Density-dependent regulation of the growth of the hookworms *Necator americanus* and *Ancylostoma ceylanicum*

S. M. B. NOROZIAN-AMIRI *and* J. M. BEHNKE*

MRC Experimental Parasitology Research Group, Department of Life Science, University of Nottingham, University Park, Nottingham NG7 2RD

(*Received 7 July 1993; revised 19 November 1993; accepted 20 November 1993*)

SUMMARY

Laboratory bred DSN hamsters were exposed to varying doses of infective larvae of *Ancylostoma ceylanicum* (orally) or *Necator americanus* (percutaneously) and were autopsied at times which corresponded to a period immediately before cessation of growth of worms or soon afterwards. A total of 829 (404 male and 425 female) *A. ceylanicum* and 1582 (781 male and 801 female) *N. americanus* were measured. At worm burdens of fewer than 100, the length of *A. ceylanicum* appeared to increase with infection intensity and no evidence was found that growth was retarded under crowded conditions. In an experiment comparing directly low (mean worm burden = 22) and heavy infections (mean worm burden = 180) significant negative associations between both weight and width, and worm burden were detected, but length again increased with worm burden. In contrast, 5 experiments with *N. americanus* indicated negative relationships between measures of worm size (length, width, wet and dry weight) and worm burden. It was concluded that *N. americanus* is subject to regulation by density-dependent processes within the host while *A. ceylanicum* is not sensitive to the same degree.

Key words: Nematoda, *Necator americanus*, *Ancylostoma ceylanicum*, hookworms, crowding, growth.

INTRODUCTION

Hookworms typically cause chronic infections in their hosts with the duration of patency being measured in terms of months for *Ancylostoma* species affecting dogs (Sarles, 1929*a*, *b*; Carroll & Grove, 1984) and years in the case of *Necator americanus* (Beaver, 1988) and *A. duodenale* (Kendrick, 1934) in humans. Whilst the former species are known to elicit potent immunity in canine hosts (Miller, 1971), infections are maintained for many months under field conditions (McCoy, 1931). Human hookworms are carried throughout life by the people living in endemic regions although worm burdens may stabilize in the middle-aged sector of some communities, or continue to accumulate with increasing age in others (Behnke, 1987; Haswell-Elkins *et al.* 1988; Bundy, 1990; Pritchard *et al.* 1990). These patterns, together with the restoration of pre-treatment intensities of infection within several years of chemotherapy (Behnke, 1987; Quinnell *et al.* 1993), do not support a dominant role for age-associated, immune-dependent protection in regulating human hookworm burdens in the majority of subjects (Behnke, 1987; Bradley *et al.* 1992).

While host-protective immunity is unlikely to dominate regulation of hookworm infections in man, the temporal stability of hookworm populations implies that some regulatory processes are involved. In this context density-dependent processes governing the growth and feeding activities of hookworms may aid in reducing parasite fitness and hence the extent of pathology succumbed by the host. Density-dependent constraints on the development of parasites are well documented in the literature (Keymer, 1982), particularly among the tapeworms where strong negative relationships between increasing parasite burdens and measures of worm size give rise to the so-called 'crowding effect' (Read, 1951; Moss, 1971). Although density-dependent reduction in *per capita* fecundity of hookworms is well established (Sarles, 1929*b*; Krupp, 1961), density-dependent constraints on growth are less well studied. For ethical reasons, such relationships can only be monitored in human infections through examination of worms of mixed age voided after chemotherapy, and consequently data on density-dependent constraints on the size of worms of known age cannot be readily obtained. Moreover, adequate studies based on animal experiments, involving the canine and feline hookworms, have not been reported to-date.

In this paper we report on experiments in which laboratory hamsters of matched age and genetic background were infected with known doses of infective larvae of *A. ceylanicum* or *N. americanus*. Hookworms, recovered at specific times after infection, were examined for evidence of density-dependent constraints on growth. Our results clearly

* Reprint requests to Dr J. M. Behnke.

establish that the growth and size attained by the laboratory strain of *N. americanus* in hamsters is dependent on the magnitude of the worm burden and indicate that hookworm species may differ markedly in their response to crowding.

MATERIALS AND METHODS

Parasites and hosts

N. americanus has been maintained since 1983 (courtesy of Dr Rajasekariah of CIBA-Geigy Hindustan Ltd, Bombay, India) by regular passage through hamsters as described originally by Sen (1972) and Behnke *et al.* (1986*a*, *b*). In experiments involving *N. americanus*, neonatal litters (1–3 days old) were exposed to doses of larvae percutaneously. *A. ceylanicum* was passaged through adult hamsters using techniques described by Garside & Behnke (1989). For experimental purposes 6 to 8-week-old animals were exposed to oral infection with the stated dose of larvae and were killed by chloroform anaesthesia at stated times after infection. All the animals used in this work were syngeneic DSN hamsters originally purchased from Intersimian Ltd, Oxford, UK, but now maintained, under conventional conditions, with access to food and water *ad libitum*, as a closed breeding colony in the Department of Life Science at Nottingham University.

Recovery of worms and measurement of size

Adult worms were recovered from the small intestine within minutes of the death of the host. The entire small intestine was opened longitudinally, taking care not to damage worms, under Hanks's saline maintained at 37 °C in a Petri dish on a bench incubator. Worms were picked off individually and placed into tissue-culture plates with large (2 ml) flat-bottomed wells, containing 1 ml of Hanks's saline. Female worms were incubated in Hanks's saline for 6 h in order to stimulate shedding of their eggs. More than 90% of eggs are generally shed within the first hour of incubation (Norozian-Amiri & Behnke, unpublished observations). Immature worms were recovered by a modified Baerman technique, in which the entire small intestine was suspended in a gauze and incubated for a minimal period of 4 h at 37 °C in 50 ml beakers containing Hanks's saline. After incubation the contents of each beaker were emptied into a Petri dish and worms were counted as they were individually picked out with a Pasteur pipette. After recovery worms were fixed in 10% formalin and stored in individual tubes each containing worms from a single host.

Worms were drawn to scale under appropriate magnification with the aid of a binocular dissecting microscope adapted with a camera lucida. Line drawings were then converted into units of length by using a digitizer pad and an IBM computer with a programme for conversion of lengths traced into discrete units (courtesy of Dr R. Ramsey). Each drawing was measured twice and the mean value used. A maximum of 10 male and 10 female worms were measured from each hamster, although all available intact worms were measured when fewer were recovered. Worms were individually weighed on an automatic electrobalance (CAHN 25-Ventron Corporation, California, USA) accurate to the nearest μg. Wet weights were determined on worms removed from tubes and blotted briefly on tissue paper before transfer to the weighing pans. Worms were then dried at 30–32 °C for 15 min and weighed again individually as above.

Statistical analysis of results

Values for measurements of size of worms, isolated from individual hamsters, were averaged by sex and are presented as mean values (MWR) $\pm$ standard error (S.E.M.) for clarity. Correlational relationships were determined on the mean values so that for each analysis n = number of hamsters infected with worms of the relevant sex. Correlations are illustrated by best-fit, linear regression lines. The total number of animals exposed to infection was greater than n in cases where some hamsters, usually those with low intensity infections, were found to have worms of a single sex. Non-parametric statistical procedures were used to analyse the data sets throughout. Correlations between variables were tested by the Spearman Rank Order Correlation Test and the statistic r_s is given, as appropriate. Probabilities (P) of 0·05 or less were considered significant. However, multiple analyses could not be avoided in certain cases and where these were undertaken, the cut-off value for significance was lowered to $P = 0{\cdot}025$ or 0·01 (depending on no. of tests) in order to avoid Type I errors. The Mann–Whitney U test was used to compare values between 2 groups. The results of Exp. 4 were analysed by a two-way ANOVA (Meddis, 1984) specifying infection intensity (low versus high) and sex (male versus female) as the two factors. A one-way ANOVA was used to test predictions about the relative ranking of measures of size in relation to worm category (female, male, low and high intensity infection). For all *a priori* hypotheses examined the test statistic z is given as appropriate.

EXPERIMENTAL DESIGN AND RESULTS

The rate of growth of worms after exposure to a uniform larval inoculum

Two experiments were carried out to measure the growth rates of both species of hookworms. These were necessary in order to identify suitable times after infection for optimal quantification of possible density-dependent constraints on growth. In the

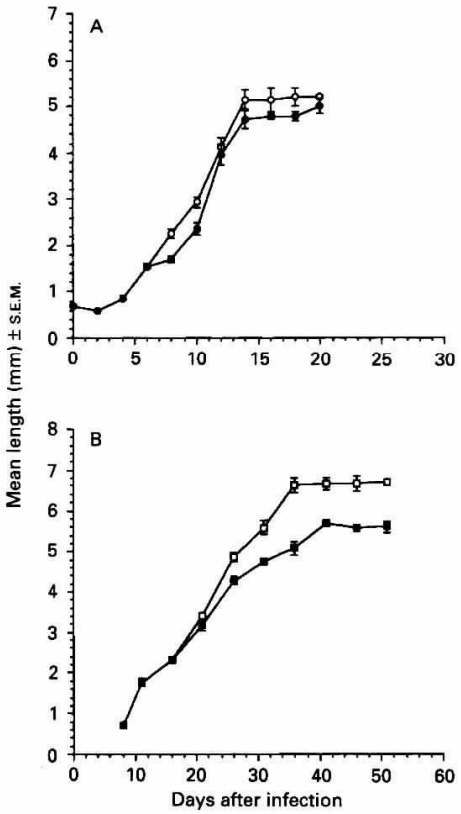

Fig. 1. Growth of *Ancylostoma ceylanicum* (A, Exp. 1; ○, female; ●, male) and *Necator americanus* (B, Exp. 2; □, female; ■, male) following exposure of hamsters to 50 L3.

first experiment a sample of 10 L3 of *A. ceylanicum* provided data for day 0. Ten male hamsters were infected with 50 L3 of *A. ceylanicum* and one animal was killed on each of the days shown in Fig. 1A. Ten worms were measured from each hamster killed on days 2–6 post-infection (p.i.) (Total = 30 worms) and 5 of each sex in the period 8–20 days p.i. (Total = 35 males and 35 females). In the second experiment mixed litters of hamsters were exposed to 50 L3 of *N. americanus* but only male hamsters were used. On days 8, 12 and 16 p.i. 10 worms were measured from each animal (Total = 30) and thereafter 10 of each sex (Total = 70 males and 70 females). The results are illustrated in Fig. 1B.

A. ceylanicum developed more rapidly than *N. americanus* with no further increase in length evident after day 14 p.i. *N. americanus* continued to increase in length until day 36 p.i., more than 3 weeks later. In both species male worms were smaller than females but the difference was only marginal in the case of *A. ceylanicum* (male worms 92·1% length of females on day 18 p.i. [$U = 0, n_1, n_2 = 5, 5, P = 0{\cdot}004$]) whilst in *N. americanus* the difference was greater (male worms 83·9% length of females on day 46 p.i. [$U = 2{\cdot}5; n_1, n_2 = 10, 10, P < 0{\cdot}001$]). However, the final size attained was greater in *N. americanus* with female worms averaging $6{\cdot}65 \pm 0{\cdot}18$ mm (day 46 p.i.) in comparison to *A. ceylanicum* females which were on average $5{\cdot}2 \pm 0{\cdot}19$ mm long (day 18 p.i.). Similarly, male *N. americanus* (average day 46 p.i. = $5{\cdot}58 \pm 0{\cdot}08$ mm) were longer than those of *A. ceylanicum* (average day 18 p.i. = $4{\cdot}79 \pm 0{\cdot}1$ mm).

Density-dependent effects on the size of A. ceylanicum

Three experiments were carried out to determine whether the growth and final size attained by *A. ceylanicum* was constrained by worm density. In Exp. 3, thirty-one male hamsters were segregated into 4 groups, one of which had 7 animals and the remainder 8 each. Hamsters were infected with 10, 25, 52 or 75 ($n = 7$) L3 of *A. ceylanicum* and were killed for assessment of worm burdens on day 23 p.i. Two hamsters exposed to the low intensity inoculum (25 L3) did not have female worms at autopsy. The total number of worms measured was 485 (237 males and 248 females). The mean lengths of male worms and female worms were similar to those found in Exp. 1 (Table 3, 4·62 *vs* 4·79 for males and 5·13 *vs* 5·2 mm for females) and there was a significant difference in length between the sexes ($U = 55, n_1, n_2 = 29, 31, P < 0{\cdot}01$). The difference between the size of the sexes was greater when the wet weights were compared (Table 3, $U = 0, n_1, n_2 = 29, 31, P < 0{\cdot}001$). No significant correlations were detected between the number of worms present and the length of female worms or the weight of male or female worms (Fig. 2) but there was a significant positive relationship between worm density and the length of male worms ($r_s = 0{\cdot}604, n = 31, P < 0{\cdot}001$). The relationships were similar when only female worms were considered as a measure of worm density (Table 2).

In Exp. 4, twelve male hamsters were infected with larval doses ranging from 5 to 100 L3. This time the experiment was terminated on day 12 p.i., before the attainment of full adult size, since density-dependent constraints on growth should be readily apparent at this stage: experiments terminated after day 16 when the size of worms has achieved a maximum value (see Fig. 1A) might allow an opportunity for the growth of initially smaller worms, showing delayed development, to catch up with larger worms. The total number of worms measured was 208 (99 males and 109 females). Again, a significant difference in the length and weight of the sexes was recorded (Table 3, for length $U = 17, n_{1,2} = 12, 12, P < 0{\cdot}001$; for weight $U = 40$, $n_{1,2} = 12, 12, P < 0{\cdot}05$) and the length for each sex accorded well with the length of 12-day-old worms in Exp. 1 (Fig. 1A, 3·84 *vs* 3·96 for males and 4·09 *vs*

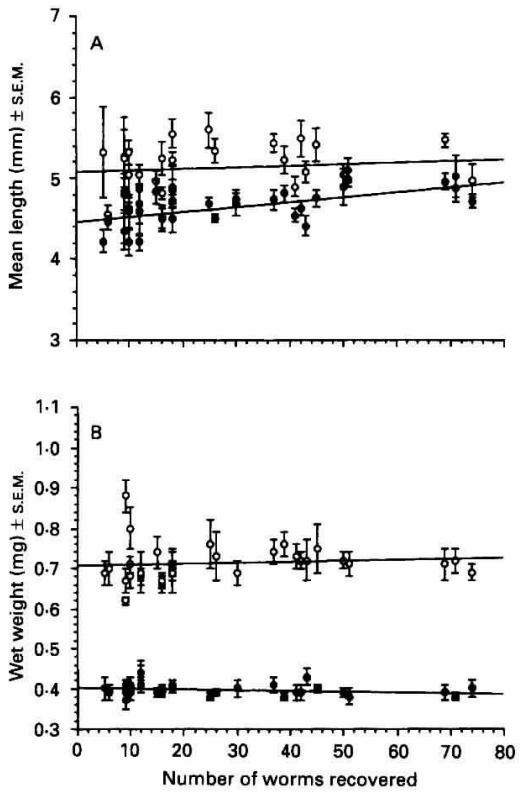

Fig. 2. The relationship between the number of *Ancylostoma ceylanicum* recovered and the length (A) and wet weight (B) of male (●) and female (○) worms 23 days after infection (Exp. 3, $n = 29$ for all measurements on female worms and 31 for males).

4·12 mm for females). No significant correlations were found between the number of worms present and the length of male worms, or the weight of male or female worms (Table 2). This time worm burden and the length of female worms showed a significant positive relationship which was marginally stronger when only the female worm burden was taken into account.

In a final experiment with *A. ceylanicum* two contrasting intensities of infection were examined. Four hamsters were given 25 L3 and three 250 L3 and both groups were killed on day 17 p.i. This time, in addition to length and wet weight, the width and dry weight of worms were also recorded. The total number of worms measured was 136 (68 of each sex). The results are shown in Table 1. The mean worm burden of the group given the low intensity inoculum was $21·8 \pm 1·3$ and that exposed to the higher inoculum $180·3 \pm 2·6$. Therefore an 8-fold difference in parasite burden was established and density-dependent effects should have been clearly apparent. For each measure of size three specific hypotheses were tested. The first, that the parameter should be greater in female compared with male worms, was

Table 1. Exp. 5: comparison of male and female *Ancylostoma ceylanicum* 17 days after exposure of hamsters to low (25 L3) or heavy (250 L3) oral infection

	Mean ± S.E.M.				Hypothesis*							
	Male worms		Female worms		1 F > M		2 L > H		3 F-L > F-H > M-L > M-H			
Measure of worm size	Low intensity	High intensity	Low intensity	High intensity	z	P	z	P	z	P		
Length (mm)	4·098 ± 0·045	4·3 ± 0·053	5·027 ± 0·036	5·18 ± 0·188	3·13	< 0·001	1·03	> 0·1†	3·27	< 0·001†		
Width (μm)	10·325 ± 0·131	9·5 ± 0·115	12·925 ± 0·287	11·767 ± 0·033	3·13	< 0·001	1·55	0·1 > P > 0·05	3·5	< 0·001		
Wet weight (mg)	0·285 ± 0·004	0·270 ± 0·004	0·427 ± 0·004	0·4 ± 0·003	3·14	< 0·001	1·42	0·1 > P > 0·05	3·44	< 0·001		
Dry weight (mg)	0·105 ± 0·002	0·098 ± 0·001	0·156 ± 0·001	0·15 ± 0·001	3·14	< 0·001	1·56	0·1 > P > 0·05	3·51	< 0·001		

* Three hypotheses were tested. Hypothesis 1 predicted that specific measures of size should be greater (>) in female (F) compared with male (M) worms. Hypothesis 2 predicted that measures of size should be greater for worms from low (L) intensity infections compared with those from high (H). Hypothesis 3 predicted that measures of size should follow the relationship females from low > females from high > males from low > males from high.

† In the case of length, Exps 3 and 4 indicated that a positive rather than a negative relationship might be expected with increasing worm burden and hence the hypothesis examined was low intensity < high intensity. In all other cases, there were no *a priori* reasons for expecting other than low > high. Similarly for hypothesis 3 the prediction that females from high > females from low > males from high > males from low intensity infections was tested.

Table 2. Summary of statistical analysis of the relationship between measures of worm size and worm burden

(For further details including *n*, see text.)

		Spearman rank order correlation coefficients for relationships between worm burden and measures of worm size†							
		Length		Width		Wet weight		Dry weight	
Experiment	Measure of intensity	Males	Females	Males	Females	Males	Females	Males	Females
3	Total worm burden	0·604*****	0·155	N.D.‡	N.D.	−0·215	0·336	N.D.	N.D.
A. ceylanicum	Female worm burden	0·563****	0·144	N.D.	N.D.	−0·277	0·267	N.D.	N.D.
4	Total worm burden	0·231	0·821****	N.D.	N.D.	0·135	0·26	N.D.	N.D.
A. ceylanicum	Female worm burden	0·245	0·854*****	N.D.	N.D.	0·235	0·313	N.D.	N.D.
6	Total worm burden	−0·599****	−0·817*****	N.D.	N.D.	−0·422	−0·704****	−0·634****	−0·733*****
N. americanus	Female worm burden	−0·611****	−0·855*****	N.D.	N.D.	−0·292	−0·568****	−0·522***	−0·553***
7	Total worm burden	−0·686*****	−0·71*****	−0·964*****	−0·937****	−0·02	−0·11	−0·736*****	−0·714*****
N. americanus	Female worm burden	−0·698*****	−0·674*****	−0·964*****	−0·937****	−0·119	−0·167	−0·647****	−0·633****
8	Total worm burden	−0·477	−0·753****	N.D.	N.D.	−0·854*****	−0·921*****	−0·887*****	−0·945*****
N. americanus	Female worm burden	−0·404	−0·674	N.D.	N.D.	−0·776*****	−0·863*****	−0·809*****	−0·902*****
9	Total worm burden	−0·81***	−0·738**	−0·63	−0·563	−0·862****	−0·819***	−0·874****	−0·881****
N. americanus	Female worm burden	−0·635	−0·838****	−0·573	−0·633	−0·729**	−0·691	−0·53	−0·731**
10	Total worm burden	−0·189	−0·408**	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
N. americanus	Female worm burden	−0·179	−0·423**	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

† Statistical significance: * $P = 0{\cdot}05$; ** $0{\cdot}05 > P \geqslant 0{\cdot}025$; *** $0{\cdot}025 > P \geqslant 0{\cdot}01$; **** $0{\cdot}01 > P \geqslant 0{\cdot}001$; ***** $P < 0{\cdot}001$. ‡ N.D., Not measured.

Table 3. Summary of average measures of size in Experiments 3–10

(For statistical analysis see text.)

				Mean ± S.E.M.							
	Average worm burden	Average female worm burden	Days after infection	Length (mm)		Width (μm)		Wet weight (mg)		Dry weight (mg)	
Experiment*				Male	Female	Male	Female	Male	Female	Male	Female
4 *A. ceylanicum*	46·3 ± 9·4	25·0 ± 5·0	12	3·838 ± 0·042	4·092 ± 0·044	N.D.†	N.D.	0·127 ± 0·003	0·139 ± 0·004	N.D.	N.D.
5 *A. ceylanicum*	21·8 ± 1·32	10·5 ± 1·0	17	4·098 ± 0·045	5·027 ± 0·036	10·325 ± 0·131	12·925 ± 0·287	0·285 ± 0·004	0·427 ± 0·004	0·105 ± 0·002	0·156 ± 0·001
	180·3 ± 2·6	79·7 ± 7·3	17	4·3 ± 0·053	5·18 ± 0·188	9·5 ± 0·115	11·767 ± 0·033	0·27 ± 0·004	0·4 ± 0·003	0·098 ± 0·001	0·15 ± 0·001
3 *A. ceylanicum*	27·4 ± 3·6	13·4 ± 1·7	23	4·619 ± 0·039	5·13 ± 0·049	N.D.	N.D.	0·398 ± 0·003	0·714 ± 0·008	N.D.	N.D.
9 *N. americanus*	101·8 ± 11·2	53·6 ± 5·2	30	4·541 ± 0·093	5·842 ± 0·156	9·075 ± 0·255	10·45 ± 0·216	0·219 ± 0·015	0·316 ± 0·02	0·053 ± 0·004	0·103 ± 0·006
7 *N. americanus*	69·0 ± 6·3	36·3 ± 3·0	36	5·065 ± 0·058	6·29 ± 0·103	9·842 ± 0·18	12·343 ± 0·242	0·25 ± 0·004	0·388 ± 0·006	0·075 ± 0·002	0·133 ± 0·003
8 *N. americanus*	18·8 ± 3·5	9·9 ± 2·1	36	5·521 ± 0·067	6·947 ± 0·15	N.D.	N.D.	0·278 ± 0·01	0·483 ± 0·02	0·108 ± 0·004	0·177 ± 0·004
6 *N. americanus*	54·0 ± 2·9	27·8 ± 1·2	46	5·563 ± 0·059	6·722 ± 0·053	N.D.	N.D.	0·292 ± 0·011	0·49 ± 0·008	0·111 ± 0·003	0·194 ± 0·007
10 *N. americanus*	18·6 ± 2·4	9·6 ± 1·4	72	5·374 ± 0·048	6·515 ± 0·078	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

* The experiments are arranged in order of increasing duration to autopsy for each species in order to enable changes in parameters to be related to age of worms vertically down columns. † N.D., Not measured.

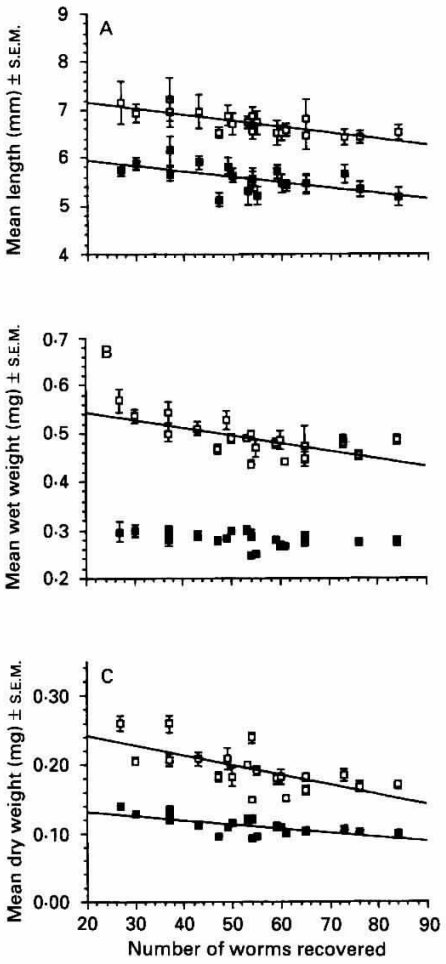

Fig. 3. The relationship between the number of *Necator americanus* recovered and the length (A), wet weight (B) and dry weight (C) of male (■) and female (□) worms 46 days after infection (Exp. 6, $n = 20$ in all cases).

tested by a two-way ANOVA. All four comparisons yielded highly significant differences between male and female worms indicating that female worms were longer, broader and heavier than males. However, the predictions that worms should be broader and heavier in low intensity infections were not significant although high z values ($P < 0{\cdot}1$) did not entirely eliminate such a possibility. However, the prediction that measurement of size should follow the order: females from low intensity infections > females from high > males from low > males from high intensity infections was also tested in a one-way ANOVA. For width, wet and dry weight these were all highly significant. Since there appeared to be a positive relationship between worm length and worm burden in previous experiments, the *a priori* hypothesis that worms were longer in more heavily infected animals was tested. Comparison of the mean values indicates that worms were indeed longer in the more heavily infected hamsters although the difference between the groups was not significant in a two-way ANOVA. The specific prediction that the lengths of worms from the four groups should follow the relationship: females from high intensity infection > females from low > males from high > males from low intensity infections was tested and found to be highly significant.

Density-dependent effects on the size of N. americanus

Five experiments were carried out with *N. americanus*. In Exp. 6, 24 neonatal hamsters of mixed sex were exposed to 50, 75, 100 or 120 L3 and were killed on day 46 p.i. A total of 397 worms (197 males and 200 females) were measured. Female worms were longer ($U = 0, n_{1,2} = 20, 20, P < 0{\cdot}001$), and heavier (wet weight $U = 11{\cdot}5, n_{1,2} = 20, 20, P < 0{\cdot}001$; dry weight $U = 0, n_{1,2} = 20, 20, P < 0{\cdot}001$) than male worms (Table 3). Significant negative relationships were evident between worm density and the length and dry weight of male and female worms (Fig. 3, Table 2). The relationship between wet weight was significant for females but not for males ($P = 0{\cdot}064$).

Exp. 7 comprised 24 hamsters of mixed sex infected with 50, 100, 125, 150 or 200 L3 and killed on day 36 p.i. The total number of worms measured was 476 (236 males and 240 females). As expected female worms were larger than males in all measures considered (Table 3, $U = 0$ in all cases and $P < 0{\cdot}001$). Significant negative correlations were found between worm burden, and length, width and dry weight, for both male and female worms (Fig. 4 and Table 2).

Since the infectivity of the inoculum used in Exp. 7 was high a second experiment was carried out with the same interval between infection and autopsy but this time involving a range of lower intensity inocula. In Exp. 8 sixteen male hamsters were used and the doses of L3 were 6, 10, 25 and 50. A total of 216 worms (112 males and 104 females) were measured. One hamster exposed to 5 L3 had only male worms present. As in earlier experiments female worms were significantly larger in all measures recorded (Table 3, length $U = 0, n_{1,2} = 15, 16, P < 0{\cdot}001$; wet and dry weight $U = 0$, $n_{1,2} = 15, 16, P < 0{\cdot}001$ in both cases). Significant negative correlations were found for both sexes for measures of weight and for female worm length in relation to worm burden (Table 2). The relationship between the length of male worms and worm burden was also negative and only just outside the upper limit for significance ($P = 0{\cdot}062$). However, the analysis was repeated on the raw data (i.e. before calculation of average values for each hamster). The assumption was made that there were no major differences between the hamsters and considered legitimate because age- and sex-matched

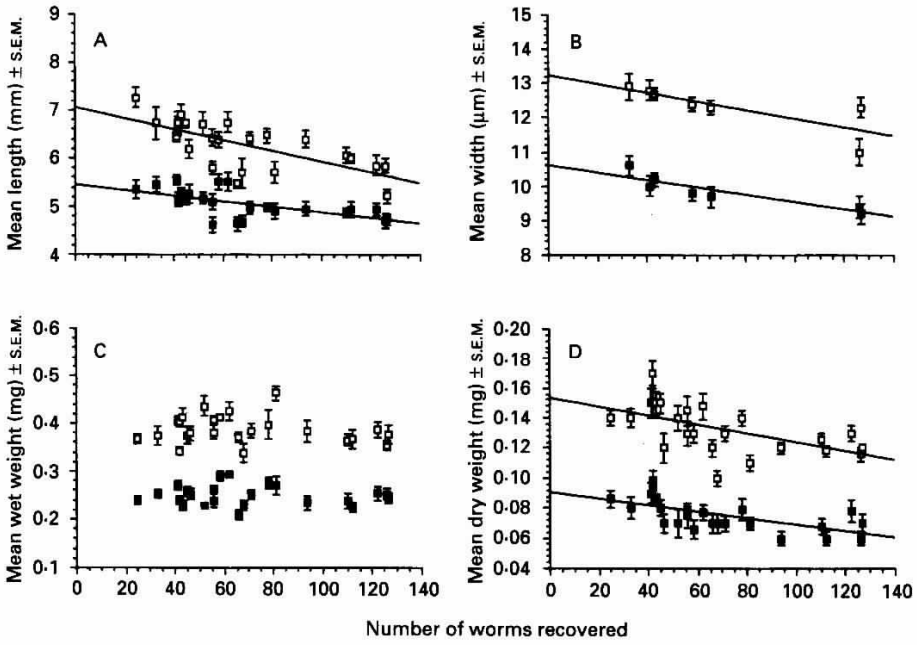

Fig. 4. The relationship between the number of *Necator americanus* recovered and the length (A), width (B), wet weight (C) and dry weight (D) of male (■) and female (□) worms 36 days after infection (Exp. 7, $n = 24$ in A, C and D, and 7 in B).

Table 4. Relationships between the various measures of size employed in the study

				Female worms			Male worms		
	Measure 1	*versus*	Measure 2	r_s	n	P	r_s	n	P
Exp. 4	Length	*vs*	Wet weight	0·6	12	0·04	0·67	12	0·018
Exp. 6	Length	*vs*	Wet weight	0·77	20	< 0·001	0·531	20	0·016
	Length	*vs*	Dry weight	0·795	20	< 0·001	0·7	20	0·001
	Wet weight	*vs*	Dry weight	0·889	20	< 0·001	0·755	20	< 0·001
Exp. 7*	Length	*vs*	Dry weight	0·817	24	< 0·001	0·465	24	0·022
	Length	*vs*	Width	0·775	7	0·041	0·929	7	0·003
	Dry weight	*vs*	Width	0·881	7	0·009	0·649	7	0·115
Exp. 8	Length	*vs*	Wet weight	0·761	15	0·001	0·741	16	0·001
	Length	*vs*	Dry weight	0·741	15	0·002	0·617	16	0·011
	Wet weight	*vs*	Dry weight	0·887	15	< 0·001	0·945	16	< 0·001

* In Exp. 7 wet weight showed no relationship to any of the other measures used. Moreover there was no evidence in this experiment that wet weight had any association with worm burden.

syngeneic hamsters were employed for this experiment. On re-analysis the relationship between worm burden and the length of male worms was highly significant ($r_s = -0{\cdot}4612, n = 108, P < 0{\cdot}001$).

In Exp. 9 male hamsters infected with 50, 150 or 200 L3 were killed 30 days p.i. before the cessation of growth. A total of 160 worms (80 from each sex) were measured. All measurements of size were significantly greater in female relative to male worms (Table 3, length $U = 0, n_{1,2} = 8, 8, P < 0{\cdot}0001$; width $U = 3{\cdot}5, n_{1,2} = 8, 8, P = 0{\cdot}001$; wet weight $U = 6, n_{1,2} = 8, 8, P < 0{\cdot}002$; dry weight $U = 0$, $n_{1,2} = 8, 8, P < 0{\cdot}001$). There were significant negative correlations for worms of both sexes between length, wet and dry weight, and worm burden. Neither sex of worms, however, showed a significant relationship between width and worm burden (Table 2). However, the correlation coefficients for width were high and on re-analysis using raw data, as described for Exp. 8 and with the same assumptions, all the relationships became significant. Thus total worm burden showed a significant negative correlation with width of female worms ($r_s = -0{\cdot}31$, $n = 79, P = 0{\cdot}005$) and width of male worms ($r_s = -0{\cdot}262, n = 80, P = 0{\cdot}019$). The relationships between female worm burden and width of males ($r_s = -0{\cdot}351, n = 80, P = 0{\cdot}001$), width of females

($r_s = -0{\cdot}384, n = 79, P < 0{\cdot}001$), length of males ($r_s = -0{\cdot}315, n = 80, P = 0{\cdot}004$), wet weight of female worms ($r_s = -0{\cdot}557, n = 80, P < 0{\cdot}001$) and dry weight of females ($r_s = -0{\cdot}309, n = 80, P = 0{\cdot}005$) were also all significant.

In the final experiment, 25 hamsters of mixed sexes were exposed to 50, 100, 125, 150 or 200 L3 and were killed 72 days p.i. The total number of worms measured was 133 (156 males and 177 females). Only length was recorded and this was significantly greater in female compared with male worms (Table 3, $U = 0, n_1, n_2 = 24, 25, P < 0{\cdot}001$). The relationship between worm burden and length was significant for female worms but not for males (Table 2).

Relationship of individual measures of size to each other

Finally it was necessary to establish that the measures of size employed in this study were related to each other and did not represent independent variables with unrelated associations with worm burden. Such analyses were undertaken only in the larger experiments and the results are summarized in Table 4. With the number of analyses illustrated the likelihood of Type I errors has to be considered but as can be seen only one of the 20 analyses failed to reach significance. Most showed high correlation coefficients with the strongest relationship between wet and dry weight of male worms in Exp. 8. In Exp. 7 wet weight failed to show a significant relationship with any of the other measures and it is pertinent that there was no significant correlation of this measure with worm burden (Table 2).

DISCUSSION

Density-dependent regulation of parasite populations is a widespread phenomenon, described for many different parasite species and depending on host–parasite combination considered to operate at several levels from density-dependent effects on parasite establishment, growth, fecundity to host mortality (Keymer, 1982; Michael & Bundy, 1989). The results described in this paper show conclusively that *N. americanus*, a widespread human parasite in the tropics, is subject to density-dependent constraints affecting its size as reflected in each of the four measures which were employed to quantify the effect. Of the 60 relationships between worm burden and measures of size analysed in Table 2, all, without exception, had negative signs and 42 (70%) were significant. Because so many analyses were undertaken some correlations may have occurred through Type I errors, although even this possibility is offset by the low probability values which were obtained for the null hypothesis (32 were $P < 0{\cdot}01$). Moreover many of the correlations which were not significant had high r_s values but probably lacked significance because of low n. In two experiments we considered it legitimate to re-examine the correlations employing the raw data, rather than by-host-averaged values, because in both age- and sex-matched syngeneic animals were used (Exps 8 and 9). In both cases the relationships between worm burden and measures of size (Exp. 8, female length; Exp. 9, width) were found to be significant on re-analysis. Therefore, we are confident in concluding that *N. americanus* was subject to density-dependent constraints affecting growth.

In contrast, *A. ceylanicum* was not affected and indeed worms appeared longer under high, compared with low, density. Of the 16 analyses in Table 2 only 2 were negative and the only significant correlation coefficients were for positive rather than negative relationships. The last experiment with *A. ceylanicum* (Exp 5) compared low with high intensity (8-fold difference in worm burden) exposure directly, rather than over a range of worm burdens and, surprisingly, the length of worms of both sexes was again longer among worms developing under high density. However, other measures of size were reduced, including width and weight. It has to be emphasized that the parasite burdens in these animals were high and probably induced considerable pathology in the intestine, but the conclusion that worms were longer albeit thinner and lighter is inescapable. Overall, this study in hamsters has provided for the first time detailed quantitative data which showed clearly that *N. americanus* was subject to regulation by density-dependent processes within the host and which indicated that *A. ceylanicum* was not sensitive to the same degree.

There are few published studies with which we can compare our data because density-dependent effects on hookworm size have been relatively neglected. This may seem particularly surprising because the stability of hookworm burdens in naturally infected human populations implies that they must be tightly regulated (Bradley *et al.* 1992) and yet evidence for a role of host immunity is, at best, equivocal (reviewed by Behnke (1991)). Thus we might have expected other possible contributing processes to have been thoroughly explored. Japanese workers in the 1950s investigated crowding effects but the literature is largely untranslated and difficult to interpret (see Komiya & Yasuraoka (1966) for English summary). In agreement with our findings Nagayoshi & Mudaguchi (1956) compared *N. americanus* expelled by individuals harbouring low intensity (< 125) and high intensity worm burdens (> 3500) and found that the former were, on average, 11·48 mm and the latter 7·9 mm, but no statistical evaluation was made available. Similar results were apparently found for *A. duodenale*. In contrast, Quinnell *et al.* (manuscript in preparation) reported that the weight of *N. americanus* showed little variability and no significant differences were

detected in the weight of worms from persons carrying light compared with heavy infections, but this latter study sought associations between measures of size and worm burden among a population where the average worm burden was only 25 worms/host and the highest intensity 263 worms. The canine species *A. caninum* has also been investigated. Firstly, Yazima & Machida (1958) reported that female *A. caninum* were longer and heavier in the anterior of the intestine where worm densities were lower than in the midgut and the effect was more prominent among female worms. Little quantitative data were provided on comparisons between hosts carrying different intensity worm burdens. Sarles (1929*b*) found no consistent effect of parasite density on length in dogs experimentally infected with *A. caninum* and carrying fewer than 27 compared with 84–284 worms.

Whilst the hamster is susceptible to infection and supports a chronic infection, the host–parasite relationship of *N. americanus* clearly differs in some respects from that in the human host. The marked age resistance (Rajasekariah *et al.* 1985) and low fecundity of worms, particularly in female hamsters (Behnke & Pritchard, 1987), may indicate that the worms are not entirely adapted to this host, that they represent a select genotype of the wild parasite or that despite their adaptation they are subject to more intense host-mediated regulation than worms in man. The density-dependent loss of worms from the 5th week of infection supports the last idea (Rose & Behnke, 1990). However, whether the immune response is involved is still uncertain. *N. americanus* certainly initiates potent antibody responses in the hamster (Behnke & Prichard, 1987), as does the parasite in its natural host (Ogilvie *et al.* 1978; Pritchard *et al.*1992), but in neither case has evidence been provided for a host-protective role for antibodies. However, density-dependent regulation may be mediated by other mechanisms and the most likely candidate in this case is density-dependent induction of pathology in the gut, ultimately exhausting the ability of the mucosa to provide sufficient nutrients for all the worms in heavily infected hamsters. The role of pathological changes in this context is currently under investigation. *A. ceylanicum*, like *A. caninum* and *A. duodenale*, bites deeper into the intestine (Bonne, 1942) than *N. americanus* and it is conceivable that in so doing the worms are less likely to be limited by local resources. Certainly *A. ceylanicum* is considerably more pathogenic in hamsters than *N. americanus* and causes more severe blood loss (Behnke, 1990).

However, the failure to detect regulatory influences on *A. ceylanicum*, other than at extremely high intensities of infection, also indicates that this species may be more robust than *N. americanus*, at least in the hamster host. This would be in keeping with the finding that *A. ceylanicum* matured more quickly than *N. americanus*, stopped growing at a smaller size and the sexes showed only a minor size difference. All these are compatible with an opportunist life-style, i.e. rapid maturation, little additional investment in allowing females to grow larger, lack of susceptibility to regulation and presumably intense egg output without investment in the long-term effects on the host. In comparison *N. americanus* grew more slowly, female worms achieved a significantly larger size than males and the worms were subject to density-dependent regulation. These features are consistent with a life-style involving greater sensitivity to the environment within the host, with an organism causing chronic infections and ultimately achieving its biological potential in the long term rather than in the short term.

We thank Professors P. N. R. Usherwood and D. Wakelin for providing facilities for this work within the Department of Life Science, Mr D. Fox for the supervision of our animal housing facilities, Mrs J. Brown, Miss J. Street, Mr A. Brown for technical assistance and Dr R. Ramsay for providing a computer program facilitating measurement of worm lengths. We are particularly indebted to Dr F. Gilbert for advice with statistical analysis. S.M.B.N.-A. was supported by a Post-Graduate Scholarship provided by the Ministry of Health and Education in Medicine of Iran, for which we are grateful. We also thank the Sir Samuel Scott of Yews Trust and the Daniel Falkner Charitable Trust for their financial support of this project.

REFERENCES

BEAVER, P. C. (1988). Light, long-lasting *Necator* infection in a volunteer. *American Journal of Tropical Medicine and Hygiene* **39**, 369–72.

BEHNKE, J. M. (1987). Do hookworms elicit protective immunity in man? *Parasitology Today* **3**, 200–6.

BEHNKE, J. M. (1990). Laboratory animal models. In *Hookworm Disease. Current Status and New Directions* (ed. Schad, G. A. & Warren, K. S.), pp. 105–28. London: Taylor and Francis.

BEHNKE, J. M. (1991). Immunology. In *Human Parasitic Diseases, Vol. 4, Hookworm Infections* (ed. Gilles, H. M: & Ball, P. A. J.), pp. 93–155. Amsterdam: Elsevier.

BEHNKE, J. M., PAUL, V. & RAJASEKARIAH, G. R. (1986*a*). The growth and migration of *Necator americanus* following infection of neonatal hamsters. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **80**, 146–9.

BEHNKE, J. M., WELLS, C. & BROWN, J. (1986*b*). An improved technique for experimental infections with skin penetrating nematode larvae (*Necator americanus*). *International Journal for Parasitology* **16**, 461–4.

BEHNKE, J. M. & PRITCHARD, D. I. (1987). *Necator americanus* in neonatally infected hamsters. The time-course of infection and antibody response to the surface antigens of L4 and adult worms. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **81**, 967–72.

BONNE, C. (1942). Invasion of the wall of the human intestine by ancylostomes. *American Journal of Medicine* **22**, 507–9.

BRADLEY, M., CHANDIWANA, S. K., BUNDY, D. A. P. & MEDLEY, G. F. (1992). The epidemiology and population biology of *Necator americanus* infection in a rural community in Zimbabwe. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **86**, 73–6.

BUNDY, D. A. P. (1990). Is the hookworm just another geohelminth? In *Hookworm Disease: Current Status and New Directions* (ed. Schad, G. A. & Warren, K. S.), pp. 147–64. London: Taylor and Francis.

CARROLL, S. M. & GROVE, D. I. (1984). Parasitological, hematologic and immunologic responses in acute and chronic infections of dogs with *Ancylostoma ceylanicum*: a model of human hookworm infection. *Journal of Infectious Diseases* **150**, 284–94.

GARSIDE, P. & BEHNKE, J. M. (1989). *Ancylostoma ceylanicum*: observations on host–parasite relationship during primary infection. *Parasitology* **98**, 283–9.

HASWELL-ELKINS, M. R., ELKINS, D. B., MANJULA, K., MICHAEL, E. & ANDERSON, R. M. (1988). An investigation of hookworm infection and reinfection following mass anthelmintic treatment in the South Indian fishing community of Vairavankuppam. *Parasitology* **96**, 565–77.

KENDRICK, J. R. (1934). The length of life and rate of loss of hookworms, *Ancylostoma duodenale* and *Necator americanus*. *American Journal of Tropical Medicine and Hygiene* **14**, 363–79.

KEYMER, A. (1982). Density-dependent mechanisms in the regulation of intestinal helminth populations. *Parasitology* **84**, 573–87.

KOMIYA, Y. & YASURAOKA, K. (1966). The biology of hookworms. In *Progress of Parasitology in Japan*, Vol. 3, pp. 1–114. Tokyo: Meguro Parasitological Museum.

KRUPP, I. M. (1961). The effects of crowding and super-infection on habitat selection and egg production of *Ancylostoma caninum*. *Journal of Parasitology* **47**, 957–61.

MCCOY, O. R. (1931). Immunity reactions of the dog against hookworm (*Ancylostoma caninum*) under conditions of repeated infection. *American Journal of Hygiene* **14**, 268–303.

MEDDIS, R. (1984). *Statistics Using Ranks. A Unified Approach*. New York: Basil Blackwell Publishers Ltd.

MICHAEL, E. & BUNDY, D. A. P. (1989). Density dependence in establishment, growth and worm fecundity in intestinal helminthiasis: the population biology of *Trichuris muris* (Nematoda) infection in CBA/Ca mice. *Parasitology* **98**, 451–8.

MILLER, T. A. (1971). Vaccination against the canine hookworm diseases. *Advances in Parasitology* **9**, 153–83.

MOSS, G. E. (1971). The nature of the immune response of the mouse to the bile duct cestode *Hymenolepis microstoma*. *Parasitology* **62**, 285–94.

NAGAYOSHI, K. & MUDAGUCHI, T. (1956). Studies on bionomics of hookworms. 1. Effect of crowding on the length of worms. *Tokyo Iji Shinshi* **73**, 367–8.

OGILVIE, B. M., BARTLETT, A., GODFREY, R. C., TURTON, J. A., WORMS, M. J. & YEATES, R. A. (1978). Antibody responses in self-infections with *Necator americanus*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **72**, 66–71.

PRITCHARD, D. I., QUINNELL, R. J., SLATER, A. F. G., MCKEAN, P. G., DALE, D. D. S., RAIKO, A. & KEYMER, A. E. (1990). The epidemiological significance of acquired immunity to *Necator americanus*: humoral responses to parasite collagen and excretory–secretory antigens. *Parasitology* **100**, 317–26.

PRITCHARD, D. I., WALSH, E. A., QUINNELL, R. J., RAIKO, A., EDMONDS, P. & KEYMER, A. E. (1992). Isotypic variation in antibody responses in a community in Papua New Guinea to larval and adult antigens during infection, and following reinfection, with the hookworm. *Necator americanus*. *Parasite Immunology* **14**, 617–31.

QUINNELL, R. J., SLATER, A. F. G., TIGHE, P., WALSH, E. A., KEYMER, A. E. & PRITCHARD, D. I. (1993). Reinfection with hookworm after chemotherapy in Papua New Guinea. *Parasitology* **106**, 379–85.

RAJASEKARIAH, G. R., DEB, B. N., DHAGE, K. R. & BOSE, S. (1985). Site of resistance to *Necator americanus* in hamsters. *Acta Tropica* **42**, 333–40.

READ, C. P. (1951). The 'crowding effect' in tapeworm infections. *Journal of Parasitology* **37**, 174–8.

ROCHE, M. & LAYRISSE, M. (1966). The nature and causes of hookworm anemia. *American Journal of Tropical Medicine and Hygiene* **15**, 1031–100.

ROSE, R. A. & BEHNKE, J. M. (1990). *Necator americanus* in the DSN hamster: density dependent expulsion of adult worms during primary infection. *Parasitology* **100**, 469–78.

SARLES, M. P. (1929*a*). The length of life and rate of loss of the dog hookworm, *Ancylostoma caninum*. *American Journal of Hygiene* **10**, 667–82.

SARLES, M. P. (1929*b*). The effect of age and size of infestation on the egg production of the dog hookworm, *Ancylostoma caninum*. *American Journal of Hygiene* **10**, 658–66.

SEN, H. G. (1972). *Necator americanus*: behaviour in hamsters. *Experimental Parasitology* **32**, 26–32.

YAZIMA, F. & MACHIDA, K. (1958). On the ecological relations of parasite state of *Ancylostoma caninum*, especially on the density effect of population. *Kiseichugaku Zasshi* **7**, 631–40.