Parasite-induced anorexia: leptin, insulin and corticosterone responses to infection with the nematode, *Nippostrongylus brasiliensis*

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SUMMARY

The nematode parasite, *Nippostrongylus brasiliensis*, induces a biphasic anorexia in its rat host. The mechanisms, underlying this anorexia and its possible advantages to the host or parasite are unknown. We have investigated the effect of acute (12–24 h) and chronic (2–17 days) infections on plasma concentrations of leptin, insulin and corticosterone, and on hypothalamic expression of neuropeptide Y, galanin and corticotrophin-releasing factor genes. Plasma leptin was elevated in infected rats relative to uninfected *ad libitum*-fed controls and pair-fed controls in 12 h infections initiated at dark onset and in infections of 2 days' duration. At other times prior to parasite expulsion, plasma leptin in infected and pair-fed rats was lower than that of uninfected *ad libitum*-fed controls, reflecting the existing state of negative energy balance. Elevated plasma leptin concentrations in infected rats at day 2 post-infection were accompanied by reduced neuropeptide Y gene expression in the hypothalamic arcuate nucleus compared with both *ad libitum* control and pair-fed animals, and by lowered corticotrophin-releasing factor gene expression in the paraventricular nucleus relative to pair-feds. Twelve hour infections were characterized by a substantial increase in plasma corticosterone that was independent of reduced food intake, and in 12 h infections initiated at dark onset, where plasma leptin was elevated, there was also increased plasma insulin concentration in infected rats. In longer infections, differences between the groups in plasma insulin and corticosterone concentration were only observed at day 4 post-infection. In summary, perturbations to leptin, insulin and corticosterone signals early in infection may have a causative role and might feed back onto hypothalamic gene expression, whereas subsequent changes in these parameters are more likely to be secondary to negative energy balance.

Key words: parasite-induced anorexia, *Nippostrongylus brasiliensis*, hypothalamus, neuropeptide Y, galanin, corticotrophin-releasing factor.

INTRODUCTION

Anorexia, or reduced food intake in the presence of unlimited availability, commonly accompanies infection by parasitic helminths and may be a major component of the energetic 'cost' to the host of such host–parasite interactions (Coop & Holmes, 1996). This has clear consequences both for productivity in livestock and for human health. The *Nippostrongylus brasiliensis*/rat model used in the present study is characterized by 2 distinct anorexic episodes that occur around days 2 and 8 post-infection (p.i.). These episodes coincide with the lung phase and the intestinal phase of the infection, respectively (Horbury, Mercer & Chappell, 1995; Ovington, 1985). Although several studies have addressed peripheral satiety signals in the parasitized mammalian host, relatively little is known of the mechanisms that underlie the initiation and maintenance of anorexia. Indeed, our recent study of *N. brasiliensis* infections in the laboratory rat (Horbury *et al.* 1995) was the first to examine neuropeptide systems in the hypothalamus, these being key regulators of energy balance in a critical integratory centre within the central nervous system. Parasite-induced anorexia is normally reversible once the parasite burden is eliminated, but it is not clear whether anorexia is a protective response conferring host advantage, or a deleterious pathological phenomenon akin to human wasting illnesses (Schwartz, Seeley & Woods, 1997). In the latter, failure to consume sufficient calories to maintain energy balance is a major factor in the wasting syndrome and, as above, the questions that have been raised relate to the causes of reduced food intake and the absence of effective compensatory responses once weight loss occurs. Whatever the origins of parasite-induced anorexia, the pathology that results from infection with *N. brasiliensis* leads to a reduction in food assimilation efficiency. Fol-

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Table 1. Food intake and plasma concentrations of leptin, insulin and corticosterone in rats infected with *Nippostrongylus brasiliensis* (INF) at either dark onset (DO) or light onset (LO) and their matched control (CON) and pair-fed (PF) groups

($n = 5$ except at 12 h DO where $n = 8$. Infections were allowed to proceed for 12 or 24 h.)

	Time post-infection*			
	12 h DO	12 h LO	24 h DO	24 h LO
Food intake (g)				
CON	18·2 ± 1·0	7·0 ± 1·6	21·0 ± 0·9	23·6 ± 1·2
INF	12·1 ± 0·7	6·0 ± 0·8	17·4 ± 1·7	14·2 ± 4·5
Anorexia (%)†	33·6 ± 3·9	14·3 ± 12·0	17·1 ± 8·1	39·8 ± 19·0
Leptin (ng/ml)				
CON	0·82 ± 0·20[a]	1·70 ± 0·44	1·60 ± 0·25	1·28 ± 0·12
INF	2·61 ± 1·21[b]	1·20 ± 0·20	0·89 ± 0·14	0·88 ± 0·10
PF	1·16 ± 0·61	1·65 ± 0·19	1·17 ± 0·27	0·77 ± 0·44
Insulin (uU/ml)				
CON	20·9 ± 2·4[ac]	27·2 ± 4·5	27·2 ± 2·2	21·9 ± 3·2[b]
INF	57·4 ± 14·5[bc]	27·0 ± 3·7	29·5 ± 3·6	22·3 ± 1·7[b]
PF	22·1 ± 2·7	31·5 ± 4·5	19·6 ± 3·9	10·5 ± 2·1
Corticosterone (ng/ml)				
CON	225·1 ± 38·9[ac]	461·8 ± 77·0[a]	108·7 ± 28·3[abc]	144·7 ± 40·3
INF	832·4 ± 168·2[bc]	717·5 ± 82·0[b]	292·8 ± 48·6	88·0 ± 9·5
PF	303·5 ± 74·3	360·9 ± 68·0	218·7 ± 75·5	200·5 ± 36·4

* [a] Different from INF; $P < 0{\cdot}05$; [b] Different from PF; $P < 0{\cdot}05$; [c] ANOVA on Ranks.
† Percentage reduction of food intake of infected animals compared with controls.

lowing restoration of normophagia and induction of a compensatory hyperphagia, growth rates are slower in formerly parasitized rats than in their pair-fed controls (Horbury *et al*. 1995).

Neuropeptide Y (NPY) is a potent stimulant of food intake and a key player in the physiological compensatory response to negative energy balance. The activity of the NPYergic hypothalamic projection running from the arcuate nucleus (ARC) to the paraventricular nucleus (PVN) increases in response to food deprivation, food restriction and a number of other imposed energetic manipulations (Leibowitz, 1991; White, 1993). In *N. brasiliensis* infections of the laboratory rat we have already demonstrated increased NPY gene expression in the ARC of animals with established anorexia (i.e. 37% reduction in cumulative food intake after 8 days and 10–15% weight loss; Horbury *et al*. 1995). Furthermore, NPY gene expression in these animals inversely correlated with cumulative food intake. However, whereas elevated NPYergic activity would normally be associated with relative hyperphagia, this was not displayed by the parasitized rat until after the spontaneous immune-mediated expulsion of the worm burden. Therefore, we conclude that the presence of the parasite has a regulatory influence on mechanisms of energy homeostasis.

In the human wasting illnesses, it has been speculated that the disease process may initiate an inappropriate negative feedback or satiety signal, such that sustained anorexia and weight loss are not accompanied by compensatory responses. In established parasite-induced anorexia, negative energy balance induces an increase in NPYergic activity but the normal behavioural sequelae of this (i.e. elevated food intake) are not observed. It may be that the predicted response to increased NPYergic activity is blocked by the action of physiological signals on other hypothalamic neuropeptide systems, or that other peripheral signals induced by parasitic infection exert a more powerful regulatory influence. Two such peripheral systems that may be involved in negative feedback with NPY are insulin (Schwartz *et al*. 1992) and leptin, the recently described adipose tissue hormone (Zhang *et al*. 1994; Campfield, Smith & Burn, 1996), while glucocorticoids also contribute to the regulation of hypothalamic signals including NPY (White, 1993). The present study focuses on leptin, insulin and corticosterone signals and on 3 hypothalamic neuropeptides in the parasitized laboratory rat. The neuropeptides examined are all powerful regulators of energy balance with sites of action in or adjacent to the PVN. NPY and galanin stimulate food intake, while corticotrophin-releasing factor (CRF) inhibits food intake and functions in a physiological context to oppose the actions of NPY. The study addresses the issue of whether plasma leptin concentrations reflect or influence the energy balance status of the host at different stages of the infection.

MATERIALS AND METHODS

The life-cycle of *N. brasiliensis* was maintained by serial passage through outbred male Sprague–Dawley rats (150–200 g; Harlan, Bicester, UK) as

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described previously (Horbury *et al*. 1995). Nematode larvae were originally obtained from Professor R. Maizels, University of Edinburgh. Rats were divided into 3 groups in which individuals were weight-matched. Thus each rat to be infected (INF) had a weight-matched partner in both of the control (CON) and pair-fed (PF) groups. Individuals in the latter group were fed the same quantity of food that their identified INF partner had eaten in the previous 24 h, or throughout the duration of the experiment where this was less than 24 h. INF rats were injected subcutaneously (s.c.) with 45 L_3 larvae/g body weight suspended in tap water and were then housed singly with free access to food (RM1: 14·7 % crude protein; 14·8 MJ/kg gross energy; Special Diets Services) and water. CON rats were allowed to feed *ad libitum* throughout the experiment. CON and PF rats were injected with the same volume of water as their identified INF partner. Body weight and food intake were measured once each day at the end of the dark phase (08.00–09.00 h). Unless specified to the contrary, animals were infected and killed in the middle of the light phase (12 h light:12 h dark). Blood was collected in lithium–heparin tubes for hormone determinations in plasma. Brains were removed rapidly, frozen on dry ice and stored at −70 °C.

Leptin levels were determined in plasma samples using an established ELISA (Hardie *et al*. 1996), and expressed as recombinant murine leptin equivalents (PeproTech, Inc.). A detection limit of 100 pg leptin/ml was consistently achieved. Plasma insulin and corticosterone were measured by RIA, and hypothalamic neuropeptide gene expression by *in situ* hybridization using coronal forebrain sections with ^{35}S-labelled cRNA probes as described previously (Mercer, Lawrence & Atkinson, 1996*a*). PreproNPY mRNA was quantified in 2 sections from the mid-region of the ARC (adjacent to Bregma – 3·3 mm) and galanin and CRF mRNA were measured in the PVN.

All data were analysed by One Way Analysis of Variance or, where specified and appropriate, One Way Analysis of Variance on Ranks, both followed by multiple comparison tests using SigmaStat 1.0 (Jandel GmbH, Germany). Differences were considered statistically significant if $P < 0{\cdot}05$. Data are presented as means ± S.E.

RESULTS

The anorexia induced by *N. brasiliensis* infections of the rat is of variable severity both between individuals infected at the same time from the same stock of infective larvae and between different experiments (see, for example, 24 h experiments, Table 1 and 1 day experiment, Table 2.) This variability necessitates the use in each experiment of pair-fed controls matched to individual infected animals.

To investigate the possible involvement of leptin, insulin and corticosterone in the acute phase of infection, rats were infected at either dark onset (DO) or light onset (LO), and experiments terminated 12 or 24 h later. The outcome of these infections with regard to food intake and degree of anorexia is detailed in Table 1. Significant changes in plasma leptin were only observed in the 12 h DO experiment, where concentrations were elevated in INF rats (Table 1). In the remaining experiments (12 h LO, 24 h DO and 24 h LO), plasma leptin concentrations in INF and PF groups never exceeded those of the respective *ad libitum*-fed controls (CON). Plasma leptin concentration was correlated with food intake only in INF rats in the 24 h LO experiment (Table 1; positive correlation; $r = 0{\cdot}92$; $n = 5$; $P < 0{\cdot}05$). This relationship did not attain statistical significance in any other group in these acute experiments. Changes in plasma insulin levels were observed in the 2 experiments (12 h DO and 24 h LO) with the greatest proportional anorexia (Table 1). Plasma corticosterone was elevated in INF rats relative to both CON and PF in both 12 h experiments, and relative to CON animals in the 24 h DO experiment, at which time corticosterone was also elevated in the PF group. At 6 h after infection in the second half of the light phase, there was no change in plasma concentrations of leptin, insulin or corticosterone (data not shown). Hypothalamic gene expression was quantified in the 12 and 24 h experiments with the greatest proportional anorexia. Neither infection nor pair-feeding altered the expression of hypothalamic NPY, galanin or CRF mRNA in 12 h DO or 24 h LO experiments, and there was no obvious relationship between hypothalamic gene expression and food intake in these groups.

To examine established infections, rats were maintained for 1, 2, 4, 8 or 17 days post-infection (p.i.). The effects of infection on cumulative food intake, intake over the final 24 h of each infection and plasma hormone concentrations are presented in Table 2. In experiments terminated at either 1 or 8 days p.i., plasma leptin concentrations from INF and PF rats were significantly lower than their respective controls ($P < 0{\cdot}05$; day 1, ANOVA; day 8, ANOVA on Ranks; Fig. 1), but they did not differ from each other. By day 17 p.i., daily food intake of INF and PF rats was similar to CON, and plasma leptin concentrations were normalized, although 17-day cumulative food intake was still depressed (Table 2). Evidence of depressed plasma leptin concentrations in INF and PF rats earlier in the course of the 17-day experiment was obtained by tail vein blood sampling on day 6 p.i. (data not shown). In contrast to the above, plasma leptin was elevated in day 2 INF rats compared to both CON and PF groups ($P < 0{\cdot}05$; ANOVA on Ranks; Fig. 1). When cumulative food intake of individuals was related to

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Table 2. Food intake and plasma concentrations of insulin and corticosterone in rats infected with *Nippostrongylus brasiliensis* for 1, 2, 4, 8 or 17 days (INF) and their matched control (CON) and pair-fed (PF) groups

($n = 5$ except at day 2 p.i. where $n = 8$. Rats were infected and infections terminated in the middle of the light phase.)

	Days post-infection*				
	1	2	4	8	17
Cumulative food intake (g)					
CON	18·8 ± 0·4	44·6 ± 2·3	91·0 ± 2·5	215·0 ± 5·2	432·2 ± 14·6
INF	16·2 ± 1·4	32·6 ± 2·8	65·6 ± 3·2	149·2 ± 20·8	287·0 ± 36·7
Anorexia (%)†	13·8 ± 7·2	26·9 ± 6·3	27·9 ± 3·5	30·6 ± 9·7	33·6 ± 8·5
Food intake in final 24 h (g)					
CON	18·8 ± 0·4	22·2 ± 1·3	22·2 ± 1·3	26·4 ± 0·9	26·0 ± 0·7
INF	16·2 ± 1·4	17·2 ± 1·4	17·4 ± 1·2	23·4 ± 2·1	26·2 ± 2·2
Anorexia in final 24 h (%)‡	13·8 ± 7·2	22·5 ± 6·5	21·6 ± 5·6	11·4 ± 8·1	−0·8 ± 8·6
Insulin (uU/ml)					
CON	N.D.	28·7 ± 4·7	37·5 ± 5·5	53·8 ± 14·5	50·4 ± 9·8
INF	N.D.	27·7 ± 2·8	47·7 ± 6·7[a]	37·6 ± 8·4	34·2 ± 8·4
PF	N.D.	26·0 ± 5·0	21·4 ± 5·7	36·3 ± 2·9	30·8 ± 5·8
Corticosterone (ng/ml)					
CON	N.D.	109·2 ± 16·6	252·3 ± 26·4[a]	312·3 ± 51·7	136·8 ± 34·2
INF	N.D.	165·3 ± 43·7	257·3 ± 26·6[a]	309·2 ± 32·0	69·2 ± 11·4
PF	N.D.	192·3 ± 40·4	361·1 ± 14·3	338·2 ± 38·0	124·4 ± 26·2

N.D., Not determined.
* [a] Different from PF; $P < 0·05$.
† Percentage reduction of cumulative food intake of infected animals compared with controls.
‡ Percentage reduction of food intake of infected animals in final 24 h compared with controls.

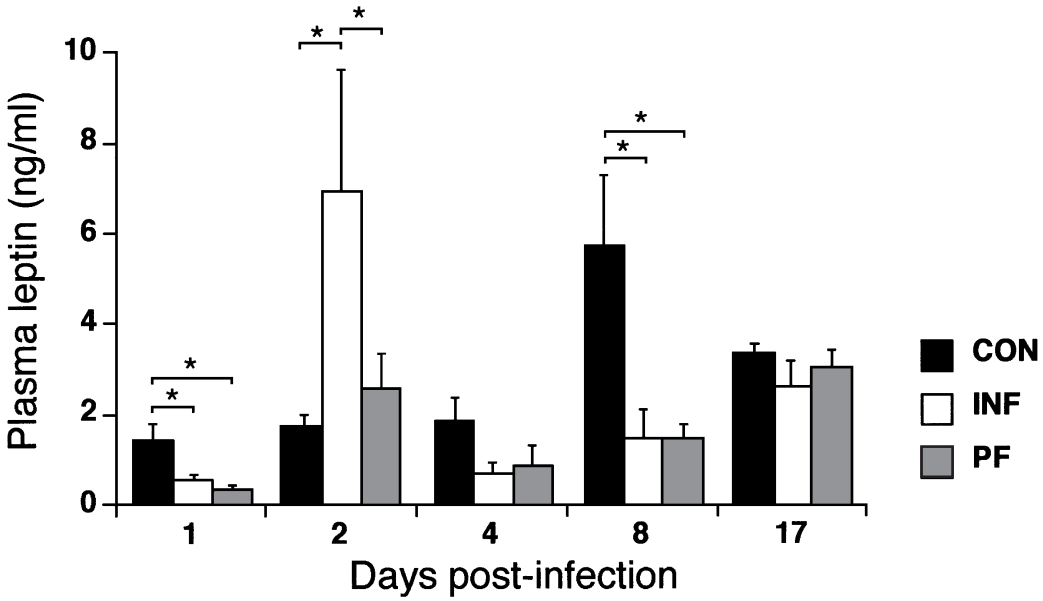

Fig. 1. Plasma concentration of leptin (ng murine leptin equivalents/ml) in rats 1, 2, 4, 8 or 17 days after infection in the middle of the light phase with *Nippostrongylus brasiliensis* (INF) and in matched control (CON) and pair-fed (PF) groups. Data are means ± S.E. where $n = 5$ except at 2 days where $n = 8$. * $P < 0·05$.

leptin concentration, statistical significance was only attained in the 2-day infection; there was a positive correlation in PF ($r = 0·83$; $n = 8$; $P < 0·05$) but not INF rats. Differences in insulin and corticosterone concentrations between the groups only attained statistical significance on day 4 p.i. (Table 2). Hypothalamic neuropeptide gene expression was examined at day 2 and day 8 p.i., coincident with the

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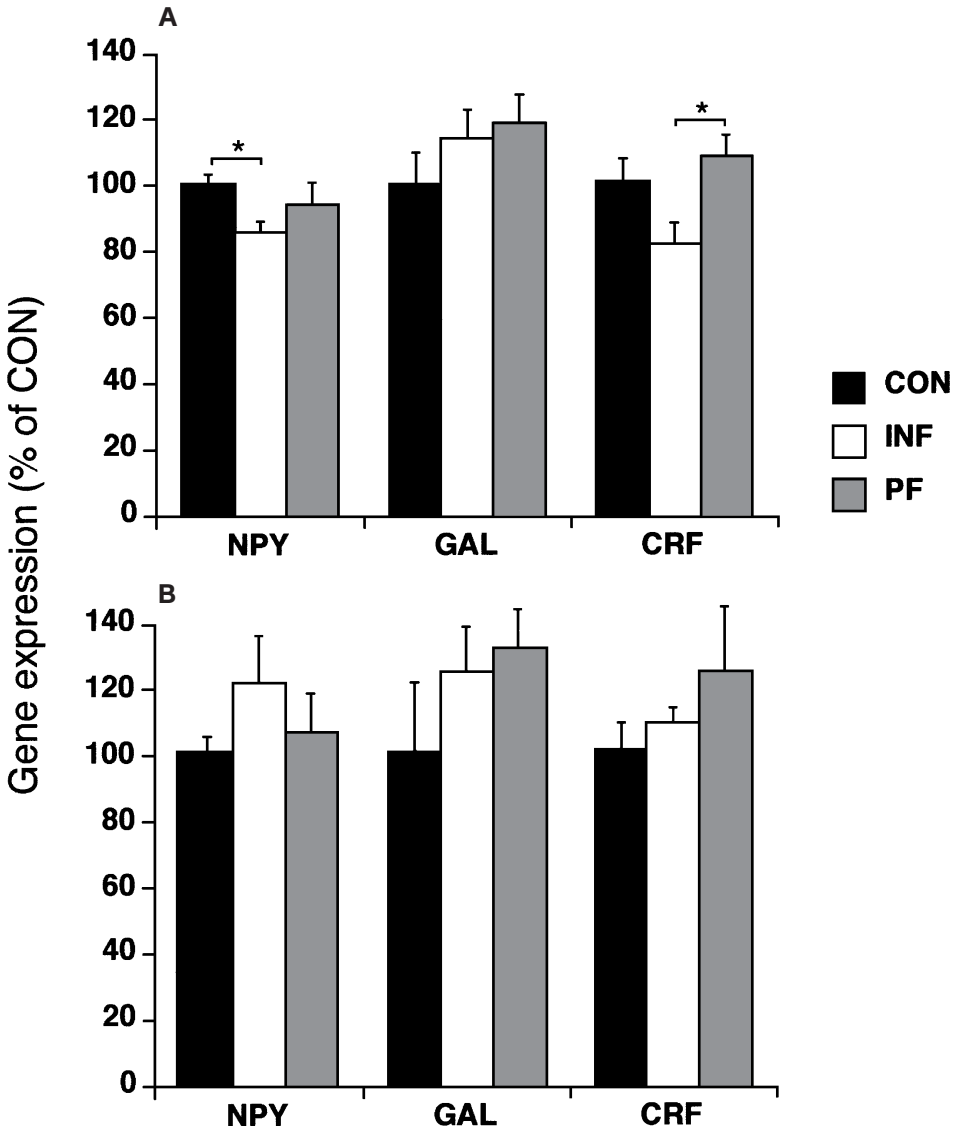

Fig. 2. Hypothalamic neuropeptide Y (NPY), galanin and corticotrophin-releasing factor (CRF) gene expression in rats infected with *Nippostrongylus brasiliensis* (INF) (A) for 2 days and (B) for 8 days and their matched control (CON) and pair-fed (PF) groups. NPY gene expression was measured in the hypothalamic arcuate nucleus, galanin and CRF mRNAs in the hypothalamic paraventricular nucleus. Gene expression data are expressed as a percentage of uninfected controls. Data are means ± S.E. where $n = 8$ or 5 at 2 days and 8 days, respectively. $^{*}P < 0{\cdot}05$.

timing of the 2 phases of anorexia induced by infection with *N. brasiliensis* (Horbury *et al*. 1995). On day 2 of infection, NPY mRNA in the ARC of INF rats was significantly lower than CON (ANOVA on Ranks), while CRF gene expression in the PVN of INF rats was lower than that of PF animals (Fig. 2A). Messenger RNA levels did not correlate significantly with cumulative food intake, and neither infection nor pair-feeding affected NPY, galanin or CRF gene expression at 8 days p.i. (Fig. 2B).

DISCUSSION

The two fundamental issues to be addressed in the laboratory model of parasite-induced anorexia afforded by *N. brasiliensis* infection of the rat are first, the mechanisms involved in the initial induction of anorexia, and secondly, given the power of the compensatory responses that are normally activated by negative energy balance, how this anorexia is maintained until the expulsion of the parasite. In the particular example of *N. brasiliensis* infection of the rat the possibly unique biphasic nature of the observed anorexia is suggestive of multifactorial mechanisms. Acute and comparatively severe imposed manipulations of energy balance in the rat, such as 48 h food deprivation, are accompanied by reductions in plasma concentrations of insulin (Mercer, Lawrence & Atkinson, 1996*b*) and leptin (Hardie *et al*. 1996), and by increases in plasma corticosterone (Mercer *et al*. 1996*b*). Within the hypothalamus of such animals, NPY gene expression in the ARC is elevated, while CRF mRNA in the PVN tends to

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decline (Mercer *et al*. 1996*b*). In the present study, examination of plasma concentrations of leptin, insulin and corticosterone in INF and PF animals revealed perturbations to all 3 hormonal signals in the initial 48 h of infection that apparently were not secondary to the relative hypophagia/restriction, and could thus have a causative role. Hypothalamic gene expression studies revealed differences only in infections terminated after 2 days. Specifically, these differences were in NPY gene expression between CON and INF animals, and in CRF gene expression between INF and PF animals; both were coincident with elevated plasma leptin in the INF group. The reported interaction between leptin and these hypothalamic systems, and in particular the ability of leptin to down-regulate NPY gene expression (Mercer *et al*. 1997; Schwartz *et al*. 1996) hints at a functional relationship. A similar trend, in which elevated plasma leptin and relatively low NPY gene expression were coincident, was observed in the 12 h DO experiment. In a previous study of NPY gene expression in the ARC of rats at day 2 p.i. (Horbury *et al*. 1995), we observed an increase in this mRNA species in PF animals and a similar trend in the INF group. However, it should be noted that these data were obtained from rats with a cumulative 48 h anorexia of 72 %, a much larger energy deficit than that expressed in the present study (i.e. 27 %). The limited energy deficit encountered by INF and PF rats in the 12 h and 24 h experiments would not be expected in isolation to markedly affect hypothalamic gene expression.

Twelve hours after infection, the plasma of rats was characterized by marked changes in the profiles of leptin, insulin and corticosterone, possibly indicative of a stress response to the migration of larvae. The perturbations to these signals appeared to be influenced by the timing of the infection within the light/dark cycle, although the mechanisms underlying this are not clear. A possible relationship with the feeding cycle appears likely since the dark phase coincides with the period of maximum food intake, but effects on plasma hormones must be independent of the relative hypophagia that results from the parasitic infection, since these parameters are unaffected in the PF groups. Particularly striking was the elevated plasma corticosterone in INF rats in both 12 h experiments. In the 12 h DO experiment these changes were accompanied by increases in both leptin and insulin concentrations. Although trends in the plasma leptin data in the 24 h infections reflected a state of negative energy balance, insulin titres in INF rats were similar to CONs and did not show the decreases or trends in that direction exhibited by the PF rats. Corticosterone titres were relatively variable and did not suggest major differences between INF and PF animals at 24 h p.i. This suggests that and infection-induced activation of the adrenal axis is relatively short-lived.

In the second part of the study, with the exception of the 2 day experiment, there were either significant reductions or downward trends in plasma leptin in both INF and PF rats until normophagia was re-established at day 17 p.i. Thus the concentration of leptin in the plasma of infected rats generally reflected the prevailing state of negative energy balance. The elevated leptin concentration on day 2 p.i. was not accompanied by changes in the other plasma hormones. This phase in the parasite life-cycle is typified by heavy larval worm burdens in the lungs and the first of the 2 anorexic phases (Horbury *et al*. 1995). Differences between INF and PF rats in insulin and corticosterone concentration were also observed on day 4 p.i. just prior to the initiation of the second anorexic episode.

Anorexia frequently accompanies infections with a number of organisms and several cytokines which regulate the host response to infection and mimic many of the metabolic consequences of such infections when administered exogenously. Cytokines have been implicated in the human wasting illnesses, and must be regarded as possible mediators of parasite-induced anorexia. For example, lipopolysaccharide (LPS) administration, a model of systemic infection, releases interleukin (IL)-1, IL-6 and tumour necrosis factor (TNF) (Langhans, 1996), of which IL-1 and IL-6 are released during the lung phase of infection with *N. brasiliensis* (around day 2 p.i.; Benbernou *et al*. 1992). When administered exogenously, these cytokines induce anorexia and weight loss in rodents, and IL-1 and TNF increase both leptin mRNA in adipose tissue and plasma leptin levels (Grunfeld *et al*. 1996). These data have led to the suggestion that the anorexia of infection may be mediated via cytokine induction of leptin synthesis (Grunfeld *et al*. 1996), although the occurrence of LPS-induced anorexia in *ob/ob* (leptin-deficient) and *db/db* (leptin receptor-deficient) mice argues against this (Faggioni *et al*. 1997). Similarly, the assignment of the leptin receptor to the class-I cytokine receptor family (Tartaglia *et al*. 1995), and similarities in signal transduction pathways between leptin and other cytokines (Tartaglia, 1997) raises the possibility that release of cytokines themselves may generate signals that mimic the effects of leptin on energy homeostasis (Schwartz *et al*. 1997).

The significance of the increases in plasma leptin observed exclusively in parasitized animals early in the infection in the present study is not clear. At the later times studied, plasma leptin simply reflected the energetic status of the host. Interestingly, these observations may find parallels in bacterial peritonitis where an early transient increase in plasma leptin occurs (Moshyedi *et al*. 1998). However, this latter leptin response was attenuated by TNF blockade whereas the anorexia was unaffected. Similarly, in parasite-induced anorexia, direct induction of anorexia by leptin released early during

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the infection appears unlikely since in preliminary experiments the obese (*fa/fa*) Zucker rats also expressed anorexia when infected with *Nippostrongylus* (our unpublished observations) despite the hyperleptinaemia (Hardie *et al*. 1996) and defective leptin receptor signalling in this obese model (Tartaglia, 1997). It may be significant, in the context of *N. brasiliensis* infections of the rat, that IL-6, which is released during the lung phase, does not apparently induce leptin expression, but is a known mediator of anorexia in acute-phase protein responses (Moshyedi *et al*. 1998). Further investigation of the striking elevation of plasma insulin and corticosterone during the first 12 h of infection may provide more insight into the induction of anorexia, but the more chronic changes to plasma and hypothalamic parameters described here and in our previous study (Horbury *et al*. 1995) all appear to be secondary to the anorexia that is maintained until parasite expulsion.

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