An experimental field approach to parasitism and immune defence in voles

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SUMMARY

The fox tapeworm *Echinococcus multilocularis* is typically perpetuated in a cycle with red foxes as definitive hosts and various rodent species as intermediate hosts. In this study, foxes were baited with a highly efficient drug against cestodes (praziquantel) in 5 blocks of 1 km^2. Voles, *Arvicola terrestris*, the most abundant intermediate host species, were trapped in the 5 baited blocks and in 5 non-baited control blocks. Baiting the foxes reduced the prevalence of *E. multilocularis* in fox faecal samples in the baited blocks, but voles trapped in the two blocks did not differ in their infection rates. However, voles from the baited blocks had significantly smaller spleen masses and were more likely to be infested with mites than those from the control blocks, possibly reflecting different immunological activities. Our study suggests that the environmental contamination with *E. multilocularis* eggs, and perhaps those of other tapeworms, influences the immune system of the intermediate host species *A. terrestris* in the wild.

Key words: *Arvicola terrestris*, *Echinococcus multilocularis*, urban fox, rodent, infection pressure.

INTRODUCTION

Human Alveolar Echinococcosis, one of the most lethal helminth zoonoses, is caused by infection with the tapeworm *Echinococcus multilocularis* (Amman & Eckert, 1995). Red foxes (*Vulpes vulpes*) are the main definitive hosts of this worm and the source of human infections. Since red fox populations have increased recently in several European countries (Christensen, 1985; Møller-Nielsen, 1990; Breitenmoser *et al*. 2000), their growing populations, especially in urban areas, may increase health risks for urban citizens.

In Europe, *E. multilocularis* is typically perpetuated in a cycle including foxes and various rodent species as intermediate hosts (Eckert & Deplazes, 1999). Adult tapeworms in foxes produce eggs for 1–4 months and these eggs reach the environment in fox faeces (Nonaka *et al*. 1996). When ingested by an intermediate host, the oncosphere hatches in the gut. It penetrates the intestinal mucosa, enters venous or lymphatic vessels and then develops predominantly in the liver, with subsequent metastasis formation in other sites (see Schantz & Gottstein, 1986). In the intermediate host, production of protoscoleces takes place within 20–35 days, and infective protoscoleces can be found 40–60 days after infection (Eckert, 1998). A definitive host must ingest metacestodes which contain infective protoscoleces to complete the cycle.

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The most important intermediate hosts of *E. multilocularis* in Europe are *Microtus arvalis* and *Arvicola terrestris* (Eckert *et al*. 2001*a*). Although foxes prefer small microtine rodents as prey, they switch diet when *A. terrestris* are highly abundant (Weber & Aubry, 1993). Recent analyses of urban foxes in Zürich found that *A. terrestris* was the most frequent potential intermediate host (Contesse *et al*. 2004). Little is known about parasite prevalences in intermediate hosts, although compared to prevalences in foxes (20–60%), they appear to be low (1–6%) (Houin *et al*. 1982; Pétavy & Deblock, 1983; Eckert *et al*. 2001*b*). However, a study in Zürich found prevalences of up to 20% in *A. terrestris* and in urban foxes during winter, rates as high as 47% in the urban and 67% in adjacent recreational areas (Hofer *et al*. 2000).

The prevalence of *E. multilocularis* in wild foxes can be reduced by regular distribution of baits containing praziquantel, a highly efficient drug against adult cestodes (Schelling & Frank, 1997; Tackman *et al*. 2001). Recently, we have demonstrated that the *E. multilocularis* egg contamination in urban areas can be reduced to very low levels by the manual distribution of anthelmintic baits at monthly intervals. This is even possible within small defined urban patches situated in a highly endemic area (Hegglin *et al*. 2003). However, little attention has been paid to the role of the intermediate hosts in transmission dynamics, although several studies have indicated its importance (Giradoux *et al*. 1997; Eckert *et al*. 2001*a*).

Studies on the natural life-cycle of the parasite *E. multilocularis* and its transmission dynamics are rare.

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In the framework of an ongoing experimental study (Hegglin *et al*. 2003) foxes were baited with praziquantel in 5 blocks of 1 km² and voles from these blocks were compared with voles from 5 non-baited control blocks. If baiting successfully reduced parasite prevalence in foxes, 2 different environmental treatments were created in which the voles were exposed to different infection risks with *E. multilocularis*, and possibly other tapeworms. We investigated immunological activity by measuring spleen mass. The mammalian spleen is an important component of the immune system; it helps the body to resist parasites (Kopp, 1990), it induces immune responses (Steininger & Barth, 1999) and an increase in spleen mass occurs in response to infections and passive immunizations (John, 1995; Skarstein, Folstrad & Liljedal, 2001). In early *E. multilocularis* infections in the intermediate host, the suppression of larval growth is critical for the final outcome of the disease (Gottstein, 1992), and infected laboratory rodents show marked activation of cell-mediated immunity (e.g. Ali-Khan, 1978*b*; Fotiadis *et al*. 1999). During parasite growth, there is a decline in peritoneal lymphocyte, monocyte and eosinophil cell numbers and an increase in spleen mass (Ali-Khan, 1974, 1978*a*; Devouge & Ali-Khan, 1983). We also measured the prevalence of ectoparasitic mites, enabling us to examine relationships between infections with different parasites.

MATERIALS AND METHODS

Study site

The city of Zürich is surrounded by forests and agricultural land. There is a high density of 11·2 foxes per km² and the urban transmission cycle of the parasite *E. multilocularis* occurs predominantly in areas adjacent to the city (Deplazes *et al*. 2002; Gloor, 2002; Stieger *et al*. 2002). We therefore selected 5 areas near Zürich and marked out within each of these areas 2 blocks of 1 km² (Fig. 1). One was an experimental block, where baits containing praziquantel were distributed monthly, and the other was a control block. Blocks were allocated to a treatment without prior knowledge. They were arranged so that control and baited blocks alternated geographically. This ensured there was no possibility that baited or control blocks were clumped together; i.e. blocks within a treatment would not have shared a particular infection history (Fig. 1). In particular, we stress that although voles in our study may have been already infected, this would be equally likely for a vole exposed to either of our treatments. We used commercial baits (Impfstoffwerk Dessau Tornau GmbH, Rosslau, Germany), each weighed 13·5 g, and the matrix consisted of Altrofox 91 (Impfstoffwerk Dessau Tornau GmbH). This matrix is the same as in the widely used rabies vaccine bait Rabifox

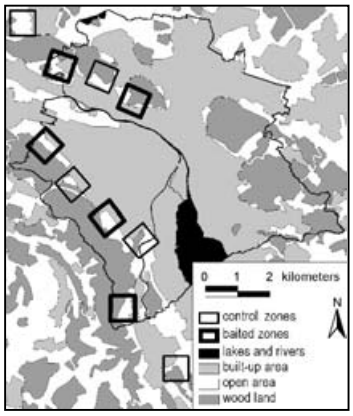

Fig. 1. Study site: City of Zurich and adjacent area.

(Impfstoffwerk Dessau Tornau GmbH). The baits contained 50 mg of the anthelmintic praziquantel (Droncit Bayer AG, Leverkusen, Germany), a highly efficient drug against adult cestodes.

There was a distance of at least 750 meters between an experimental block and a control block, to minimize the chance of individual foxes using 2 study blocks. The blocks all shared similar portions of urban area (1/3) and agricultural landscape and woodland (2/3) in a pattern typical for the area.

Anthelmintic treatment of foxes

Preliminary experiments confirmed the good acceptance of bait items (Hegglin *et al*. 2004). From April 2000, pellets were distributed monthly in the baited blocks at doses of 50 baits per km². They were distributed where they were likely to be found by foxes, for example near compost heaps, on a path frequently used by foxes, on a vole tumulus or close to dens (for details see Hegglin *et al*. 2003).

Faecal samples were collected at least monthly in all blocks. A method of identifying fox faeces was established. Several criteria, such as size, shape and smell of the dung, were used to distinguish fox faeces from dog faeces (Stieger *et al*. 2002). Only faecal samples collected within a radius of 750 metres from the centre of the study blocks were analysed. This maximized the likelihood that only faeces of territorial foxes living within the blocks were used. *E. multilocularis* was detected by screening samples with a coproantigen ELISA (Em-ELISA) (Deplazes *et al*. 1999).

Arvicola terrestris

The *A. terrestris* trap sites were located mainly on grassland. In every sampling block, one or two grassland patches that showed the highest relative densities of voles were selected (see Giradoux *et al*. 1995). From May to October 2000, four trapping sessions were conducted on every trap site at around 6-week intervals. Between 20 and 30 traps were set

inside vole galleries per trapping session. We used unbaited Topcat traps (Topcat GmbH, Wintersingen, Switzerland). A trapping session was ended if 10 voles were caught. Voles were collected after death and stored individually at −20 °C. Body weight to the nearest 0·01 g and body length to the nearest 1 mm, from the tip of the nose to the first vertebra of the tail, were recorded. The presence of ectoparasitic mites (*Laelaps* species) was recorded by carefully combing the coat against the grain and examining the plastic bag the vole was stored in. At necropsy, the spleen was removed, cleaned of other tissue and weighed to the nearest 1 mg. In females, the presence and number of embryos and placental scars was recorded and it was noted whether they were lactating. The liver in particular, but also other organs such as lung, kidney, spleen and reproductive and intestinal tracts, were examined for lesions or irregularities. Metacestodes of *E. multilocularis* were identified morphologically and by using modified PCR (Dinkel *et al*. 1998). Identification of lesions caused by other cestodes was based on gross morphology and by comparing hook morphology and length.

Statistics

Data were analysed using ANCOVA and logistic regression (SPSS 10 PC-Version, 1999). To evaluate the infection rate of foxes in baited and control zones, we pooled the data of faecal samples collected in the two periods from April to June 2000 and from July to October 2000. The small number of faecal samples collected in some months prevented analysis of shorter time-intervals. To evaluate parasite prevalence in voles trapped in the two treatments, we pooled the data from May to June 2000 and from July to October 2000. To reveal effects on a finer timescale, we used 2-month intervals (May and June, July and August, September and October) to carry out further analyses with the vole data. Spleen mass was log transformed. Body length was used as a covariate. Since body length influenced spleen mass significantly, we removed the effects of body length by taking the residuals of a linear regression of transformed spleen mass on body length.

RESULTS

Prevalence of E. multilocularis *in fox faecal samples*

Logistic regression with the factors BAIT (treatment of the foxes), AREA (5 different areas that each included a baited and a control block) and 2 time-periods, SEASON (April to June and July to October) was used to investigate whether baiting the foxes reduced the portion of coproantigen-positive faeces. The proportion decreased during the year (April–June 28·9 ± 5·2% *vs*. July–October 14·2 ± 2·5%) and was lower in baited zones (baited 14·0 ± 0·26% *vs*. control 26·3 ± 4·5%) (AREA, Wald $\chi^2 = 6{\cdot}2$, 4 D.F., $P = 0{\cdot}186$; BAIT, Wald $\chi^2 = 4{\cdot}5$, 1 D.F., $P = 0{\cdot}035$; SEASON, Wald $\chi^2 = 7{\cdot}9$, 1 D.F., $P = 0{\cdot}005$; SEASON BY BAIT, Wald $\chi^2 = 3{\cdot}0$, 1 D.F., $P = 0{\cdot}084$). Although the interaction season by bait was not significant, it was on the borderline. However, our data only represent the early stages of the developing impact of baiting the fox population. The interaction was later strongly significant in a larger study (Hegglin *et al*. 2003).

Arvicola terrestris

Of 534 voles, 213 were male and 321 female. Of the females, 95 (29·6%) showed signs of reproduction. Male voles are either larger than, or the same size as, females (Heske & Ostfield, 1990). The sexually active females caught all weighed more than 60 g, and their body lengths exceeded 120 mm. Animals below both these thresholds (71, 13·1%) were considered juvenile and omitted from the analysis. Of 469 adult voles, we detected liver lesions in 157 (33·5%) and *E. multilocularis* infections in 41 (8·7%). In 17 of the infected animals, we found 14 to 244 400 protoscoleces; this means that 3·6% of the adult voles were actually infective when captured. Most of the lesions not caused by *E. multilocularis* were metacestodes of *Taenia taeniaeformis* (*Strobilocercus fascioloaris*) (60, 12·8%). Only 5 (1·06%) animals were infected with both *E. multilocularis* and *T. taeniaeformis*. Metacestodes of *T. crassiceps* were detected in 4 (0·8%) voles, and of these 2 (0·4%) were simultaneously infected with *T. taeniaeformis*. Two *T. crassiceps* metacestodes were in subcutaneous cysts, once in the pleural cavity and once in the pericardium. Two lesions (0·4%) were caused by unidentified *Taenia*. In 50 (10·6%) voles, the cause of the lesions which were PCR negative for *E. multilocularis* could not be determined.

We found *E. multilocularis* infected voles in all but 1 of the 10 blocks. Prevalence varied from 3·3 to 16·2% per block. A binary logistic regression with the main factors (BAIT), (AREA), vole sex (SEX), the dichotomous variable (MITE), whether a vole was infected with mites, the three 2-month periods (TIME PERIOD) and, as covariates, body length and transformed spleen mass, revealed no significant effects on *E. multilocularis* prevalence in voles (all $P > 0{\cdot}05$; overall prevalence 8·74 ± 1·31%).

ANCOVA was used to investigate the influence of the factors: treatment of the foxes (BAIT), vole sex (SEX), the dichotomous variables (INFECTION) and (MITE) and the three 2-month periods (TIME PERIOD) on spleen mass. Body length was entered as a covariate. The analysis also included (AREA), to remove the variance in spleen size due to variation amongst the areas. All 4-way and 3-way interactions were non-significant, and were therefore removed from the final model, as were all non-significant

Table 1. Analysis of covariance of log(spleen mass) (overall adjusted $R^2 = 0{\cdot}160$)

Source of variation	Type III Sum of squares	D.F.	Mean square	*F*	Significance
BODY LENGTH (covariate)	0·35	1	0·35	11·50	0·001
AREA	0·98	4	0·25	8·07	<0·001
BAIT	0·24	1	0·24	8·02	0·005
TIME PERIOD	0·08	2	0·04	1·33	0·265
MITE	<0·001	1	<0·001	0·01	0·926
SEX	<0·001	1	<0·001	0·15	0·703
INFECTION	0·08	1	0·08	2·65	0·105
BAIT * TIME PERIOD	0·51	2	0·25	8·31	<0·001
Error	9·98	328	0·03		

2-way interactions. Spleen mass was positively related to body length ($\beta = 0{\cdot}176$) and varied between areas. Spleen mass decreased with time in the baited blocks only (Table 1; Fig. 2). Furthermore, infected voles had slightly, albeit not significantly, heavier spleens than uninfected voles (residual mass $0{\cdot}069 \pm 0{\cdot}040$ *vs.* $-0{\cdot}007 \pm 0{\cdot}010$), but infection stage (whether protoscoleces were found) had no effect.

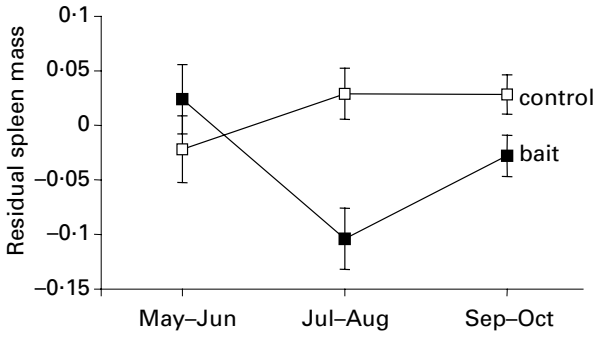

Fig. 2. Mean relative spleen mass ± S.E. of voles at 3 different time-periods ($n = 342$).

Ectoparasitic mites

For the frequency of mite infestation, there were no significant 4-way or 3-way interactions. These were therefore removed from the final model, as were all non-significant 2-way interactions. Infestation was affected significantly by the interaction of BAIT and TIME PERIOD (logistic regression, $\chi^2 = 6{\cdot}09$, 1 D.F., $P = 0{\cdot}048$); other effects SEX ($\chi^2 = 4{\cdot}61$, 1 D.F., $P = 0{\cdot}032$), BAIT ($\chi^2 = 5{\cdot}17$, 1 D.F., $P = 0{\cdot}023$), TIME PERIOD ($\chi^2 = 40{\cdot}95$, 2 D.F., $P < 0{\cdot}001$), INFECTION ($\chi^2 = 2{\cdot}40$, 1 D.F., $P = 0{\cdot}12$), AREA ($\chi^2 = 8{\cdot}82$, 4 D.F., $P = 0{\cdot}066$). In the later two time-periods, voles from baited blocks were more likely to be infested than those from control blocks (May–June $44{\cdot}4 \pm 8{\cdot}4\%$ *vs.* $41{\cdot}9 \pm 7{\cdot}6\%$; July–August $40{\cdot}0 \pm 9{\cdot}1\%$ *vs.* $14{\cdot}8 \pm 4{\cdot}58\%$; September–October $74{\cdot}2 \pm 5{\cdot}42\%$ *vs.* $62{\cdot}3 \pm 4{\cdot}73\%$ respectively). Males were more likely to be infested than females ($58{\cdot}3 \pm 4{\cdot}2\%$ *vs.* $43{\cdot}8 \pm 3{\cdot}5\%$ respectively). Infection with *E. multilocularis* also slightly decreased the frequency of mite infestation (infected $30{\cdot}3 \pm 8{\cdot}1\%$ *vs.* non-infected $49{\cdot}1 \pm 2{\cdot}7\%$).

DISCUSSION

The proportion of fox faecal samples infected with *E. multilocularis* collected in blocks baited with praziquantel was lower than in control blocks (see also Hegglin *et al.* 2003). As a consequence it would be expected that fewer eggs of this and presumably of other tapeworms were deposited in baited blocks than in control blocks. Since egg counts in faecal samples of foxes declined in baited blocks, it is likely that the environmental contamination with eggs in these blocks also declined and hence voles in baited zones presumably had a lower contact rate with infective eggs. Although there was no reduction in the infection rate of voles, spleen mass was significantly reduced over time in baited blocks. This was not the case in non-baited control blocks. The frequency with which voles were infested by mites was higher in baited blocks. Although we have no data from before our experiment, these results are most likely due to the treatment. Block allocation was random to prevent any geographical clumping of the blocks of a treatment. It is therefore extremely unlikely that there was any systematic difference between the voles in our treatment groups.

E. multilocularis infection rates in voles are generally low; in geographically extensive studies they are usually below 1% (Eckert, 2001*b*). We found prevalences of 3·3 to 16·2% in all but 1 study block, indicating that the study was performed in a highly endemic area. This confirms the existence of locally highly endemic foci (Pétavy & Deblock, 1983; Gottstein *et al.* 1996; Hofer *et al.* 2000). Since infected voles survive for several months, some of the voles trapped were probably infected before or shortly after the beginning of baiting. Parasite eggs may survive for up to 8 months in autumn and winter

and for up to 3 months in summer (Veit *et al*. 1995). Therefore, even when final hosts are free of infection, the parasite may survive within a treated area because the voles still come into contact with infective eggs and thus represent sources of new infections.

The average spleen mass of voles in the baited blocks decreased over time, but this did not occur in the control blocks. An increase in spleen mass during infection with *E. multilocularis*, reflecting increased immune defence, has been reported previously (e.g. Ali-Khan, 1978*a*, *b*; Playford & Kamiya, 1992; Fotiadis *et al*. 1999). Not every contact with *E. multilocularis* leads to infection with the parasite. In early stages of infection the successful establishment of the parasite depends upon the competition between the larval development and the establishment of a protective host immune response (Gottstein, 1992). During this stage of an *E. multilocularis* infection, a marked activation of the cell-mediated immunity against the parasite occurs (Ali-Khan, 1978*b*; Playford & Kamiya, 1992; Fotiadis *et al*. 1999). *E. multilocularis* growth appears to be controlled by the host T-cell, and, at least in the early phase of the disease, T-lymphocytes probably play the most important role in the immune response to the parasite (Baron & Tanner, 1976; Kamiya *et al*. 1980; Playford & Kamiya, 1992). The spleen plays an important role in cell-mediated immunity as the storage organ of mature cytotoxic T-cells (Kopp, 1990). In contrast to voles in baited blocks, where the risk of infection seemed to decrease over the season, voles in control blocks had higher spleen mass probably because they were exposed to higher levels of contamination with eggs and because they would have had continuously to maintain higher levels of cell-mediated immunity to combat infection.

We found different frequencies of voles infested with mites in the two treatments. Fewer voles were infested with mites in control blocks in the second and third time-periods; there was no difference in the first period. Voles infected with *E. multilocularis* also tended to have fewer mites. Since cell-mediated immunity and T-cells play an important role in the immune defence against mites (O'brien *et al*. 1996), this supports the hypothesis that exposure to one parasite (*E. multilocularis*) could lead to an activated, general cell-mediated immunity, and consequently to an increased immune response to another parasite (mites). Elevated cell-mediated immunity and the resulting production of T-cells following infections with *E. multilocularis* (Ali-Khan, 1978*b*; Playford & Kamiya, 1992; Fotiadis *et al*. 1999) may thus simultaneously increase immune defence against several challenges. Furthermore, more males harboured mites than females. Gender differences in natural parasitic infections are frequently observed in vertebrates (reviewed by Zuk & McKean, 1996). These differences are usually attributed to ecological (i.e. different exposure to the parasites) or physiological (i.e. hormonal) differences between the sexes (Zuk & McKean, 1996; Travi *et al*. 2002).

This experimental study strongly suggests that the environmental contamination with eggs of *E. multilocularis*, and possibly other tapeworms, influences the immune system in the intermediate host *Arvicola terrestris*. Further studies are required to understand the longer term consequences of anthelmintic baiting in both the final and the intermediate host(s) of this cestode parasite.

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