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Parasite co-infection and interaction as drivers of host heterogeneity

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Abstract

We examined the hypothesis that the interaction between concomitant infecting parasites modifies host susceptibility, parasite intensity and the pattern of parasite distribution within the host population. We used a 26 year time series of three common parasites in a natural population of rabbits: two gastrointestinal nematodes (*Trichostrongylus retortaeformis* and *Graphidium strigosum*) and the immunosuppressive myxoma virus. The frequency distribution of nematodes in the host population and the relationship between host age and nematode intensity were explored in rabbits with either single or dual nematode infections and rabbits infected with the nematodes and myxoma virus. The aggregation of *T. retortaeformis* and *G. strigosum* among the rabbits varied with the nature of the co-infection both in male and female hosts. The two nematodes exhibited different age–intensity profiles: *G. strigosum* intensity increased exponentially with host age while *T. retortaeformis* intensity exhibited a convex shape. The presence of a secondary infection did not change the age–intensity profile for *G. strigosum* but for *T. retortaeformis* co-infection (either both nematodes or myxoma-nematodes) resulted in significantly greater intensities in adult hosts. Results suggest that multi-species infections contributed to aggregation of parasites in the host population and to seasonal variation in intensity, but also enhanced differences in parasitism between sexes. This effect was apparent for *T. retortaeformis,* which appears to elicit a strong acquired immune response but not for *G. strigosum* which does not produce any evident immune reaction. We concluded that concomitant infections mediated by host immunity are important in modifying host susceptibility and influencing heterogeneity amongst individual hosts.

Keywords: Co-infection; Age–intensity relationship; Frequency distribution; Gastrointestinal nematodes; Myxoma poxvirus; Immunity; Seasonality

1. Introduction

Species interactions, either through direct interaction or mediated through an indirect mechanism, are fundamentally important processes in shaping community structure and generating biodiversity (Bonsall and Hassell, 1997). In the parasite communities of animal populations, direct interactions occur when parasites compete for the same resource, either space or food, while indirect interactions occur, for example, when the host's immune response to one parasite affects the host's ability to control a second parasite species. Specifically, one parasite can enhance the immune response to a second through cross-immunity (negative interaction), or alternatively cause immuno-suppression (positive interaction) (Behnke et al., 2001; Cox, 2001). In the latter case, the extreme scenario is seen when a second parasite subverts the immune response leading to reduced resistance and increased susceptibility to the first parasite (Graham et al., 2007). This interaction can be illustrated by the co-infection of a host with a virus and a helminth. Viruses usually cause acute, highly inflammatory infections that promote a response mediated by cellular mechanisms, while helminth infections promote chronic but less harmful responses and stimulate a humoral reaction mediated by antibodies and other molecules (Quinnell and Keymer, 1990; Abbas et al., 1996; Cox, 2001). The mechanism of regulation of the virus and the helminth often activates different effectors that can be mutually

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inhibitory (Jankovic et al., 2001; Neurath et al., 2002). As a consequence of this, host susceptibility to the helminth infection is likely to change and potentially affect worm intensities and transmission rates, and have important non-linear effects on host-parasite interaction and parasite dynamics (Graham et al., 2007; Hudson et al., in press). An alternative indirect route of interaction may also occur when a parasite secretes compounds that influence another parasite species or may induce physiological changes in the host that alter the survival of the second species but this route seems relatively uncommon in natural systems (Behnke et al., 2001; Cox, 2001).

One inference from these observations is that immune-mediated species interactions can drive variation in susceptibility and infectiousness between individual hosts and consequently shape the parasite community of a host population (Hershow et al., 1997; Nacher et al., 2002; Elliott et al., 2004; Andreansky et al., 2005; Graham et al., 2005; Thorburn et al., 2006; Cattadori et al., 2007). For example, if variation in susceptibility is influenced not only by past and current exposure to the focal parasite but also to the presence and history of infection of the second species, this will increase variation in intensity between hosts and the overall pattern of parasite distribution in the whole host population (Boag et al., 2001).

One signal that indicates that a helminth is regulated by an acquired immune response is a convex age–intensity relationship, a profile sometimes referred to as a Type III response (Hudson and Dobson, 1995; Hudson et al., 2006). This age–intensity curve is well illustrated by models that describe the development of acquired immunity as a response to the accumulated exposure to the parasite's infective stages (Woolhouse, 1992, 1998). However, the convex profile can also be generated by other host-parasite processes, such as parasite-induced host mortality, age related effects or frailty in the data, and disentangling their role can be challenging (Hudson and Dobson, 1995; Cattadori et al., 2005). Nevertheless, the evidence of a shift in the peak intensity of infection with host age, as a function of changes in the force of infection, the rate at which susceptible hosts acquire infection (McCallum, 2000), or an increase in parasite intensity in breeding females, the periparturient rise in nematode burdens, provides good support for the hypothesis that parasites are immuno-regulated (Soulsby, 1965; Woolhouse, 1998; Cattadori et al., 2005; Cornell et al., unpublished data). If a host is infected with two parasites, the first species immune-regulated and the second that reduces resistance to the first, then we expect that the age–intensity profile of the first will be modified (Cattadori et al., 2007). If the first parasite is not regulated by immune mechanisms, then there will be no apparent changes in the age–intensity profile, assuming all other factors remain constant.

In this paper we investigate the hypothesis that concomitant infections of indirectly competing species that are regulated by host immunity will generate variation in resistance between hosts leading to an increased mean intensity of infection but reduced variance within the co-infected groups; over the whole host population this will promote increased variance. To address this hypothesis we examined time series data from a free-living population of European rabbits (*Oryctolagus cuniculus*) and the most common parasites of this lagomorph, two gastrointestinal nematodes, *Trichostrongylus retortaeformis* and *Graphidium strigosum,* and the poxvirus myxoma, that causes myxomatosis. We selected these parasites because they differ in their age–intensity relationships. *Trichostrongylus retortaeformis* causes a Type III convex age–intensity profile and previous studies have suggested that this is generated by an acquired immune response (Michel, 1952a,b; Cattadori et al., 2005, 2007; Cornell et al., unpublished data). In contrast, *G. strigosum* exhibits a Type I profile where intensity increases constantly with host age and does not show any clear sign of acquired immunity (Supplementary Figs. S3 and S4). Myxoma has a strong immunosuppressive effect, so we expected it to reduce the immune regulation of other parasites (McFadden et al., 1995; Nash et al., 1999; Zuniga, 2002; Seet et al., 2003). In our case, we predicted that rabbits co-infected with myxoma would have greater intensities of the nematode *T. retortaeformis* but no such effects would be observed in hosts with *G. strigosum.* We examined both the frequency distribution and the host age–intensity relationship for each nematode in rabbits with single and dual nematode infections and rabbits co-infected with myxoma and the nematodes, with respect to sex and breeding status.

2. Materials and methods

2.1. The parasite–host system

Parasite and host data were obtained from a population of European rabbits sampled with a .22 rifle by walking transects across an area of 400 ha in central Scotland, every month from 1977 to 2002 (Boag et al., 2001). Wild rabbits represent a pest in the UK and no special permission is required to harvest this lagomorph, other than the permission of the landowner. From each rabbit, we recorded the intensity of two nematodes, *T. retortaeformis* and *G. strigosum*, and the presence of myxoma virus from characteristic internal and external lesions (Boag, 1988; Best and Kerr, 2000; Boag et al., 2001). We also quantified the intensity of three less common gastrointestinal helminths and the prevalence of the hepatic protozoan *Eimeria stiedai*, but rabbits co-infected with these parasites were excluded from the current study. In this paper, and to be consistent with epidemiological terminology, we define intensity as the total number of worms in a host, intensity is zero when a host has no nematodes; this contrasts with the definition suggested by Bush et al. (1997). Rabbit sex, mass, body size and reproductive status were recorded and the age structure of the sampled population was reconstructed using body mass as a proxy for age. The age classification was initially based on myxoma-free individuals and rabbits

were classified into eight age-mass classes that corresponded to three major age categories: kittens (classes 1–3), juveniles (classes 4–5) and adults (classes 6–8) (Cattadori et al., 2005). The accuracy of this age classification was supported by an analysis of the relationship between host age and body measurements, body length, body-tail length and foot length (Cattadori et al., 2005). Eye lens mass is commonly considered a reliable indicator of age in rabbits (Lord, 1959; Taylor, 1959; Wheeler and King, 1980; Kolb, 1994). The eye lens was collected from individuals sampled between 2002 and 2004 and the relationship between host's biometrics (mass and body measurements) and eye lens mass examined (Myers and Gilbert, 1968; Wheeler and King, 1980; Kolb, 1994). Body mass was strongly correlated with ln-transformed eye lens mass (Pearson correlation: $r = 0.95$, $P < 0.001$, $df = 421$) and both variables exhibited strong relationships with body measurements (Supplementary Fig. S1). This additional comparison further supported the predictability of the mass-age classification used for the rabbit population sampled in this study area. The age reconstruction was also tested on myxoma-infected rabbits and similar age-biometric relationships were found between myxoma-positive and myxoma-free hosts (Supplementary Fig. S2 and Table S1). Consequences of myxomatosis are the loss of body mass, vision impairment and reduced feeding abilities (Fenner and Fantini, 1999). Despite this, and because our age-mass categories were relatively large, a misclassification of the myxoma-infected individuals appeared negligible. Moreover, while we are aware that seasonal changes in breeding conditions or diet may affect rabbit mass (Sibly et al., 1990), the large sample size (>3500 rabbits) used to estimate the age classes reduced the within-class variability and the number of misclassifications.

Trichostrongylus retortaeformis and *G. strigosum* are gastrointestinal nematodes with a direct life cycle and free-living larval stages. Infection occurs following ingestion of the infective L3 (Anderson, 2000). *Trichostrongylus retortaeformis* infects the small intestine and *G. strigosum* the stomach. The free living stages of both nematodes are relatively sensitive to low temperature and dry conditions (Crofton, 1948; Soulsby, 1982; Marquardt et al., 2000). Infections are highly seasonal and coincide with the period of rabbit reproduction that in our population occurs mostly between April and August (Cattadori et al., 2005). The two nematodes cause distinct infection patterns: *T. retortaeformis* intensity initially increases with host age, peaks and then decreases in older individuals. This convex age–intensity relationship is consistent with previous suggestions that rabbits develop an acquired immune response as a function of the accumulated exposure to this nematode (Michel, 1952a,b; Cattadori et al., 2005; Cornell et al., unpublished data). In contrast, the intensity of *G. strigosum* rises exponentially with host age and shows no apparent sign of immune- or density-dependent constraints (Supplementary Figs. S3 and S4). Previous analyses of the adult rabbits from this data set suggest a cross-immunity of *T. retortaeformis* on *G. strigosum* infection but also a weak positive effect of *G. strigosum* on *T. retortaeformis* (Lello et al., 2004).

Myxoma is a poxvirus introduced into the UK in 1953 as a bio-control agent (Fenner and Fantini, 1999). Initially, the virus caused massive mortalities but attenuated strains were identified in wild populations as early as 1955 (Hudson and Mansi, 1955). In Europe, the virus is mechanically transmitted by the European rabbit flea (*Spilopsyllus cuniculi*) (Muirhead-Thompson, 1956). Myxoma is highly immunosuppressive (Nash et al., 1999; Zuniga et al., 1999; Zuniga, 2002; Seet et al., 2003). Viral replication initially occurs in the skin and subsequently moves into the lymph nodes (Zuniga, 2002). Prevalence is seasonal and in our population, myxoma outbreaks occurred between July and January. Preliminary analyses indicated that myxomatosis reduced the aggregation of the gastrointestinal helminths in rabbits (Boag et al., 2001). Since myxoma outbreaks usually occur after the peak of the breeding period, when the majority of kittens and adults have already been exposed to nematodes, we assume that the viral infection follows the initial nematode infection (Cattadori et al., 2007).

2.2. Single and concomitant infections

We used three subsets of data: the first included rabbits infected with a single nematode species, either *T. retortaeformis* or *G. strigosum*, the second considered rabbits infected with both nematodes, and the third included rabbits co-infected with myxoma and the two nematodes. Due to seasonal differences in the dynamics of infection of these parasites, few rabbits were found to have myxoma and a single nematode infection, and over 26 years only four individuals were sampled with only the virus infection, so not all comparisons were possible. Our data allowed us to examine three different patterns of infection: single nematode infection, dual nematode infection and micro-macroparasite infection. In addition, the marked parasitological characteristics of the three parasites, the absence of a direct interaction among them, and the possibility of assessing these patterns in relation to host age, between genders and breeding period, allowed us to disentangle the effect of age from an immunological response and from seasonal breeding. Since we used the same population of rabbits, we were able to compare individuals sharing similar environmental conditions and influenced by the same demographic processes, and therefore avoid confounding effects due to spatial differences between populations.

Analyses were based on an examination of the pattern of infection for *T. retortaeformis* and *G. strigosum* using the frequency distributions of parasites in the host population and the host age-parasite intensity relationship. For the frequency distributions, the nematode intensity was classified into 16 intensity classes where class 0 represented no nematode infection and each subsequent class corresponded to an incremental step of 500 nematodes per host for

T. retortaeformis and 50 nematodes per host for *G. strigosum*. The degree of parasite aggregation was estimated using the parameter k from the negative binomial distribution, which provides an inverse estimate of aggregation. The corrected moment estimate of k was fitted to the whole data set (Elliot, 1977) but since worm aggregation may have changed over the 26 years of sampling, k was also estimated every year for each sex and type of infection. One issue faced in the analyses was that of rabbits with no infection (Lotz and Font, 1994; Behnke et al., 2005). Different assumptions were investigated, such as the removal of all zeros or the partitioning of zeros by type of infection (taking into account seasonal variations in host age classes and sex ratio). Different assumptions did affect the variability of nematode intensity among hosts, so to avoid additional variability due to the constraints of the data, the zero cases were included when analysing each type of infection and removed when comparing infection types.

For the age–intensity relationship we examined the mean nematode intensity $[\ln(x+1)]$ by host age using a weighted cubic spline fitted to each month of sampling, from January to December (Cattadori et al., 2005). Since the fitted curves were weighted by the number of samples in each age class, we have reliable evidence to show that the shape of the profiles is not due to a sampling artefact. For both nematode species, the age–intensity profiles were similar for the breeding period, from April to July, and then again for the non-breeding period, from August to March, thus for the single and dual nematode infections, the age–intensity relationship was examined by combining the data in these two main periods. Similarly, since myxoma outbreaks usually occur between July and January, we focused on this period and compared the profile of nematode intensity by host age in rabbits co-infected with myxoma and rabbits with no myxoma lesions. To identify differences between sexes, the age–intensity curves were determined for both females and males.

A generalised linear model (GLM) was used to examine changes in nematode intensity as a function of host characteristics (age, sex, breeding status), time and infection type (i.e., both within and between infection types). Different error distributions were tested to identify the minimal adequate model through a stepwise backward deletion routine from the maximal model including all factors and their second and third order interaction (Crawley, 2002). The best fitted minimal models are presented and discussed.

3. Results

Single nematode infections of either *T. retortaeformis* or *G. strigosum* were recorded in 41% of the hosts sampled, 27% had a dual nematode infection and 28% of the population had no infections. Myxoma-positive rabbits represented 4% of the samples: 2% had myxoma-dual nematode infections and the other 2% had myxoma and a single infection of either *T. retortaeformis* or *G. strigosum*.

3.1. Frequency distribution

The estimates of the aggregation parameter k of the negative binomial varied for both nematodes with respect to the nature of the co-infection. However, since k also varies with the mean, we can only obtain a qualitative assessment of the way in which aggregation varies with co-infection (Wilson et al., 2002). Additionally, because the myxoma-nematode dataset considered rabbits sampled in a different time-frame with respect to the single or dual nematode dataset, no statistical comparison could be made among the three types of infection with respect to the patterns of aggregation. Over the 26 years, the aggregation of *T. retortaeformis* was relatively high in the dual nematode infections, decreased in the micro-macroparasite co-infections, and was lowest in the single infections (Fig. 1). Males and females showed similar patterns. The negative binomial parameter k was also estimated every year for each sex and type of infection. While the general trend reflected the pattern seen in the full data set, the annual k parameters were higher, suggesting that the annual distribution of *T. retortaeformis* in the rabbit population is less aggregated and shows some inter-annual variation (Table 1). The estimates of k for *G. strigosum,* over the 26 years of data, decreased from single through dual to myxoma-dual nematode co-infections (Fig. 1). Again, the annual k parameters were similar to the general trend but the values were higher and exhibited some annual variation (Table 1).

3.2. Age–intensity relationship

Since an examination of the frequency distribution fails to consider age-related infection processes, we examined the age–intensity relationship of the two nematodes in relation to the nature of the co-infection.

3.2.1. Single nematode infections

Outside the host breeding period, *T. retortaeformis* exhibited a clear type III convex age–intensity relationship in both rabbit sexes (Fig. 2). This profile was less pronounced during the breeding period in that older rabbits exhibited higher parasite intensities than non-breeding adults. Adult breeding females showed significantly higher mean nematode intensities than adult breeding males (mean intensity $\pm$ SEM, $n =$ cases in adult females versus adult males: 525.08 ± 156.04, $n = 38$ versus 146.51 ± 50.64, $n = 37$, GLM nematode intensity in adult classes by sex: $P < 0.01$, $\mathrm{df} = 73$). This latter pattern was consistent with a periparturient rise in worm intensity in breeding females (Fig. 2). The best GLM suggested that *T. retortaeformis* intensity was significantly affected by sex, age and breeding conditions (Table 2).

Analyses were repeated for rabbits with single *G. strigosum* infections (Fig. 2). Intensity increased significantly with age in both males and females. Host sex and breeding period, as interacting variables, were important in explaining the changes in intensity observed (Table 2). These

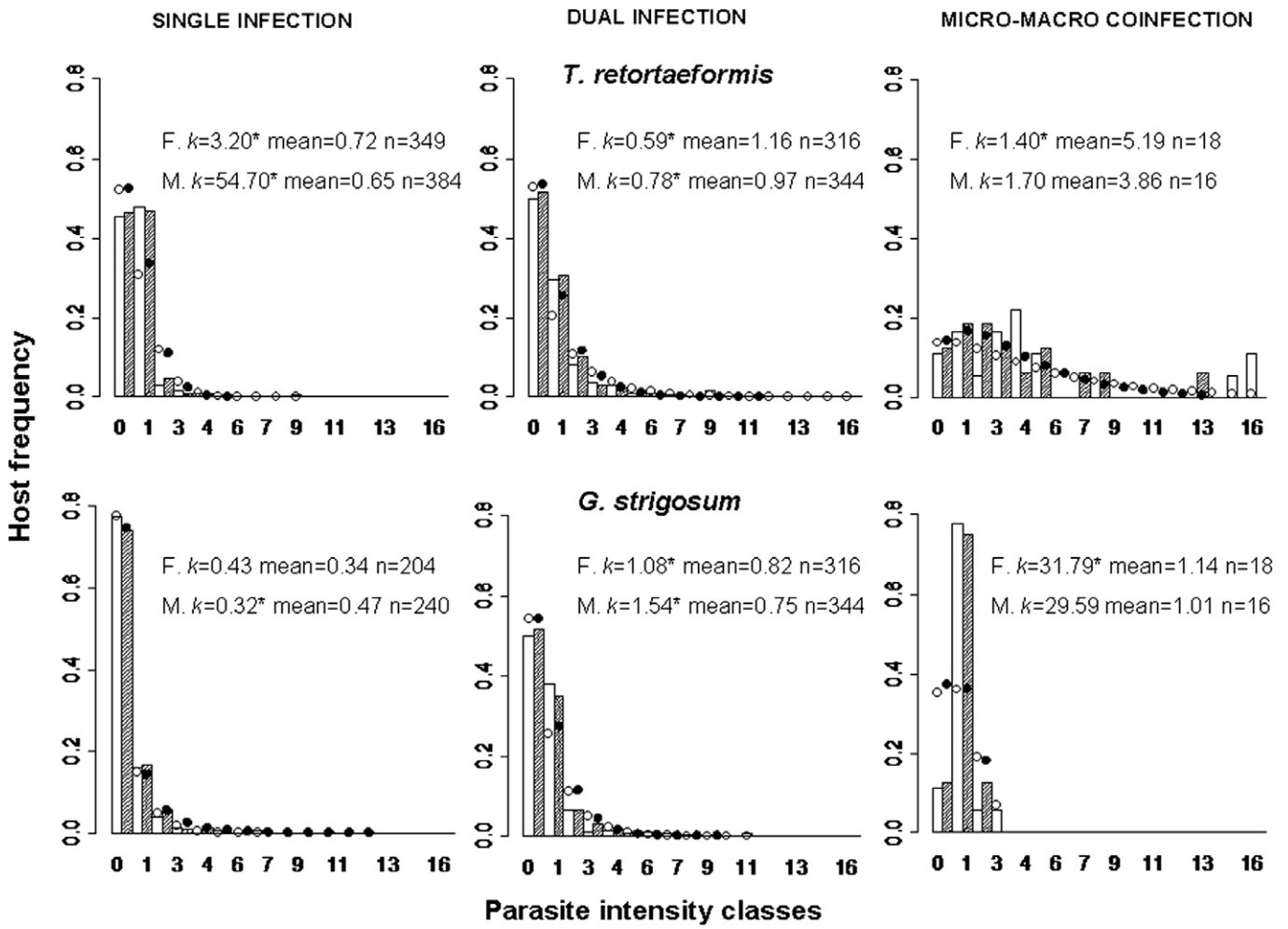

Fig. 1. Frequency distribution of female and male rabbits infected with *Trichostrongylus retortaeformis* and *Graphidium strigosum* in single and concomitant infections. Observed frequencies (bars) and expected fit of negative binomial distribution (dotted line) are presented. The estimated parasite aggregation parameter k and the mean parasite intensity are reported for females (F) and males (M), Chi-squared test between observed and fitted data, $^{*}P < 0.01$. Parasite intensities are reported by classes, from 1 to 16; they represent increments of 500 worms for *T. retortaeformis* and 50 worms for *G. strigosum*.

Table 1
Mean of the annual aggregation estimates for k of the negative binomial distribution for female and male rabbits with respect to the type of infection

	Single infection		Dual infection		Myxoma and nematodes	
	Trichostrongylus retortaeformis	*Graphidium strigosum*	*T. retortaeformis*	*G. strigosum*	*T. retortaeformis*	*G. strigosum*
Female	20.678 ± 4.536 (13.42 ± 1.73)	8.489 ± 1.561 (8.52 ± 1.11)	4.622 ± 3.608 (12.42 ± 1.62)	13.688 ± 2.645 (14.48 ± 1.57)	40.921 ± 57.106 (3.00 ± 0.58)	23.652 ± 4.249 (4.00 ± 0.00)
Male	25.758 ± 3.656 (15.75 ± 1.86)	9.094 ± 2.362 (11.56 ± 1.22)	8.227 ± 2.864 (14.48 ± 1.57)	16.822 ± 3.150 (14.48 ± 1.57)	20.627 ± 8.471 (2.75 ± 0.48)	26.083 ± 2.094 (3.00 ± 0.58)

The mean k, weighted by the number of samples in each year, and the SEM are presented. The annual mean rabbit sample size $\pm$ SEM, is shown in parenthesis. We used 26 years of data but distributions with only one frequency class were not included.

results, in combination with the pattern described by the cohorts of rabbits born monthly from February to August (Supplementary Figs. S3 and S4), provide further evidence to suggest that other mechanisms of regulation, rather than acquired immunity, modulate the dynamics of *G. strigosum*. Indeed, seasonal changes in exposure probably play a role in the regulation of this parasite.

3.2.2. Dual nematode infection

Initially, we compared the age–intensity profiles between hosts with single and dual nematode infections. Rabbits co-infected with *G. strigosum* exhibited higher *T. retortaeformis* intensities compared with hosts infected only with *T. retortaeformis* (respectively: mean $\pm$ SEM, $n =$ cases, with zeros in both data sets: 347.85 ± 32.77,

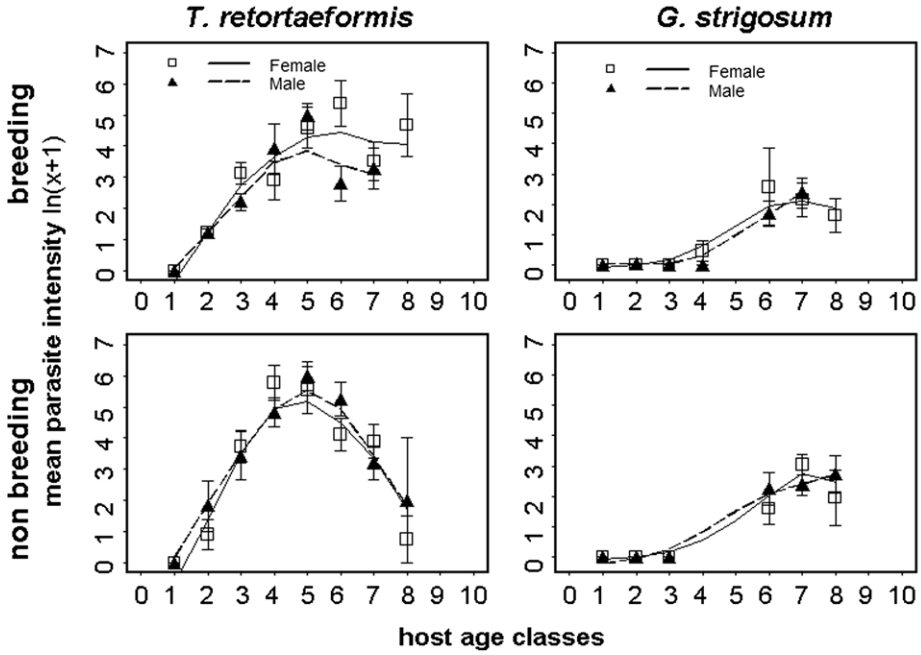

Fig. 2. Host age–parasite intensity profiles for *Trichostrongylus retortaeformis* and *Graphidium strigosum* compared with host sex during and outside the breeding period in rabbits carrying a single nematode infection. A weighed cubic spline curve is fitted to the relationship between the $\ln(x+1)$ mean parasite intensity and host age classes (symbols). The standard error bars of the mean parasite intensities are shown.

$n = 660$ versus 133.65 ± 14.19, $n = 733$; without zeros: 708.58 ± 60.60, $n = 324$ versus 246.77 ± 24.83, $n = 397$, Fig. 3). The results suggested that host age, sex, breeding period and their second order interaction contributed to explain the differences in *T. retortaeformis* intensity between single and dual infections (the zeros were removed from this analysis, Table 2).

We also compared the pattern of *G. strigosum* infection between single and dual infected hosts. Intensities were similar whether *T. retortaeformis* was present or not (respectively: mean ± SEM, n = cases, with zeros in both data sets: 13.33 ± 2.28, $n = 443$ versus 22.57 ± 2.29, $n = 660$; without zeros: 55.18 ± 8.23, $n = 107$ versus 45.98 ± 4.29, $n = 324$, Fig. 3). Host age was the only significant variable affecting *G. strigosum* intensities (GLM with zeros removed: $P < 0.001$, df = 429), suggesting that the presence of *T. retortaeformis* does not significantly modify the intensity of this stomach dwelling nematode.

A detailed examination of rabbits with the dual nematode infection revealed that during the breeding period *T. retortaeformis* intensity was significantly greater in adult females than in adult males (mean ± SEM, n = cases: $1{,}300.25 \pm 237.68$, $n = 59$ versus 338.26 ± 93.25, $n = 50$; GLM: $P < 0.01$, df = 107). Examination of these adult females (age classes 6, 7 and 8) showed that breeding females were responsible for the high intensities observed (nursing/pregnant versus non-nursing/pregnant females mean ± SEM, n = cases: $1{,}377.65 \pm 265.84$, $n = 51$ versus 806.87 ± 440.04, $n = 8$; GLM: $P < 0.01$, df = 57). Changes in *T. retortaeformis* intensity in dual infected rabbits were influenced by host sex, age and breeding condition (Table 2).

Graphidium strigosum intensity increased in both sexes in the breeding period and out of the breeding period (Fig. 3). Host age and breeding condition influenced *G. strigosum* intensity while rabbit sex had no significant effect (Table 2).

3.2.3. Virus and dual-nematode co-infection

Rabbits infected with myxoma were usually recorded from July to January, so analyses focussed on this period and the intensity of *T. retortaeformis* or *G. strigosum* was compared between hosts with and without myxoma. The age–intensity profile of *T. retortaeformis* changed dramatically with the viral co-infection; nematode infection was much higher in myxoma-positive compared to myxoma-negative rabbits for both sexes (Table 2, Fig. 4). The lack of a clear type III convex curve for *T. retortaeformis* in myxoma-negative rabbits could be an artefact of combining adults exposed to different rates of infections, from the late breeding season, when the force of infection is high, compared to the non-breeding season, when infection is relatively low. Host age and sex contributed to explain the variance between myxoma-negative and myxoma-positive rabbits (Table 2). No apparent effect of myxoma on *G. strigosum* was observed (Fig. 4). Host age was the only significant variable that explained the changes in nematode intensity (GLM: $P < 0.001$, df = 257); myxoma presence did not contribute.

4. Discussion

We examined the hypothesis that concomitant infections interact by affecting host susceptibility and resistance to parasites and this leads to increased variation in intensities

Table 2
Best generalized linear models (GLM; Poisson error distribution) according to type of infection for *Trichostrongylus retortaeformis* or *Graphidium strigosum* intensity, as the response variable (columns), and host characteristics and breeding period as independent variables (rows)

	Single infection		Dual infection		Single versus dual nematode	Myxoma+ versus Myxoma−
	T. retortaeformis Coefficient, *P*, df	*G. strigosum* Coefficient, *P*, df	*T. retortaeformis* Coefficient, *P*, df	*G. strigosum* Coefficient, *P*, df	*T. retortaeformis* Coefficient, *P*, df	*T. retortaeformis* Coefficient, *P*, df
Age	19.433, 0.001, 731	8.515, 0.001, 441	49.046, 0.001, 658	10.061, 0.001, 658	0.091, 0.001, 718	79.888, 0.011, 255
Sex	76.805, 0.151, 729	1.381, 0.098, 440	−19.145, 0.001, 657	−5.195, 0.102, 656	0.120, 0.001, 719	−291.721, 0.051, 256
Breeding period	−70.302, 0.032, 730	0.804, 0.993, 439	−213.079, 0.317, 656	2.165, 0.001, 657	−1.005, 0.100, 716	
Age * Breeding period	47.475, 0.001, 728	−1.172, 0.042, 438	127.866, 0.001, 655	−2.957, 0.001, 655	0.395, 0.001, 713	
Sex * Breeding period	−135.050, 0.014, 727	1.099, 0.033, 437	−225.278, 0.010, 654		−0.865, 0.001, 714	
Type of infection					1.410, 0.001, 717	
Age * Type of infection					−0.126, 0.041, 715	
Myxoma presence						1761.995, 0.001, 257

The coefficients of the GLM, the significance of the test, *P*, and the degrees of freedom, dfs are shown. A positive sign for the age coefficient means: intensity increases with age; for the sex coefficient: intensity increases from females to males; for the time coefficient: intensity increases from non-breeding to breeding period. * = Second order interaction.

between hosts. We investigated long-term data on parasites in a free-living system and provide evidence to support the predictions that co-infections, mediated by the host's immune response, increased intensity of *T. retortaeformis*, a parasite that appears to be immuno-regulated. This increase in intensity was observed when *G. strigosum* was present, despite no clear evidence of immuno-regulation of this nematode, and was even more pronounced when rabbits showed evidence of infection with the immunosuppressive myxoma virus. Moreover, the regulatory effect of the immune response during a co-infection appeared to occur in addition to the reduced immunity and rise of parasite intensity in breeding females, the periparturient rise. Overall, co-infections acted to increase between-host variability of the nematodes and altered the degree of parasite aggregation. This allowed us to identify the dual infected rabbits in the breeding season as the most highly infected individuals, and those that are likely to be responsible for most of the transmission. Importantly, *G. strigosum* did not show these patterns in co-infected rabbits, supporting the hypothesis that such effects are mainly mediated through the host's immune system. Indeed, host age, sex and breeding status also contributed to the pattern of co-infection, particularly for *T. retortaeformis,* and probably their role was partly mediated by the immune response. We did find variability in *G. strigosum* aggregation and intensity with co-infection but the results suggest that host characteristics, and probably changes in exposure, are more relevant for the dynamics of this parasite (Keymer and Anderson, 1979; Hudson et al., 2006).

A significant part of this study is the evidence that the shape of the age–*T. retortaeformis* intensity curve is generated from the development of an acquired immune response, whereas the age–intensity profile to *G. strigosum* indicates no discernable immune regulation. Previous epidemiological studies have highlighted differences in the immunological reactions to these two nematodes (Michel, 1952a,b; Cattadori et al., 2005, 2007; Cornell et al., unpublished data; Supplementary Figs. S3 and S4). These differences appear to be consistent with the patterns of co-infection currently described but we do not exclude the possibility that other processes operate and may interact to produce these relationships, particularly for *G. strigosum*.

As predicted, the immuno-suppressive effect of myxoma increased the intensity but reduced the aggregation of *T. retortaeformis*, both in female and male rabbits, such that there were more rabbits heavily infected with this nematode that also carried the virus. The lack of the oldest age classes in myxoma co-infected hosts is probably caused by host mortality induced by heavy parasite infections. Indeed, it is possible that highly parasitized hosts may be more susceptible to multiple infections, and the additional infection with the immuno-suppressive myxoma virus may have enhanced host mortality. The presence of *G. strigosum* was associated with increased aggregation of *T. retortaeformis*; by contrast, *G. strigosum* aggregation decreased both in dual-nematode infected rabbits and myxoma-nematode infected hosts without altering the intensity. These findings support a previous analysis which implied a complex interaction between these two nematode species (Lello et al., 2004). Nevertheless, we did not find any clear evidence of immune-regulation of *G. strigosum* but an increase in intensity with host age for every cohort of rabbits born between February and August (Supplementary Fig. S3), confirming the suggestion of little or no density-dependent regulation, or parasite-induced host mortality.

The high level of infection in adult rabbits with myxoma could be explained by the immunosuppressive nature of the virus. We have recently developed an infection-immunity model for the myxoma-*T. retortaeformis* interaction that explicitly quantified the immune regulation of parasite establishment and death/expulsion (Cattadori et al., 2007). Modelling showed that while myxoma can subvert the ability of the host to remove *T. retortaeformis*, some immune-mediated regulation of the nematode has to be included in the model to be consistent with field data, supporting the hypothesis that myxoma does not completely

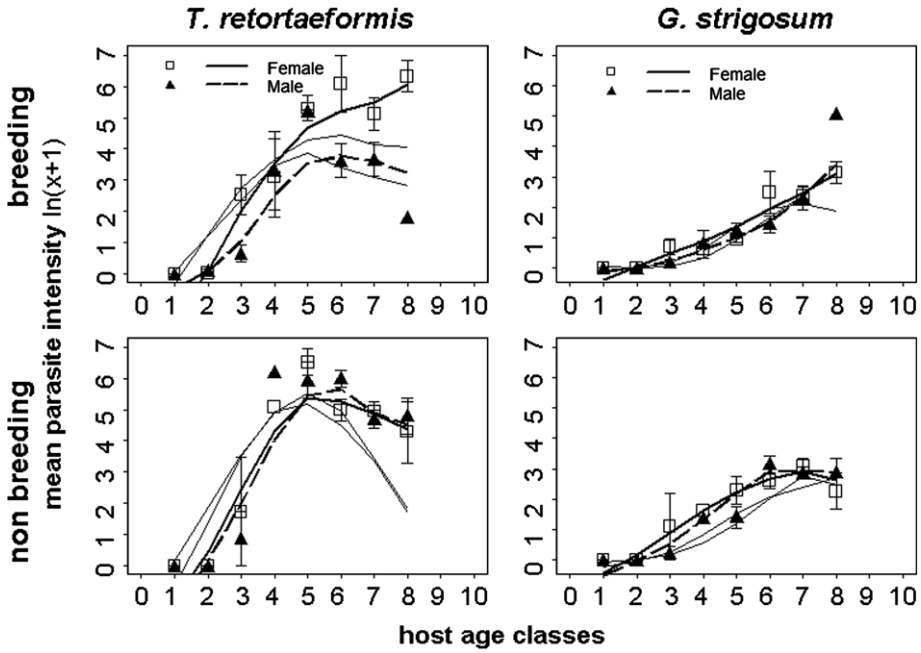

Fig. 3. Host age–parasite intensity profiles for *Trichostrongylus retortaeformis* and *Graphidium strigosum* compared with host sex during and outside the breeding period in dual nematode infected rabbits. Single infection profiles from Fig. 2 are overlaid for comparison (thin lines). Details on the dataset and fitted curves are provided in legend to Fig. 2.

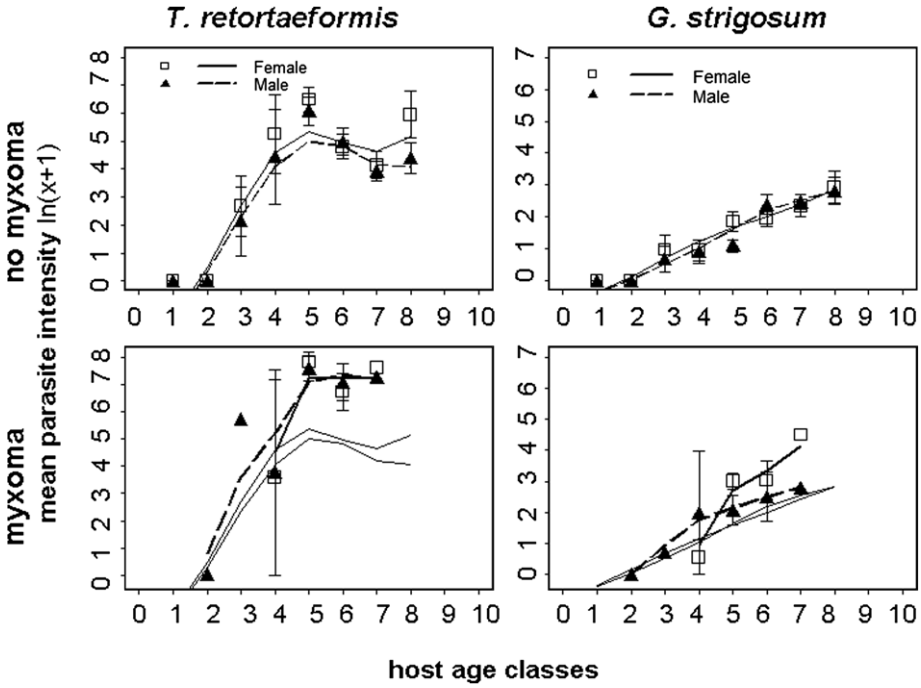

Fig. 4. Host age-parasite intensity profiles for *Trichostrongylus retortaeformis* and *Graphidium strigosum* compared with sex in myxoma-negative and myxoma-nematode co-infected rabbits for the July-January period. The myxoma-negative dual nematode infection profiles are overlaid to provide comparison (thin lines). Details on the dataset and fitted curves are in the legend to Fig. 2.

disrupt the immune response to the nematode. The reason for higher *T. retortaeformis* intensities in older rabbits with the dual nematode infection is not clear. One explanation is that these individuals are the more susceptible hosts in the population and their immune response to *T. retortaeformis* is less effective when *G. strigosum* is present. In fact, the poor host condition may facilitate infection with *G. strigosum*. Another possible explanation could be a positive effect of substances released by *G. strigosum* that directly or indirectly affect *T. retortaeformis*. For example, *G. strig-*

osum may cause alterations in the gastric environment which may facilitate the passage of *T. retortaeformis* to the small intestine. However, we do not have any clear evidence to support these speculations.

We found evidence of enhanced bias in parasitism between sexes in dual infected rabbits compared to single-infected hosts. During reproduction female rabbits, undergo immuno-suppressive hormonal and physiological changes which can affect parasite intensities (Dunsmore, 1971). The increase of gastrointestinal parasitism in breeding females, the 'periparturient rise', has been commonly recorded in the parasites of herbivores such as sheep (Marquardt et al., 2000) and in rabbits (Michel, 1952b; Dunsmore, 1966). In our study, co-infection with *G. strigosum* appeared to have further modified host conditions and amplified the periparturient rise of *T. retortaeformis* in adult females.

Our findings on the dual nematode infection are consistent with previous conclusions of a positive interaction between *G. strigosum* and *T. retortaeformis* but do not support the hypothesis of cross-immunity between these two nematodes (Lello et al., 2004). In fact, *G. strigosum* intensity increased with host age in both sexes and no clear differences were observed between the single and the dual nematode infection. Host age appeared to be the major source of variability for this nematode and this pattern was consistent for female and male rabbits during the breeding and non-breeding periods. Therefore, though we do not exclude some level of immune response to *G. strigosum*, acquired immunity does not seem the main mechanism controlling the intensity of this nematode.

In conclusion, the distinct epidemiological patterns of the two nematodes, which do not directly compete for space, and the strong immune suppressive characteristics of the virus has allowed us to disentangle the relative contribution of host age and sex from the role of immune-mediated parasite interactions in a host population exposed to similar seasonal processes. Host heterogeneities can be generated by changes in host susceptibility and exposure. This study suggests that multi-species infections can cause changes in parasite intensities and consequently are an important source of variability between hosts.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpara.2007.08.004.

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