kinetics of the hydrolysis of urea and of arginine. J Biol Chem. 1942; 142: 705-723.
25. Yoshimoto T, Oka I, Tsuru D. Purification, crystallization, and some properties of creatine amidinohydrolase from *Pseudomonas putida*. J Biochem. 1976; 79: 1381-1383.
26. Yoshimoto T, Oka I, Tsuru D. Creatine amidinohydrolase of *Pseudomonas putida*: crystallization and some properties. Arch Biochem Biophys. 1976; 177: 508-515.
27. Schumann J, Böhm G, Schumacher G, Rudolph R, Jaenicke R. Stabilization of creatinase from *Pseudomonas putida* by random mutagenesis. Protein Sci. 1993; 2: 1612-1620.
28. Kaplan A, Szabo LL. Creatinine hydrolase and creatine amidinohydrolase: II. Partial purification and properties. Mol Cell Biochem. 1974; 3: 17-25.
29. Weinland E. Über die Zersetzung stickstoffhaltiger Substanz bei Ascaris. Z Biol. 1904; 45: 517-531.