Density-dependent effects on the survival and growth of the rodent stomach worm *Protospirura muricola* in laboratory mice

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Abstract

The spirurid nematode, *Protospirura muricola*, is of intrinsic interest as a rodent model of gastric nematode infections. Since worm burdens can be very heavy in nature, density dependent processes may constrain parasite growth. Laboratory mice (BKW) were exposed to varying doses of infective larvae of *P. muricola* in the range 5 to 40 third-stage larvae (L3), in four separate experiments in which progressively higher doses were utilized. All mice were culled 60 days after infection and a total of 518 worms (226 male and 292 female worms) was recovered, measured and weighed. Overall survival was 58.9%, but survival declined significantly with increasing dose by approximately 21% (from 66% at 5 L3 per mouse to 52% at 40 L3 per mouse). The length and weight of worms correlated positively in both sexes. Total worm biomass increased linearly with increasing numbers of worms. However, whilst the length and weight of male worms declined with increasing worm burden (8.4 and 24.6% respectively), female worms were less affected, only length showing a significant reduction with increasing parasite burden (16.0%). Therefore, increasing worm burdens impeded growth of *P. muricola*, but reduction in length and weight were relatively small in relation to the overall size of this nematode. Increasing worm burdens were associated with loss of host weight and reduction in stomach weight and worm burdens in excess of 20 exerted a measurable cost to the host, which in the field, may be associated with loss of overall host fitness.

Introduction

Density-dependent effects on the growth and reproductive capacity of helminths have been well documented in many different species in naturally infected populations (Anderson & May, 1978; Keymer, 1982) but are perhaps best known from experimental cestode infections. For example the rat tapeworm, *Hymenolepis diminuta*, shows a marked inverse relationship between the number of parasites harboured and their individual lengths and weights, and this has been recognized for some time (Chandler, 1939; Read, 1951; Kennedy & Behnke, 2001). In contrast, nematodes are generally considered to be more resilient and although their growth in experimental systems can be constrained with increasing parasite burden (Michael & Bundy, 1989; Norozian-Amiri & Behnke, 1994), the effects on length and weight are less marked than in cestode infections. The mechanistic basis of density-dependent effects on helminth size and reproductive effort has been the subject of debate and controversy, and has spawned hypotheses ranging from competition for limited resources, regulatory factors of parasite origin to host immune responses (Andreassen *et al.*, 1999; Bush & Lotz, 2000; Roberts, 2000; Paterson & Viney, 2002).

During field work in the south of the Sinai peninsula of Egypt, we encountered a relatively high prevalence of the spirurid nematode *Protospirura muricola* in the dominant wild rodent in the region, the spiny mouse *Acomys dimidiatus* (Behnke *et al.*, 2000). *Protospirura muricola* is a large stomach nematode reaching over 5 cm in length (females), commonly encountered in the tropics

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in a variety of hosts, including rodents, and often accumulating in hosts with resultant heavy worm burdens (Baylis, 1928; Campos & Vargas, 1978; Behnke *et al.*, 2000). Some naturally infected spiny mice can harbour over 100 worms, with total biomass exceeding 2% of host body weight (Behnke *et al.*, unpublished observations; Behnke *et al.*, 2000) and occupying most of the stomach lumen. On the basis of observations made in the field we suggested that this species may be costly for the host (Behnke *et al.*, 2000; Barnard *et al.*, 2003), but currently virtually nothing is known about the host–parasite relationship of *P. muricola*.

In this paper, employing a strain of *P. muricola* recently isolated from *A. dimidiatus* in the Sinai and under laboratory conditions, we examine the hypothesis that density-dependent constraints on the growth of *P. muricola* may limit the total biomass that can develop and survive to maturity in mice. We test the predictions that parasite survival, worm length and weight should decline with increasing numbers of worms in the stomach. We also present preliminary observations on the effects of this parasite on host body weight and the weight of the stomach within which the worms reside.

Materials and methods

Animals

All infections were initiated in 6- to 8-week-old male BKW laboratory mice (*Mus musculus*), purchased from B and K Universal, which have been found to be suitable hosts for the maintenance of this species under laboratory conditions. The mice were fed on Teklad Rat/Mouse/Breeding Diet, supplied by Harlem Laboratory Animal Feed limited, provided with both food and water *ad libitum* and maintained on a 12 h light: 12 h dark regime.

Maintenance, infection and recovery of Protospirura muricola

The isolate of *P. muricola* was obtained in 1997 from *A. dimidiatus*, from the St Katherine's Protectorate in the Sinai region of Egypt. Eggs from adult female worms were allowed to embryonate and were fed to *Tribolium confusum* beetles in flour. The third-stage larvae (L3) developed in the haemocoel of the insect after 4–5 weeks and these were dissected and used to infect mice for passage, by conventional oral gavage. In our experience, this strain of *P. muricola* develops only in the stomach of its host. At the end of each experiment mice were culled by exposure to carbon dioxide, weighed, and the body cavity opened. The stomach was removed and placed in warm Hanks' saline in a Petri dish and carefully opened using forceps, to ensure that the worms inside were not damaged. Worms were removed with forceps and the stomach was carefully washed out with Hanks' saline to free it of any remaining contents. It was then dabbed gently on a paper towel to remove excess liquid and weighed.

Experimental design

Four experiments were carried out in each of which a range of infection doses was employed. In the first experiment we explored doses not exceeding 15 L3, but in subsequent experiments the range was extended gradually to 40 L3. The step-wise exploration with increasing doses was required on ethical grounds, because this species grows to a relatively large size and little is known about its potential pathogenicity to the host (but see Foster & Johnson, 1939). The doses employed and the number of mice surviving to day 60 post-infection, when all mice were culled, are shown in table 1.

Statistical analysis

Throughout, multivariate or univariate GLIM was used, implemented by the software package SPSS for windows, version 9.0.0. To assess whether survival of worms was affected by the dose of larvae administered, the worm recovery of each mouse was converted to a percentage of the dose administered, the frequency distributions were checked for normality and analysis was by ANOVA with percentage recovery as the dependent variable, the experiment as a factor and the dose of larvae administered as a covariate. Residuals from a one-way ANOVA, with experiment as the factor, on percentage recovery were saved to illustrate the relationship between dose and recovery with between-experiment variation taken into account.

Individual lengths and weights of worms were averaged by sex for each mouse in the study, and the analysis was conducted on these mean values. To ensure that type 1 errors were not likely, a multivariate analysis was first conducted with worm length and weight for both sexes as dependent variables, and in each case using models that incorporated either number of worms (test 1) or total biomass (test 2) as covariates. Experiment was also included as a factor (to control for between-experiment differences) and percentage of female worms as a covariate (to control for sex bias because female worms are longer than males and considerably heavier). For the first of these analyses Wilks' lambda was 0.554 for number of worms ($F_{4,43} = 8.667$, $P < 0.001$), 0.801 for percentage of female worms ($F_{4,43} = 2.671$ $P = 0.045$) and 0.285 for experiment $F_{12,114} = 5.769$, $P < 0.001$). For the second, Wilks' lambda was as 0.633 for total worm biomass ($F_{4,44} = 6.368$, $P < 0.001$) and 0.323 for experiment ($F_{12,117} = 5.19$, $P < 0.001$). Since both indicated a highly significant effect of worm density, in a second step we employed univariate tests on each variable in turn to allow estimation of the gradient of the slope. Pearsons's product moment correlation test

Table 1. Number of mice surviving to day 60 post-infection with third-stage larvae (L3) of *Protospirura muricola*.

Experiment no.	Dose of larvae						
	0	5	10	15	20	25	40
1	5*	5	5	5			
2	5	5	5			4	
3	5		5		5		2
4	5	5			5		5

*Each group originally comprised 5 mice.

was used to test the relationship between worm weight and length.

Results

Worm recovery and sex ratio

A total of 518 worms were recovered comprising 226 male and 292 female worms, giving an overall sex ratio of 56.4% females. The sex ratio did not vary between experiments (ANOVA with experiment as factor and number of worms recovered as a covariate; $F_{3,51} = 1.646$, $P = NS$) nor with increasing worm burden ($F_{1,51} = 0.049$, $P = NS$). The overall survival of worms was 58.86% of the dose administered.

Recovery of worms

Raw data for percentage recovery from all four experiments combined are illustrated in fig. 1a and this indicates that a small reduction in worm survival with increasing dose was detected. The slope of the regression was $\beta = -0.45 \pm 0.2$ and this was significantly different from zero ($t = -2.25$, $P = 0.03$). Figure 1b illustrates this relationship with minor but significant between-experiment differences taken into account (residuals from one-way ANOVA with experiment as the factor).

Relationship between worm weight and worm length

Figure 2 shows the relationship between individual worm weight and worm length for each of the sexes. For both sexes this relationship was positive and highly significant (males $r_p = 0.24$, $n = 226$, $P < 0.001$; females $r_p = 0.37$, $n = 292$, $P < 0.001$), although, as is evident from the figure, there was considerable variation in both sexes.

Relationship between number of worms recovered and total worm biomass

The relationship between the number of worms recovered from individual mice and the corresponding total biomass of worms is illustrated in fig. 3. This was essentially a linear positive relationship ($r_p = 0.98$, $n = 56$, $P < 0.001$).

Effect of worm numbers on length of male and female worms

Figure 4 shows the relationship between the lengths and weights of male and female worms, and the number of worms harboured at autopsy. The results of statistical analyses are summarized in table 2. Male worms were sensitive to the density of worms, their lengths and weights both declining with increasing worm density (by 8.4 and 24.6%). Both parameters were sensitive to the percentage of female worms present, with a negative
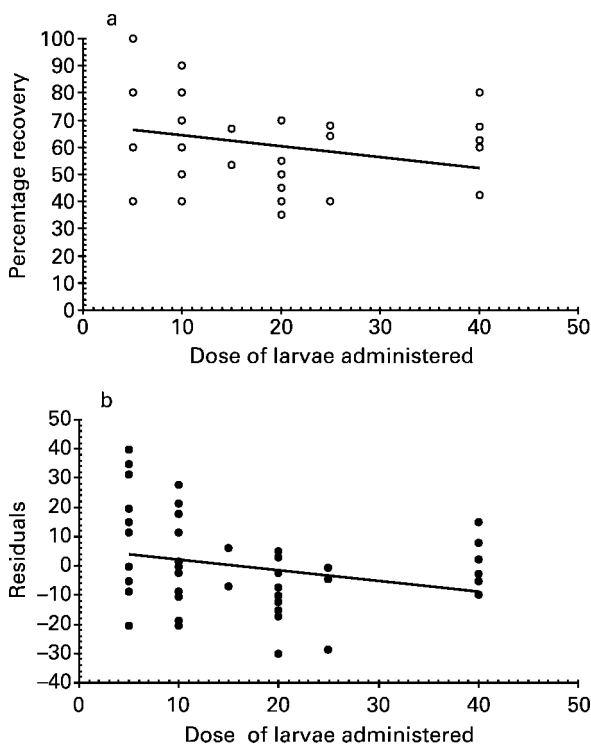

Fig. 1. Relationship between the dose of third-stage larvae (L3) of *Protospirura muricola* administered and (a) the percentage of worms surviving to day 60 post-infection (ANOVA, with experiment as factor, $F_{3,51} = 2.913$, $P = 0.04$, and dose of larvae administered as covariate, $F_{1,51} = 5.051$, $P = 0.029$, gradient $\beta = -0.45 \pm 0.2$, $t = -2.25$) and (b) the residuals of ANOVA (see text) controlling for between-experiment variation in worm burdens.
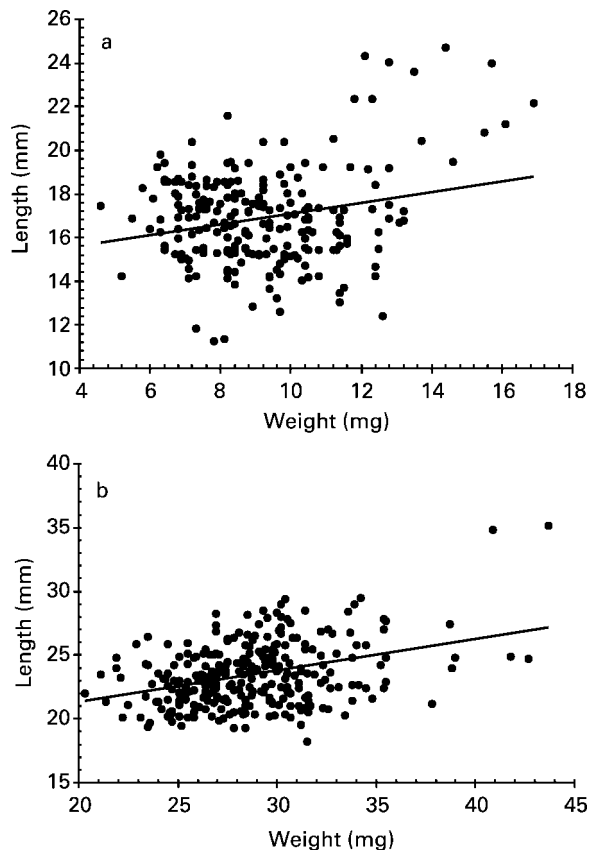

Fig. 2. Relationships between the weight and length of individual male (a) and female (b) *Protospirura muricola*
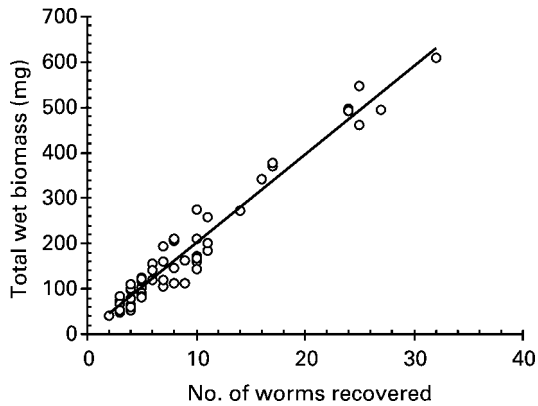
Fig. 3. Relationship between the total worm biomass (wet weight) and the number of worms of *Protospirura muricola* recovered.

gradient in relation to increasing percentage of female worms. There was also significant variation between experiments. Female worms were less sensitive. Only the length of female worms declined significantly with increasing worm burden (16.0%). There was no significant relationship with the percentage of female worms, and no difference between experiments.

Effect of total worm biomass on size of male and female worms

The relationships between total worm biomass and the lengths and weights of male and female worms are illustrated in fig. 5 and the statistical analyses are summarized in table 3. As in the case of worm number,

male worms were sensitive to crowding and both mean weight and mean length declined significantly with increasing biomass, although the gradient was shallow. There was a significant reduction in the length of female worms with increasing biomass, but not of weight and there was no significant variation between experiments.

Effect of increasing worm burden on host body weight

Mouse weight at autopsy declined with increasing worm burden whether worm recovery was used as a measure of the intensity of infection (fig. 6b) or the total worm biomass (data not shown, ANOVA with experiment as factor and total worm biomass as covariate, main effect of total worm biomass $F_{1,71} = 14.64$, $\beta = -0.008 \pm 0.002$, $P < 0.001$).

Effect of increasing worm burden on stomach weight

There was a significant loss of stomach weight with increasing worm recovery (fig. 6a) and with increasing total worm biomass (fig. 6c). Analysis of the stomach weight as a percentage of body weight did not generate significant relationships with number of worms recovered or total worm biomass (fig. 6d), most likely because body weight also declined with increasing worm burden. However, as a final step we fitted ANOVAs with experiment as factor and mouse weight as an additional covariate in order to control for differences in mouse weight. In both cases, the relationship between stomach weight and number of worms recovered and with total
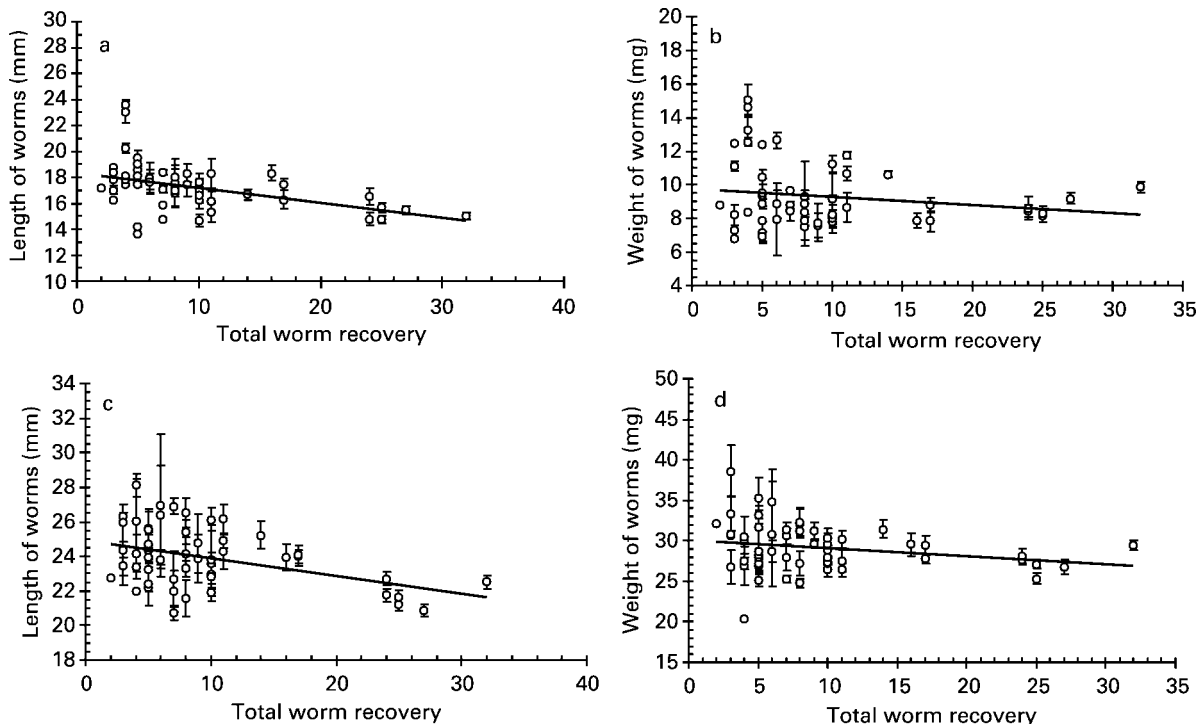

Fig. 4. Relationships between the length (a and c) and weight (b and d) of male (a and b) and female (c and d) *Protospirura muricola*, and the number of worms recovered on day 60 post-infection. For statistical analysis see table 2.

Table 2. Univariate analysis of the length and weight of male and female worms of *Protospirura muricola* by ANOVA with the number of worms and percentage of female worms as covariates and experiment as a factor (4 levels).

Parameter	Male worms		Female worms	
	Length	Weight	Length	Weight
$F_{1,48}$ for no. of worms	4.235	11.044	16.261	0.926
Gradient (β) $\pm$ SEM	-0.052 ± 0.025	-0.114 ± 0.034	-0.135 ± 0.033	-0.061 ± 0.063
t	-2.058	-3.323	-4.033	-0.962
P	0.045	0.002	<0.001	NS
$F_{1,48}$ for % females	28.92	10.997	0.762	0.192
Gradient (β) $\pm$ SEM	-0.044 ± 0.008	-0.037 ± 0.011	0.010 ± 0.01	0.010 ± 0.022
t	-5.38	-3.316	0.873	0.438
P	<0.001	0.002	NS	NS
$F_{3,48}$ for experiment	8.862	7.43	2.23	1.69
P	<0.001	<0.001	NS	NS

NS, not significant.

worm biomass, remained significant ($F_{1,70} = 6.761$, $P = 0.011$ and $F_{1,70} = 6.075$, $P = 0.016$, respectively).

Discussion

Protospirura muricola is primarily a nematode parasite of rodents living in the tropics, known mostly from records of its occurrence in Africa and South America (Baylis, 1928; Campos & Vargas, 1978). Very little is known about the host–parasite relationship of this species and experimental studies have been concerned largely with elucidating its growth and development in different intermediate and definitive hosts (Quentin,1969; Campos & Vargas, 1977). *Protospirura muricola* is a long-lived but slowly developing parasite that takes 1.5 to 2 months to mature in its rodent host. Eggs first appear in mouse faeces 62 days after infection and infections can last many months thereafter (over 200 days, Lowrie, Behnke & Barnard unpublished observations). In the present experiments, mice were culled after 60 days to assess density dependent effects on growth within the prepatent
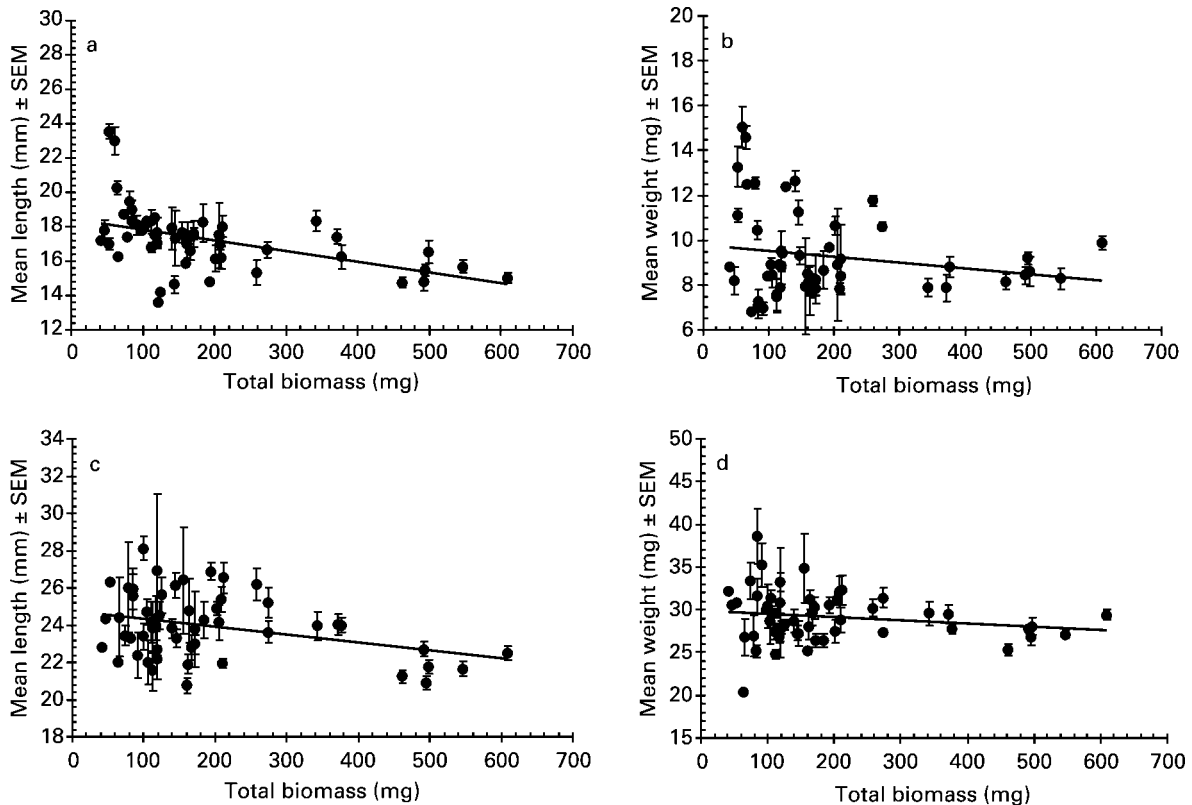

Fig. 5. Relationships between the length (a and c) and weight (b and d) of male (a and b) and female (c and d) *Protospirura muricola*, and the total worm biomass (wet weight) on day 60 post-infection. For statistical analysis see table 3.

Table 3. Univariate analysis of the length and weight of male and female worms of *Protospirura muricola* by ANOVA with total worm biomass as a covariate and experiment as a factor (4 levels).

Parameter	Male worms		Female worms	
	Length	Weight	Length	Weight
$F_{1,48}$ for total worm biomass	4.509	10.227	11.203	0.330
Gradient (β) $\pm$ SEM	-0.003 ± 0.002	-0.006 ± 0.002	0.006 ± 0.002	0.002 ± 0.003
t	-2.124	-3.198	-3.347	0.574
P	0.039	0.002	0.002	NS
$F_{3,48}$ for experiment	6.145	5.635	1.851	1.896
P	0.001	0.002	NS	NS

NS, not significant.

period. The experiments varied in the range of infection levels examined and higher worm burdens were only administered when it had been ascertained that there was no mortality associated with infection at the lower doses.

None of the density-dependent effects detected were marked. Parasite survival for 60 days after infection declined by only 14%, indicating that over the range of 5 to 40 worms, total worm burdens were not a major factor in survival. At this stage it is unknown whether some worms failed to establish or whether they were lost during the 60 day infection period. It is conceivable that at

higher worm burdens host immune mechanisms become effective or competition between worms for resources restrict numbers, but currently these are unexplored aspects of this host–parasite relationship. Nevertheless, it is noteworthy that in naturally infected hosts worm burdens can exceed 100, so wild *A. dimidiatus* can tolerate such burdens, even though they occupy the vast majority of the stomach lumen and presumably limit the feeding capacity of the host.

Surprisingly, an essentially linear relationship was found between the number of worms developing and the
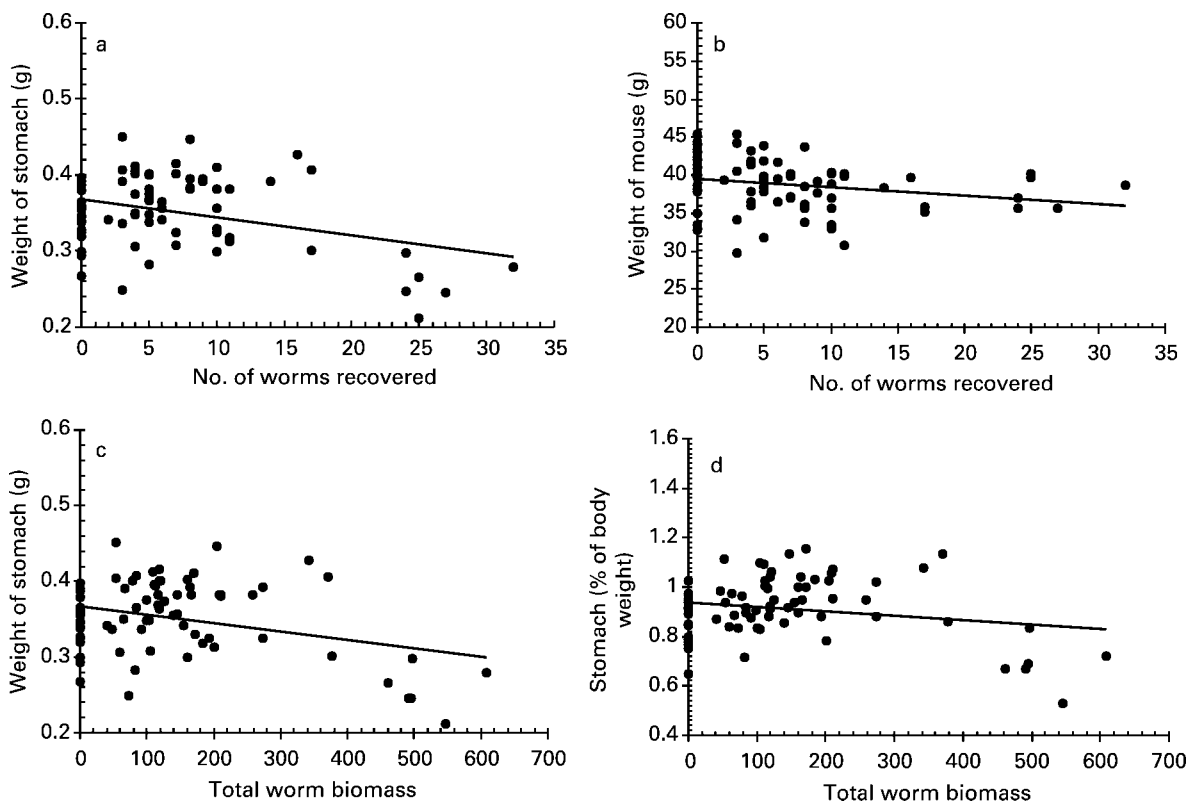

Fig. 6. The effect of *Protospirura muricola* on host stomach weight (a, c and d) and body weight (b). Statistical analysis of host body weight (b) as the dependent variable by ANOVA with experiment as factor and number of worms recovered as covariate, $F_{1,71} = 14.4$, $\beta = -0.163 \pm 0.043$, $P < 0.001$. Analysis of weight of stomach (a) by ANOVA with experiment as factor and number of worms recovered as covariate, gave $F_{1,71} = 11.68$, $\beta = -0.003 \pm 0.001$, $P = 0.001$. Analysis of weight of stomach (c) with experiment as factor and total worm biomass as covariate, gave $F_{1,71} = 10.84$, $P = 0.002$.

total worm biomass. Therefore, in contrast to cestode infections, there was no intestinal maximum holding capacity restricting further increases in biomass (Moss, 1971). It is conceivable that such a limit, represented by an expected plateau to the relationship (fig. 3), would have become evident at higher infection doses, but the total biomass in heavily infected hosts was substantial at 1.58% of host body weight, and ethical considerations limited exploration of higher levels.

A decline in the lengths of both males and females was shown with increasing numbers of worms, and of weight in the case of males, but no density-dependent decline in the weight of female worms was observed. Moreover, male worms were sensitive to the percentage of female worms present (after controlling for differences between experiments and variation in worm burden, table 2), both male length and weight declining as the percentage of female worms increased. In contrast, the relative proportion of sexes affected neither female worm lengths nor weights. Male worms are smaller than female worms (fig. 2), and their increased sensitivity to crowding is consistent with both findings. In these experiments, we did not quantify eggs in female worms, but it is possible that the egg content of female worms may have been a complicating issue. Female worms tend to shed eggs quickly when transferred to Hanks' medium (Lowrie, personal observations) and variation in the duration of incubation prior to fixation in ethanol, as well as between the egg content of individual worms, may have been responsible for generating sufficient noise to cloud any underlying relationship. In any case, all gradients were very shallow (table 2). Extrapolating from the fitted model in table 2, for male worms, those recovered from mice with high worm burdens (32 worms) were 1.56 mm shorter than those in low intensity infections (2 worms, = 8.4% reduction) and 3.42 mg lighter (= 24.6% reduction). In the case of females, the reduction in length with increasing worm burden was 4.1 mm (= 16.0%) and weight 1.8 mg (= 6.5%). Thus, from present data, density-dependent constraints on the growth of *P. muricola* were relatively small.

Observations on captive white-faced monkeys (*Cebus capucinus*) indicate that *P. muricola* can also infect primates in which infections can exceed 500 worms. Foster & Johnson (1939) described heavily infected white-faced monkeys in which worms migrated out of the stomach into the body viscera and were responsible for death of heavily infected animals. In the present system, *P. muricola* is confined to the stomach of mice but worm burdens are much lower and it could be that movement out of the stomach is initiated when crowding passes a critical threshold. Nevertheless, we provide the first experimental evidence that mice infected with *P. muricola* experience some quantifiable pathological effects. Experiments were not specifically designed to investigate the consequences of infection to the host, but they did provide an opportunity to make preliminary observations on the relationships between worm burden and host body weight and stomach weight. In both cases, values fell with increasing worm burden. Weight loss was not marked, and even the most heavily infected mice (32 worms) had a body weight within the naïve uninfected range. However, the effect of increasing worm burdens on stomach weight

was more marked, with mice harbouring more than 20 worms showing values towards the lower end of, or lower than, the range for naïve mice. The declining stomach weight suggests that there was local loss of tissues during infections and that homeostatic regulatory processes could not compensate, but whether this was driven by the parasite, perhaps through feeding on host tissues, or whether it was a component of a host response, remains to be established. In contrast to our findings, we expected stomach weight to increase, in association with a host response to the presence of the worms (Castro, 1990). However, there was little indication that, as with gastrointestinal nematodes of the small and large intestine, infection is associated with mucosal hypertrophy and hyperplasia. These aspects of the host–parasite relationship remain to be explored but currently nothing is known about whether mice respond to infection with inflammatory or other local responses.

Finally, we conclude that whilst increasing worm burdens impeded the growth of *P. muricola*, the overall reduction in size (as reflected in length and weight) was relatively small in relation to the overall size of this robust stomach worm. Nevertheless worm burdens in excess of 20 exerted a measurable cost to the host, which in the field, may be associated with loss of overall host fitness.

Acknowledgements

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