The Journal of Experimental Biology 207, 2725-2733
Published by The Company of Biologists 2004
doi:10.1242/jeb.01090

Immune response to fleas in a wild desert rodent: effect of parasite species, parasite burden, sex of host and host parasitological experience

Irina S. Khokhlova[1], Marina Spinu[2], Boris R. Krasnov[3,*] and A. Allan Degen[1]

[1]*Desert Animal Adaptations and Husbandry, Wyler Department of Dryland Agriculture, Jacob Blaustein Institute for Desert Research, Ben-Gurion University of the Negev, Beer Sheva 84105, Israel,* [2]*University of Agricultural Sciences and Veterinary Medicine, Manastur str. 3-5, Cluj-Napoca, Romania* and [3]*Ramon Science Center and Mitrani Department of Desert Ecology, Jacob Blaustein Institute for Desert Research, Ben-Gurion University of the Negev, PO Box 194, Mizpe Ramon 80600, Israel*

*Author for correspondence (e-mail: krasnov@bgu.ac.il)

Accepted 18 May 2004

Summary

We studied immune responses of the jird *Meriones crassus* to different flea species belonging to the same family. We used jirds maintained in an outdoor enclosure (enclosure; *N*=18) and parasitized by fleas *Xenopsylla conformis mycerini* and *Xenopsylla ramesis,* and also jirds born in the laboratory to previously parasitized mothers (laboratory animals; *N*=23). We asked (i) whether cross-immunity to different fleas occurs, (ii) whether there is a sex difference in immune responses to flea parasitism and (iii) whether the severity of the immune responses depends on parasite load. In the enclosure animals, immune response to antigen from the unfamiliar flea *Synosternus cleopatrae pyramidis* did not differ from those to antigens from the familiar fleas. In contrast, laboratory rodents demonstrated no difference in the immune response between *S. c. pyramidis* antigen and either the phytohemagglutinin treatment or controls, although their responses to antigens of fleas familiar to their mothers (*X. c. mycerini* and *X. ramesis*) were significantly higher than those to antigen of *S. c. pyramidis* and phytohemagglutinin. The results clearly demonstrated that (i) cross-reactivity in rodent responses to different flea species occurred for enclosure but not for laboratory jirds and (ii) immune-naïve animals whose mothers were parasitized by fleas had some degree of immunity against fleas. The only sex difference in immunological parameters was the higher level of circulating immune complexes in females than in males. Only phagocytic activity was affected by flea burden, decreasing with an increase in flea numbers.

Key words: rodent, *Meriones crassus*, flea, immune response, cross-immunity, maternal transfer, sex difference.

Introduction

Ectoparasitic arthropods are often thought of as crawling or flying hypodermic needles that suck blood and inject disease-causing agents. However, saliva of blood-feeding arthropods contains factors that help them evade host haemostatic defenses (Ribeiro, 1995; Wikel, 1996) and also has potent immunogens that influence the immune responses of the host (e.g. Roehrig et al., 1992). Ectoparasite arthropods downregulate host innate and specific acquired immune defenses, inducing host responses that impair their own ability to feed (Rechav et al., 1989). For example, a study of acquired resistance in guinea pigs to tick larvae showed that repeated infestation of the host resulted in a sharp reduction in body mass of engorged larvae (Fielden et al., 1992). This resistance is due to stimulation by ectoparasites of host immunoregulatory and effector responses, which involve antigen-presenting cells, T lymphocytes, B lymphocytes, antibodies, complement, mast cells, circulating granulocytes and cytokines (Jones, 1996). The development of an immune response to ectoparasitic arthropods such as ticks, mites, chiggers, fleas, mosquitoes and lice is well documented (Ribeiro, 1987, 1995; Rechav, 1992; Wikel et al., 1996; Wikel and Alarcon-Chaidez, 2001); however, most studies of immune responses to ectoparasites have involved livestock or laboratory animals; relatively little is known about immune responses to ectoparasites in wild animals.

Haematophagy evolved independently in different taxa of arthropods and, thus, it is commonly accepted that chemical mediators contained in their saliva are different (Ribeiro, 1995; Jones, 1996). However, salivary anticlotting, antiplatelet and vasodilatory substances can be quite similar within a parasite taxon (e.g. genus and family) (Mans et al., 2002; but see Warburg et al., 1994). This within-taxon similarity can lead to cross-resistance (= heterospecific resistance) of a host against closely related parasites. For example, cross-resistance to closely related ticks has been repeatedly reported (McTier et al., 1981; Njau and Nyindo, 1987; but see Rechav et al., 1989).

Fleas (Siphonaptera) are parasites of higher vertebrates, being most abundant and diverse on small mammals. Mammals vary in their flea species richness. For example, among 12 rodent species in the Negev desert, the number of flea species per rodent species ranged between two and eight (Krasnov et al., 1997). Host-dependent and habitat-dependent fleas were distinguished. For example, *Parapulex chephrenis* parasitized spiny mice (*Acomys cahirinus* and *Acomys russatus*) almost exclusively, independent of habitat, whereas *Synosternus cleopatrae pyramidis* was found only on sand-dwelling rodents, independent of species affinities (Krasnov et al., 1999).

Fleas are considered to be monophyletic (Traub, 1980; Smit, 1982; Whiting et al., 1997). Given the common origin and opportunistic feeding of many flea species (Marshall, 1981), cross-resistance of hosts to different species of fleas can be expected. This can be especially true for those hosts that demonstrate high richness of natural flea assemblages and/or those fleas that have a broad range of natural hosts. However, immune responses against fleas are poorly known (Jones, 1996). No datum supporting or rejecting an existence of immune cross-reaction of a host against different flea species is available except the note by Studdert and Arundel (1988) on a severe allergic reaction in cats that hunted rabbits infested with the rabbit flea *Spillopsyllus cuniculi*. The severity of these symptoms indicated that cats had a much higher response to rabbit fleas than to the cat flea *Ctenocephalides felis*, with which they were normally infested. Furthermore, differential immune responses of rodents to natural and unnatural flea species have been reported (e.g. Vaughan et al., 1989).

We studied immune responses of Sundevall's jird *Meriones crassus* to different flea species belonging to the same family (Pulicidae) (*Xenopsylla conformis mycerini*, *Xenopsylla ramesis* and *Synosternus cleopatrae pyramidis*). These fleas have different habitat preferences. *M. crassus* is parasitized either by *Xenopsylla* species, which co-occur together in some areas or by *S. c. pyramidis*, which has a distinct geographic distribution from *Xenopsylla* species (Krasnov et al., 1999). We studied rodents that originated from areas inhabited by *X. c. mycerini* and *X. ramesis* and predicted that if cross-immunity to different fleas occurs, immune responses of a rodent to a flea species that previously parasitized the rodent (familiar flea, both *Xenopsylla*) will be similar to responses to other (unfamiliar, *S. c. pyramidis*) flea species. If, however, cross-immunity does not exist, immune responses to a familiar flea species will differ from those to unfamiliar flea species.

In addition, we asked whether there is a sex difference in immune responses to flea parasitism. Sex differences in immunocompetence and susceptibility to parasites were reported for a variety of mammals, with males being generally less immunocompetent and more susceptible to parasites than females (e.g. Olsen and Kovacs, 1996; Poulin, 1996; Schalk and Forbes, 1997).

If a host immune response is an efficient tool to overcome macroparasite pressure, an increased response with an increase in parasite load can be expected (de Lope et al., 1998). However, the cost of using the immune defense system is presumably high and there are numerous trade-offs between immune defense and other concurrent needs of an organism (Sheldon and Verhulst, 1996; Schmid-Hempel and Egert, 2003). Therefore, a relatively low response of a host can be expected both when (i) parasite pressure is low and (ii) the cost of eliminating parasites is higher than the cost of limiting its pressure to a 'tolerable' level (Combes, 2001). Consequently, responses of the host are expected to peak at intermediate levels of parasite load and, thus, the curve describing the relationship between parasite load and host response level is expected to be hump-shaped. To test this prediction we studied immune responses in *M. crassus* parasitized by different numbers of fleas.

Furthermore, it is known that parasite circulating antigens, immunoglobulins, immune cells, cytokines and other cell-related products can be transferred from mammalian females during pregnancy and/or lactation to their young (Carlier and Truyens, 1995). This can induce a long-term modulation of the offspring's capacity to mount immune responses to subsequent exposure to parasites (Carlier and Truyens, 1995). Maternal transfer of immunity has been suggested for a number of protozoans and helminthes (e.g. Heckmann et al., 1967; Shubber et al., 1981; Kristan, 2002). However, we are unaware of any study on maternal transfer of immunity against ectoparasites. We did not measure directly the maternal transfer of immunity in our experiments in terms of immune parameters of placental–fetal circulation or milk. Nevertheless, the occurrence of maternal transfer of immunity could be inferred from the comparison between immune responses of rodents that were previously parasitized by fleas and those of rodents that were never parasitized but whose dams were parasitized by fleas.

Materials and methods

Rodents

Meriones crassus Sundevall is a common rodent species of southern Israel. It occupies a variety of habitats and is parasitized naturally by several flea species (Krasnov et al., 1996, 1997; Table 1). We used rodents from our laboratory colonies. Progenitors of the colonies were captured at the Ramon erosion cirque, Negev Highlands, Israel (30°35′N, 34°45′E) in 1996. In this area, *M. crassus* are permanently infested with *X. c. mycerini* and/or *X. ramesis* (prevalence of infestation about 90–100%; Krasnov et al., 1997), but never infested with *S. c. pyramidis*. Animals were maintained either in an outdoor covered circular enclosure (5 m diameter and 1.5 m height) or in an animal room.

The enclosure, established in 2000, was built of wire mesh (1 cm×1 cm) and contained 60 cm layer of natural sandy-gravel substrate, which allowed rodents to burrow. In addition, 20 wooden nest boxes with dried grass (bedding material) were placed in the enclosure. However, jirds clearly preferred to stay in burrows rather than in the nest boxes. Millet seed and alfalfa (*Medicago* sp.) leaves were provided daily *ad libitum*. The

Table 1. *Flea assemblages of* M. crassus *in different habitats in southern Israel*

Habitat type	Flea assemblages
Sand dunes of the northern Negev desert	*Synosternus cleopatrae pyramidis, Nosopsyllus iranus theodori*
Sand dunes of the central Negev desert	*Xenopsylla conformis mycerini, Xenopsylla dipodilli, N. i. theodori*
Sandy-gravel plains of the central Negev desert	*X. c. mycerini, X. dipodilli, Coptopsylla africana, N. i. theodori*
Loess dry riverbeds of the central Negev desert	*X. c. mycerini, Xenopsylla ramesis, X. dipodilli, C. africana, Stenoponia tripectinata medialis, N. i. theodori*
Loess valleys of the central Negev desert	*Xenopsylla ramesis, X. dipodilli, S. t. medialis, N. i. theodori, Rhadinopsylla masculana*

Based on data of Krasnov et al. (1997, 1998, 1999).

enclosure population was started with ten rodents (five males and five females), two of which were infested with 20 *X. c. mycerini* each and two with 20 *X. ramesis* each. At the time of experiments, there were 80 animals in the enclosure. Flea burden in the enclosure was monitored monthly on 20 randomly selected individuals during a year prior to experiments. Within flea sampling period, intensity of infestation differed among individuals (range 2–50 for *X. c. mycerini* and 2–43 for *X. ramesis*). However, intensity of infestation of the same individual over time was relatively stable (±15% on average). At the time of experiments, both flea species occurred on all rodents and flea burden averaged 15.1 *X. c. mycerini* and 10.8 *X. ramesis*, which was 30% higher than the average flea burden in the field (Krasnov et al., 1998). Nonetheless, rodents reproduced successfully and did not lose body mass. The area is characterized by hot, dry summers (mean daily air temperature of July is 34°C) and relatively cold winters (mean daily temperature of January is 12.5°C) with 100 mm of annual rainfall, all occurring in winter.

Eight pregnant females from the enclosure were captured, freed from fleas (5–20 *X. c. mycerini* and 6–18 *X. ramesis*) and transferred to the animal room. At the moment of capture, females were on the second half of pregnancy (as indicated by body mass, body shape and occurrence of blood in vaginal smears; I. S. Khokhlova, unpublished observations). Rodents were housed individually in plastic cages (60 cm×50 cm×40 cm) at 25°C with a photoperiod of 12 h:12 h (L:D). They were fed millet seeds and alfalfa *ad libitum*. Dried grass was provided as bedding material. Newly born rodents were transferred to individual cages 90 days *post partum*. These animals were never subjected to flea parasitism. Measurements were done when jirds were 5–7 months old.

Immunological studies were done on 18 jirds (8 males and 10 females) from the enclosure (hereafter referred to as enclosure animals) and on 23 never-parasitized jirds (8 males and 15 females) born in the laboratory (hereafter referred to as laboratory animals) from previously parasitized mothers (eight litters, 2–4 pups per litter). All enclosure animals were 6–11 months old. It was unknown, however, whether some of these animals were from the same litter. The intensity of infestation of these jirds by *X. c. mycerini* and *X. ramesis* ranged between 4 and 17 and 5 and 22, respectively. No other ectoparasites were found on the enclosure jirds, whereas the occurrence of endoparasites was not monitored.

Fleas

X. c. mycerini, *X. ramesis* and *S. c. pyramidis* belong to the subfamily Xenopsyllinae of family Pulicidae, although no datum on genetic distances between these fleas or about their common ancestor is available. *X. c. mycerini* and *X. ramesis* are common ectoparasites of gerbils and jirds throughout the Middle East and occur mainly in sandy-gravel and loess habitats. We recorded *X. c. mycerini* mainly on *M. crassus*, *G. dasyurus*, *Gerbillus henleyi*, whereas *X. ramesis* was found on *M. crassus*, *G. dasyurus*, *Psammomys obesus*, and *Eliomys melanurus* (Krasnov et al., 1997, 1999). *S. c. pyramidis* is widely distributed in sand massifs of Israel and is characteristic for sand-dwelling rodents. We recorded this flea on *Gerbillus andersoni allenbyi*, *Gerbillus pyramidum*, *M. crassus* and *Meriones sacramenti* in sand dunes of northern Negev (Krasnov et al., 1999) as well as on *Gerbillus nanus* and *Gerbillus gerbillus* in sandy habitats of the Arava valley (B. R. Krasnov and N. V. Burdelova, unpublished observations). It should be noted that progenitors of our *M. crassus* colony originated from non-sandy area where *S. c. pyramidis* was never found.

Fleas were obtained from our laboratory colonies started in 1998–2001 from field-collected specimens on *M. crassus* (*X. c. mycerini* and *X. ramesis*) and *G. a. allenbyi* (*S. c. pyramidis*) using rearing procedures similar to those described by Metzger and Rust (1997). An individual rodent host was placed in a cage (60 cm×50 cm×40 cm) that contained a steel nest box with a screen floor and a pan containing a mixture of sand and dried bovine blood (nutrient medium for larvae). Gravid female fleas left the host and deposited eggs in the substrate and bedding material in the nest box. Every 2 weeks, all substrate and bedding material were collected from the nest box and transferred into an incubator, where flea development and emergence took place at 25°C and 75% relative humidity. The newly emerged fleas were placed on clean animals. Colonies of fleas were maintained at 25°C and 75% RH with a photoperiod of 12 h:12 h (L:D).

Procedures

Whole body extracts of fleas

We prepared whole body extracts from newly emerged fleas

that did not feed after emergence. Fleas were collected and then frozen at –20°C. Mean body masses of newly emerged individuals *X. c. mycerini*, *X. ramesis* and *S. c. pyramidis* were 0.17, 0.16 and 0.20 mg, respectively. Frozen fleas (100 individuals) were stirred in a mortar, mixed with a small volume of phosphate-buffered saline (PBS) and filtered through plane filter paper to remove remnants of chitin. Then, the extracts were centrifuged for 10 min at 3410 ***g*** (CN-2060 microprocessor control centrifuge, Hsiangtai Machinery Industry Co. Ltd, Taiwan) and the pellets of antigen were resuspended in PBS and freeze-dried (Department of Microbiology and Immunology, Faculty of Health Science, Ben-Gurion University of the Negev, Israel). Before use, the antigen was diluted with PBS to about 50% of the initial mass of the processed fleas and sterilized by filtering through 0.2 μm filters (Schleicher & Schuell, Inc., Dassel, Germany).

Blood samples

Heparinized (50 i.u. ml^{-1}) blood samples (150 μl) from rodents were collected from the infraorbital sinus of a rodent using Pasteur pipettes. We did not anesthesize the animals before sampling because this had a negative effect on both the blood (inducing haemolysis) and the recovery duration of the animals (I. S. Khokhlova and M. Spinu, unpublished observations). Blood from each animal was sampled once weekly with each jird sampled either 2, 3 (two animals) or 4 (one animal) times. Of this blood, two samples from each animal were used for measurements (one for leucocyte blast transformation test and carbon particle inclusion test and one for white blood cell count and serum preparation). The third and/or the fourth blood samplings were used when previous samples were occasionally damaged. As far as we discerned, rodents recovered fully 1–2 min after blood sampling. The experimental design was found to be suitable and to meet requirements of the 1994 Law for the Prevention of Cruelty to Animals (Experiments on Animals) of the State of Israel by Ben-Gurion University Committee for the Ethical Care and Use Animals in Experiments (License IL-27-9-2003).

Haematological and immunological tests

White blood cell count

Part of the heparinized blood was diluted 1:10 with Türk solution, kept at room temperature for 3 min and then leukocytes were counted in a Bürker–Türk chamber, counting the elements in four corner squares. The mean value was multiplied by 10 for the dilution degree and 10 for the height of the diluted blood layer in the chamber. The values were expressed in number of cells mm^{-3}.

Leukocyte blast transformation test

The leukocyte blast transformation test measures the *in vitro* reactivity of mononuclear cells to sensitizing (*in vivo* encountered) antigens. Cell growth was quantified by means of the glucose consumption technique. Part of the blood sample (100 μl) was diluted with four times the amount of RPMI 1640. The mixture was distributed in five wells of a 96-sterile-well-plate (100 μl per well). Five variants were tested once for each individual animal, namely (1) untreated control culture, (2) phytohaemagglutinin-M (PHA) (1 μl per well) treated culture, (3–5) antigens of *X. c. mycerini*, *X. ramesis* and *S. c. pyramidis* (2.5 μl per well) treated cultures. The quantities of both PHA and antigens were established when using the same technique during preliminary studies as being the most effective *in vitro* for the tested species. The cultures were incubated for 18 h at 37.5°C and 5% CO_2. Glucose concentrations were measured in the initial medium and in all variants at the end of the incubation period, using a standard (100 mg dl^{-1}) glucose solution, by means of an orto-toluidine colorimetric test. To do this, 12.5 μl of the cultural supernatant were transferred to 0.5 ml of orto-toluidine reagent, boiled for 8 min, cooled suddenly in cold water and read in a spectrophotometer at 610 nm wavelength (Unico 2100, United Products Instruments, Inc., Dayton, NJ, USA), using the reagent as a blank. The transformation index (TI) was calculated as follows: TI%=[(MG–SG)/MG]×100, where TI=blast transformation index, MG=glucose concentration in the initial culture medium and SG=glucose concentration in the sample after incubation.

Circulating immune complex measurements

Measurement of the level of circulating immune complexes (CIC) allows evaluation of the molecular clearance capacity at a particular moment. Part of the collected blood was allowed to clot for 30 min at 37°C and then centrifuged at 1308 ***g*** for 10 min. Sera were removed and kept at –20°C until tested. A 4.2% polyethylene glycol (PEG) solution in borate buffer was used as the precipitating agent, while buffer-treated samples served as controls for borate-induced precipitation. The reaction was performed in a 96-well-plate to enhance spectophotometrical readings. Volumes of 196.7 μl of borate buffer and PEG solution, respectively, were mixed with 3.3 μl samples of the serum, for each sample, in parallel wells. The samples were allowed to precipitate at room temperature (22–23°C) for 60 min, then read spectrophotometrically at a wavelength of 450 nm in the test plate (d=0.5 cm) (multichannel spectrophotometer SUMAL PE2, Karl Zeiss, Jena, Germany). CIC concentrations, expressed in optical density units (ODU) were calculated by subtracting the value of the control (serum + buffer) from that of the PEG precipitate.

Immunoglobulin measurements

Total immunoglobulin, known as opsonins, play an important role in the ‘first line of defense’, that is innate immunity, against aggressors. At a pH 7.4, the electric charge and colloidal stability of gamma globulins are lower than those of serum albumins. Thus, concentrations as low as 24 mg l^{-1} of metal salts precipitate the immunoglobulin. A volume of 6.6 μl of serum was mixed with 193.4 μl of a 0.024% barbital buffer zinc sulphate solution and allowed to precipitate for 30 min at room temperature (22–23°C). Optical density (ODU) then was read spectrophotometrically (λ=475 nm, d=0.5 cm).

Carbon particle inclusion test (phagocytic activity)

Phagocytic cells engulf inert particles such as carbon due to the defensive capacity of these cells. 50 μl portions of heparinized blood were mixed with 2 μl of supernatant of India ink, which were obtained by centrifugation at 1308 ***g*** for 40 min (CN-2060 microprocessor control centrifuge, Hsiangtai Machinery Industry Co. Ltd, Taiwan). 15 μl of the mixture were transferred immediately to 2 ml of saline and the rest was incubated for 15 min at 37°C. Another 15 μl sample was transferred to saline and the incubation was continued to 30 min, repeating the operation. All tubes containing saline, blood and ink were centrifuged at 419 ***g*** and the supernatants were read spectrophotometrically (λ=535 nm, d=1 cm). There was a decrease in absorbance with time as carbon was phagocytized. Phagocytic activity index was calculated as the difference between the natural logarithms of the optical densities of the phagocytosis at 0–15 min and 15–30 min divided by time (15 min).

Data analysis

All dependent variables did not deviate significantly from normality (Shapiro–Wilk's tests, W=0.96–0.98, P>0.3) and their variances were homogenous (Levene's tests, $F_{3,37}$=0.001–2.46, P>0.1). Therefore, parametric statistics were applied. Because some laboratory animals (as well as presumably some of enclosure animals) were offspring of the same mother, we had to correctly account for within-litter non-independence of animals. To do this, we performed analysis of variance (ANOVA) for each dependent variable with identification number of a litter as an independent factor. These analyses were done for laboratory animals only because the respective data for enclosure animals were not available. No between-litter difference was found in any of the parameters ($F_{8,14}$=0.25–1.94, P>0.3). In other words, no difference in maternal effect on any parameter was found among mothers of laboratory animals. We therefore assumed that the same was true for enclosure animals, although we did not know sibling/non-sibling relationships among these jirds and, consequently, were unable to perform the respective analysis.

Because the same parameter (transformation index) to four antigens (PHA and antigens of three flea species) as well as the spontaneous transformation index were measured in each individual, we analyzed the effects of flea species, sex and place of birth (enclosure *versus* laboratory) of a rodent on immune responses using repeated-measures ANOVA with the transformation index being a within-subjects factor and place of birth and sex being between-groups factors. The effects of sex and place of birth on non-specific immune responses were analyzed using two-way ANOVAs with immunological parameters as dependent variables and flea burden as a continuous predictor (to remove the possible confounding effect of the difference in flea burden in the enclosure animals).

The effect of flea burden on the severity of immune responses was analyzed by simple linear regression as well as linear regression using the equation $y=b_0+b_1x-b_2x^2$. The significance of the quadratic term would signify the occurrence of the peak of immune response at intermediate flea burden.

Tukey's Honest Significant Difference (HSD) tests were applied for all multiple comparisons.

To avoid an inflated Type I error, we applied Bonferroni adjustment of alpha. Significance was accepted at the adjusted alpha level of 0.005. Data are presented as means ± S.E.M.

Results

Immune responses of rodents differed among flea species (Table 2, Fig. 1); however, there was no effect of rodent sex on within-treatment (control, PHA, antigens from three flea species) transformation index (Table 2). Also, responses of enclosure rodents were significantly higher than those of laboratory rodents. This explains the significance of the interaction term of Treatment × Place of birth.

Rodents from the enclosure and laboratory demonstrated similar low levels of spontaneous glucose consumption (Tukey's HSD test, P=0.9; see Table 2 for degrees of freedom). The transformation index under phytohemagglutinin treatment was significantly higher in enclosure jirds than in controls (Tukey's HSD test, P<0.0001), but laboratory jirds did not differ from controls (Tukey's HSD test, P=0.9). Responses to antigens from *X. c. mycerini* and *X. ramesis* were higher than those to phytohemagglutinin in both enclosure and laboratory rodents (Tukey's HSD tests, P<0.001), although no difference between responses to antigens of these two fleas was found in either in enclosure or in laboratory rodents (Tukey's HSD tests, P=0.8 and P=0.9, respectively). In addition, in the enclosure jirds, the transformation index with *S. c. pyramidis* antigen did not differ significantly from those with antigens from the two other flea species (Tukey's HSD tests, P=0.08 and P=0.2, respectively) (Fig. 1). In contrast, laboratory rodents demonstrated no difference in transformation index between *S. c. pyramidis* antigen and either the phytohemagglutinin treatment or controls (Tukey's HSD tests, P=0.8 and P=0.9, respectively). Finally, transformation indices with any antigen were significantly higher in the enclosure than in the laboratory rodents (Fig. 1, Tukey's HSD tests, P=0.4–0.9).

The concentration of immunoglobulins was similar in the

Table 2. *Summary of the repeated-measures ANOVA of the results of transformation test in dependence on rodent place of birth, rodent sex and treatment (control, PHA, antigens from three flea species)*

Effect	Sum of squares	d.f.	*F*	*P*
Place of birth	0.14	1	6.09	0.018
Sex	0.073	1	3.14	0.084
Sex × Place of birth	0.0007	1	0.03	0.867
Error	0.86	37		
Treatment	0.34	4	64.27	>0.0001
Treatment × Place of birth	0.045	4	8.55	>0.0001
Treatment × Sex	0.008	4	1.61	0.17
Treatment × Place of birth × Sex	0.027	4	5.01	>0.001
Error	0.198	148		

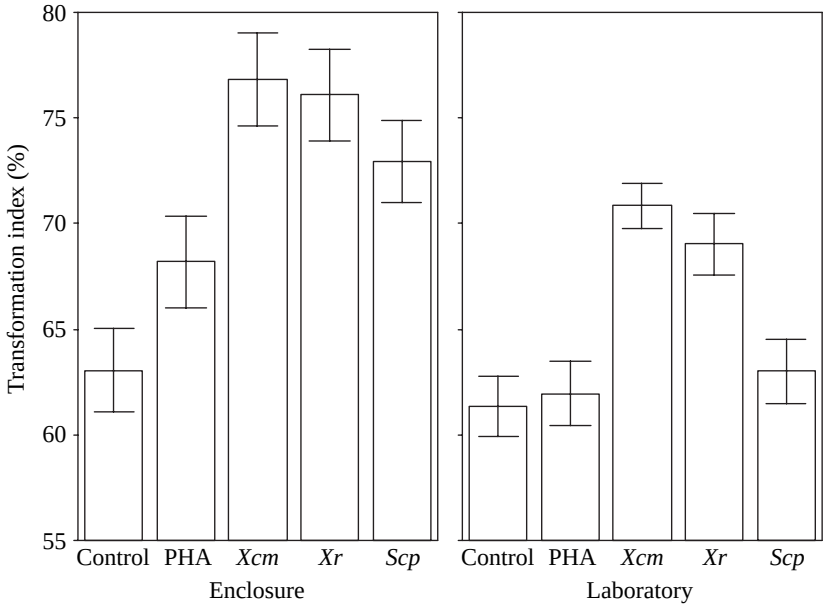

Fig. 1. Transformation index (mean ± S.E.M.) of leucocytes of *M. crassus* (*N*=41) in presence of different antigens. PHA, phytohaemagglutinin; *Xcm*, *Xr*, *Scp*, whole body extracts of *X. c. mycerini*, *X. ramesis* and *S. c. pyramidis*, respectively.

enclosure and laboratory animals as well as in males and females ($F_{1,37}$=0.10, P=0.7 and $F_{1,37}$=1.99, P=0.2, respectively). The level of circulating immune complexes also did not differ between the enclosure and laboratory rodents ($F_{1,37}$=0.46, P=0.5) but was significantly higher in females than males (adjusted least squares means 0.05±0.006 *versus* 0.02±0.009 ODU, respectively, $F_{1,37}$=8.77, P=0.005).

The number of white blood cells was significantly lower in the enclosure than in the laboratory animals (adjusted least squares means 9663.4±844.7 *versus* 13931.0±897.9 mm^{-3}, respectively, $F_{1,37}$=10.16, P=0.002), but there was no sex difference in this parameter ($F_{1,37}$=1.88, P=0.2). Phagocytic activity was significantly higher in the enclosure animals than in the laboratory animals ($F_{1,37}$=10.09, P=0.002), but did not differ between sexes ($F_{1,37}$=5.83, P=0.02).

Only phagocytic activity was affected by flea burden (Table 3); this activity decreased significantly with an increase in flea burden (Fig. 2).

Discussion

The results of the leukocyte blast transformation tests clearly demonstrated that (i) cross-reactivity in rodent responses to different flea species occurred for the enclosure but not for the laboratory jirds and (ii) immune-naïve animals whose mothers were parasitized by fleas had some degree of immunity against fleas. The former was confirmed by immune responses of the enclosure animals to flea antigens in that they did not differ between familiar (*X. c. mycerini* and *X. ramesis*) and non-familiar (*S. c. pyramidis*) fleas. The latter was supported by immune responses of naïve rodents born from parasitized mothers to antigens of *X. c. mycerini* and *X. ramesis*.

Cross-immunity (=cross-resistance) was reported for a number of parasite taxa such as protozoans (Leemans et al., 1999), gastrointestinal parasites (Smith and Archibal, 1969), blackflies (Cross et al., 1993), ticks (Kaiser et al., 1982; Rechav, 1992). Moreover, cross-immunity between distantly related parasite taxa was also reported. Rabbits infested with mites *Prosoptes cuniculi* produced antibodies reactive with both mite and tick extracts, whereas mite-free rabbits did not (den Hollander and Allen, 1986). However, some studies did not find cross-immunity against different parasite species (e.g. Rechav et al., 1989). Apparently, the occurrence of cross-

Table 3. *Summary of regressions of haematological and immunological parameters against flea burden*

	Equation					
	$y=b_0+b_1x$, d.f.=1,16			$y=b_0+b_1x-b_2x^2$, d.f.=2,15		
Parameter	r^2	F	P	r^2	F	P
Circulating immune complexes	0.02	0.2	0.6	0.28	3.5	0.07
Concentration of total immunoglobulins	0.04	0.8	0.4	0.07	0.4	0.7
White blood cell count	0.05	0.8	0.4	0.07	0.4	0.7
Phagocytic activity	0.53	13.7	0.003	0.45	6.6	0.01
Spontaneous leukocyte blast transformation	0.04	0.4	0.6	0.23	1.3	0.4
Leukocyte blast transformation in the presence of:						
PHA	0.03	0.4	0.6	0.19	2.0	0.2
X. c. mycernini antigen	0.01	0.01	0.09	0.50	3.4	0.09
X. ramesis antigen	0.05	0.4	0.5	0.40	4.1	0.06
S. c. pyramidis antigen	0.01	0.7	0.8	0.45	2.9	0.1

PHA, phytohaemagglutinin.

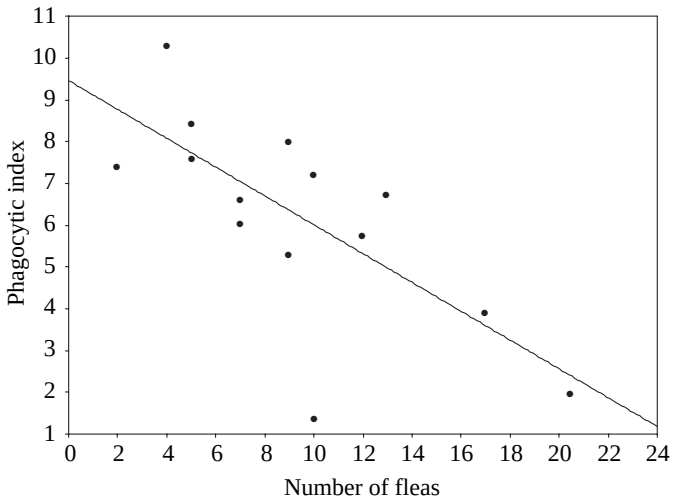

Fig. 2. Relationship between phagocytic activity in *M. crassus* (*N*=18) in dependence on flea burden.

immunity depends on both parasite and host taxon. For example, McTier et al. (1981) showed that in guinea pigs, cross-immunity occurred between two ticks of *Dermacentor* genus but not between ticks belonging to *Dermacentor* and *Amblyomma* genera. Rabbits demonstrated cross-resistance between two ticks of *Hyalomma* genus (Kumar and Kumar, 1996), but not between *Rhipicephalus* and *Ixodes* genera (Rechav et al., 1989). Furthermore, cross-immunity between two *Rhipicephalus* species was reported for rabbits (Njau and Nyindo, 1987), whereas no cross-immunity between these two ticks was found in guinea pigs (Rechav et al., 1989).

The occurrence of cross-immunity can be explained by close homology of saliva proteins in closely related ectoparasites (e.g. Mans et al., 2002). However, this is not always the case (e.g. Warburg et al., 1994). Furthermore, a variety of cross-immunity patterns was also demonstrated with the 'concealed' ectoparasite antigens (Willadsen and Kemp, 1988; Willadsen et al., 1993). Bm86 antigen from the tick *Boophilus microplus* contained in the commercial anti-tick vaccine has close homologues in both *Hyalomma anatolicum* (de Vos et al., 2001) and *Rhipicephalus appendiculatus* (Willadsen, 2001). However, a significant level of cross-protection was found between the *B. microplus* vaccine and *H. anatolicum* (F. Jongejan, unpublished; cited by Willadsen, 2001) but not between this vaccine and *R. appendiculatus* (de Vos et al., 2001). It is apparent that some still unknown factors determine cross-immunity patterns in different host-parasite systems. In addition, different components of the immune system vary interspecifically, and the determinants of such variation are unknown.

Our results demonstrate the occurrence of cross-immunity against different flea species in the enclosure but not in the laboratory jirds. Indeed, the transformation index of leukocytes of laboratory animals in presence of *S. c. pyramidis* antigen did not differ from the spontaneous transformation index. This suggests that the immunity transferred by the mother (if any, see below) was specific, aimed against certain flea species, but could not protect against other, albeit closely related species.

Significant immune responses to *X. c. mycerini* and *X. ramesis* in rodents born in the laboratory suggest a possibility that they received some protection against these fleas from their mothers. The occurrence of maternal transfer of immunity could be inferred from the difference in immune responses between the enclosure and laboratory animals, although our study lacks the direct measurements of the maternal transfer of immunity. Maternal transfer of immunity against parasites was reported (Carlier and Truyens, 1995), although the protective effect of maternal antibodies transfer to offspring is limited (e.g. Knopf and Coghlan, 1989). Indeed, the responses to antigens of both *Xenopsylla* species in the laboratory jirds were lower than those in the enclosure animals, suggesting that the protective level of maternal immunity was probably lower than the acquired immunity against the same flea species. However, the relatively short lifespan of the immune cells that could be supposedly transferred from mothers to offspring suggests higher probability of finding them in juvenile individuals rather than in young adults, as was the case in our study.

An alternative (to maternal transfer of immunity) explanation for our findings of the occurrence of the immune response to antigens of two *Xenopsylla* fleas in the laboratory jirds could be that the long association between *M. crassus* and *X. c. mycerini* and *X. ramesis* in the past induced host genotypical changes *via* selection. In particular, these changes could affect the major histocompatibility complex, which is the region of the genome that controls the immune response (Gruen and Weissman, 1997).

Overall low responses in the laboratory jirds (including relatively low phagocytic activity – see below) as well as the lack of their response to phytohemagglutinin can be explained by the lack of challenges in the controlled laboratory environment and, thus, a 'lazy' reactivity of immune cells, mainly lymphocytes and monocytes. In contrast, the enclosure animals probably had more intense change in their polymorphonuclear cells because they were likely to be exposed to permanent microbiological challenges. Therefore, their immune cells were also more active, that is functionally 'faster', than those of the animals from the laboratory. Moreover, even though enclosure jirds were permanently parasitized, there still may be some effects of maternal transfer of immune factors that facilitate their ability to mount immune response. Further manipulative experiments are needed to test this hypothesis (e.g. comparison of acquired immunity against fleas between rodents born from parasitized and not-parasitized mothers).

The only sex difference in immunological parameters was the higher level of circulating immune complexes in females than in males, indicating higher synthesis of antibodies and clearance of the antigen through complexation in females than in males. This supports the hypothesis of sexual dimorphism in immunocompetence and reduced humoral and cell-mediated immunity in males (Billingham, 1986; Schuurs and Verheul,

1990; Zuk and McKean, 1996). Lower immunocompetence in males has been explained by immunosuppressive function of androgens (Folstad and Karter, 1992) and has been reported in several vertebrate taxa (Hughes and Randolph, 2001; Uller and Olson, 2003). Moreover, sexual difference in immunocompetence is supported mainly for arthropod rather than helminth parasites (Schalk and Forbes, 1997). For example, testosterone treatments reduced both innate and acquired resistance of rodents *Clethrionomys glareolus*, and *Apodemus sylvaticus* to the feeding of the tick *Ixodes ricinus* (Hughes and Randolph, 2001). We found sex differences in the humoral component of immunity only, which suggests that androgens suppressed some but not all components of the immune defense system. Indeed, studying immune responses in *Microtus ochrogaster* and *Microtus pennsylvanicus*, Klein and Nelson (1998a) did not observe sex differences in the proliferative response of splenocytes to concanavalin (cell-mediated immunity), but found differences in humoral immunity responses (Klein and Nelson, 1998b).

Our prediction about the peak level of host immune response at the intermediate parasite burden was not supported. The only parameter that correlated with the number of parasites was phagocytic activity that decreased with an increase of flea burden. Apparently, even the weak attack of a parasite triggered the immune system. However, this system could not overcome the attack by large number of fleas, perhaps due to their additive immunosuppression effect and the cost of the immune system (Schmid-Hempel and Egert, 2003).

Finally, the difference between parasitized (enclosure) and non-parasitized (laboratory) animals in the number of white blood cells, leukocyte blast transformation index and phagocytic activity, but not in the concentration of immunoglobulins and circulation immune complexes, suggested that the immune response to flea parasitism was linked mainly to cell-mediated immunity. The same has been shown to be true for the immunity against ticks (e.g. Rubaire-Akiki and Mutinga, 1980). For example, there was no correlation between rabbit serum antibodies to soluble antigens from tick salivary gland extracts and protective immunity (Heller-Haupt et al., 1996). Yet production of anti-flea antibodies and transfer of resistance to fleas with immune serum were also reported (Greene et al., 1993; Heath et al., 1994; Jones, 1996). Thus, humoral factors can also have a role in host resistance to fleas. Furthermore, the digestion in fleas is intracellular and they lack a peritrophic membrane (Vaschenok, 1988), which lines the gut of many arthropods. It separates ingested food from the gut epithelium and, thus, may restrict penetration of ingested immune effector components (Eiseman and Binnengton, 1994). These two factors can increase the detrimental effect of soluble antigens on fleas.

We thank staff of the Department of Microbiology and Immunology, Faculty of Health Science, Ben-Gurion University of the Negev for their help in freeze-drying of antigen pellets. We thank two anonymous referees for helpful comments. This study was supported by the Israel Science Foundation (Grant no. 663/01-17.3 to B.R.K. and I.S.K.). M.S. received financial support from the Access to Research Infrastructure (ARI) program of the EU *via* the Blaustein Center for Scientific Cooperation of the Jacob Blaustein Institute for Desert Research, Ben-Gurion University of the Negev. The experiments comply with the laws of the State of Israel. This is publication no. 160 of the Ramon Science Center and no. 412 of the Mitrani Department of Desert Ecology.

References

Billingham, R. E. (1986). Immunological advantages and disadvantages of being a female. In *Reproductive Immunology* (ed. D. A. Clarke and B. A. Croy), pp. 1-9. NY: Elsevier Science Publications.

Carlier, Y. and Truyens, C. (1995). Influence of maternal infection on offspring resistance towards parasites. *Parasitol. Today* **11**, 94-99.

Combes, C. (2001). Parasitism. *The Ecology and Evolution of Intimate Interactions*. Chicago: University of Chicago Press.

Cross, M. L., Cupp, E. W., Ramberg, F. B. and Enriquez, F. J. (1993). Antibody responses of BALB/c mice to salivary antigens of hematophagous black fleas (Diptera: Simuliidae). *J. Med. Entomol.* **30**, 725-734.

de Lope, F., Moller, A. P. and de la Cruz, C. (1998). Parasitism, immune response and reproductive success in the house martin *Delichon urbica*. *Oecologia* **114**, 188-193.

de Vos, S., Zeinstra, L., Taoufik, O., Willadsen, P. and Jongejan, F. (2001). Evidence for the utility of the Bm86 antigen from *Boophilus microplus* in vaccination against other tick species. *Exp. Appl. Acarol.* **25**, 245-261.

den Hollander, N. and Allen, J. R. (1986). Cross-reactive antigens between a tick *Dermacentor variabilis* (Acari: Ixodidae) and a mite *Prosoptes cuniculi* (Acari: Psoroptidae). *J. Med. Entomol.* **23**, 44-50.

Eiseman, C. H. and Binnengton, K. C. (1994). The peritrophic membrane – its formation, structure, chemical-composition and permeability in relation to vaccination against ectoparasitic arthropods. *Int. J. Parasitol.* **24**, 15-26.

Fielden L. J., Rechav, Y. and Bryson, N. R. (1992). Acquired immunity to larvae of *Amblyomma marmoreum* and *A. hebraeum* by tortoises, guinea-pigs and guinea-fowl. *Med. Vet. Entomol.* **6**, 251-254.

Folstad, I. and Karter, A. J. (1992). Parasites, bright males and the immunocompetence handicap. *Amer. Nat.* **139**, 603-622.

Greene, W. K., Carnegie, R. L., Shaw, S. E., Thompson, R. C. A. and Penhale, W. J. (1993). Characterization of allergens of the cat flea, *Ctenocephalides felis*: detection and frequency of IgE antibodies in canine sera. *Parasite Immunol.* **15**, 69-74.

Gruen, J. R. and Weissman, S. M. (1997). Evolving views of the major histocompatibility complex. *Blood* **90**, 4252-4265

Heckmann, R., Gansen, B. and Hom, M. (1967). Maternal transfer of immunity to rat coccidiosis – *Eimeria nieschulzi* (Dieben – 1924). *J. Protozool.* **S14**, 35.

Heath, A. W., Arfsten, A., Yamanaka, M., Dryden, M. W. and Dale, B. (1994). Vaccination against the cat flea *Ctenocephalides felis felis*. *Parasite Immunol.* **16**, 187-191.

Heller-Haupt, A., Kagaruki, L. K. and Varma, M. G. R. (1996). Resistance and cross-resistance in rabbits to adults of three species of African ticks (Acari: Ixodidae). *Exp. Appl. Acarol.* **20**, 155-165.

Hughes, V. L. and Randolph, S. E. (2001). Testosterone depresses innate and acquired resistance to ticks in natural rodent hosts: a force for aggregated distributions of parasites. *J. Parasitol.* **87**, 49-54.

Jones, C. J. (1996). Immune responses to fleas, bugs and sucking lice. In *The Immunology of Host-Ectoparasitic Arthropod Relationships* (ed. S. K. Wikel), pp. 150-174. Wallingford, UK: CAB International.

Kaiser, M. N., Sutherts, R. W. and Bourne, A. S. (1982). Relationship between ticks and Zebu cattle in Southern Uganda. *Trop. Anim. Health Product.* **14**, 63-74.

Klein, S. L. and Nelson, R. J. (1998a). Sex and species differences in cell-mediated immune responses in voles. *Can. J. Zool.* **76**, 1394-1398.

Klein, S. L. and Nelson, R. J. (1998b). Adaptive immune responses are linked to the mating system of arvicoline rodents. *Amer. Nat.* **151**, 59-67.

Knopf, P. M. and Coghlan, R. L. (1989). Maternal transfer of resistance to *Schistosoma mansoni*. *J. Parasitol.* **75**, 398-404.

Krasnov, B. R., Shenbrot, G. I., Khokhlova, I. S. and Ivanitskaya, E. Y. (1996). Spatial structure of rodent community in the Ramon erosion cirque, Negev highlands (Israel). *J. Arid Environ.* **32**, 319-327.

Krasnov, B. R., Shenbrot, G. I., Medvedev, S. G., Vatshenok, V. S. and Khokhlova, I. S. (1997). Host-habitat relations as an important determinant of flea assemblages (Siphonaptera) on rodents in the Negev Desert. *Parasitology* **114**, 159-173.

Krasnov, B. R., Shenbrot, G. I., Medvedev, S. G., Khokhlova, I. S. and Vatschenok, V. S. (1998). Habitat-dependence of a parasite-host relationship: flea assemblages in two gerbil species of the Negev Desert. *J. Med. Entomol.* **35**, 303-313.

Krasnov, B. R., Hastriter, M., Medvedev, S. G., Shenbrot, G. I., Khokhlova, I. S. and Vaschenok, V. S. (1999). Additional records of fleas (Siphonaptera) on wild rodents in the southern part of Israel. *Israel J. Zool.* **45**, 333-340.

Kristan, D. M. (2002). Maternal and direct effects of the intestinal nematode *Heligmosomoides polygyrus* on offspring growth and susceptibility to infection. *J. Exp. Biol.* **205**, 3967-3977.

Kumar, R. and Kumar, R. (1996). Cross-resistance to *Hyalomma anatolicum anatolicum* ticks in rabbits immunized with midgut antigens of *Hyalomma dromedarii*. *Indian J. Anim. Sci.* **66**, 657-661.

Leemans, I., Brown, D., Hooshmand-Rad, P., Kirvar, E. and Uggla, A. (1999). Infectivity and cross-immunity studies of *Theileria lestoquardi* and *Theileria annulata* in sheep and cattle: I. In vivo responses. *Vet. Parasitol.* **82**, 179-192.

Mans, B. J., Louw, A. I. and Neitz, A. W. H. (2002). Evolution of hematophagy in ticks:common origins for blood coagulation and platelet aggregation inhibitors from soft ticks of the genus *Ornithodoros. Mol. Biol. Evol.* **19**, 1695-1705.

Marshall, A. (1981). *The Ecology of Ectoparasitic Insects*. London: Academic Press.

McTier, T. L., George, J. E. and Bennet, S. N. (1981). Resistance and cross-resistance of guinea pigs to *Dermacentor andersoni* Stiles, *D. variabilis* (Say), *Amblyomma americanum* (Linnaeus) and *Ixodes capularis* Say. *J. Parasitol.* **67**, 813-822.

Metzger, M. E. and Rust, M. K. (1997). Effect of temperature on cat flea (Siphonaptera: Pulicidae) development and overwintering. *J. Med. Entomol.* **34**, 173-178.

Njau, B. C. and Nyindo, M. (1987). Detection of immune response in rabbits infested with *Rhipicephalus appendiculatus* and *Rhipicephalus evertsi evertsi. Res. Vet. Sci.* **43**, 217-221.

Olsen, N. J. and Kovacs, W. J. (1996). Gonadal steroids and immunity. *Endocrine Rev.* **17**, 369-384.

Poulin, R. (1996). Sexual inequalities in helminth infections: a cost of being a male? *Amer. Nat.* **147**, 287-295.

Rechav, Y. (1982). Dynamics of tick populations (Acari: Ixodidae) in the Eastern Cape Province of South Africa. *J. Med. Entomol.* **19**, 679-700.

Rechav, Y. (1992). Naturally acquired resistance to ticks – a global veiew. *Insect Sci. Appl.* **13**, 495-504.

Rechav, Y., Heller-Haupt, A. and Varma, M. G. R. (1989). Resistance and cross-resistance in guinea-pigs and rabbits to immature stages of ixodid ticks. *Med. Vet. Entomol.* **3**, 333-336.

Ribeiro, J. M. C. (1987). Role of saliva in blood feeding in arthropods. *Ann. Rev. Entomol.* **32**, 463-478.

Ribeiro, J. M.C. (1995). Blood-feeding arthropods: live syringes or invertebrate pharmacologists? *Infect. Agents Dis.* **4**, 143-152.

Roehrig, J. T., Piesman, J., Hunt, A. R., Keen, M. G., Happ, C. M. and Johnson, B. J. B. (1992). The hamster immune-response to tick-transmitted *Borrelia burgdorferi* differs from the response to needle-inoculated, cultured organisms. *J. Immunol.* **149**, 3648-3653.

Rubaire-Akiki, C. M. and Mutinga, M. J. (1980). Immunological reactions associated with rabbit resistance to *Rhipicephalus appendiculatus* (Neumann) infestations. *Bull. Anim. Health Prod. Africa* **28**, 49-59.

Schalk, G. and Forbes, M. R. (1997). Male biases in parasitism of mammals: effects of study type, host age, and parasite taxon. *Oikos* 78, 67-74.

Schmid-Hempel, P. and Egert, D. (2003). On the evolutionary ecology of specific immune defence. *Trends Ecol. Evol.* **18**, 27-32.

Schuurs, A. H. M. N. and Verheul, H. A. M. (1990). Effects of gender and sex steroids on the immune response. *J. Steroid Biochem.* **35**, 157-172.

Sheldon, B. C. and Verhulst, S. (1996). Ecological immunology: costly parasite defences and trade offs in evolutionary ecology. *Trends Ecol. Evol.* **11**, 317-321.

Shubber, A. H., Lloyd, S. and Soulsby, E. J. L. (1981). Infection with gastrointestinal helminths – effect of lactation and maternal transfer of immunity. *Parasitol. Res.* **65**, 181-189.

Smit, F. G. A. M. (1982). Classification of the Siphonaptera. In *Synopsis and Classification of Living Organisms*, vol. 2 (ed. S. Parker), pp. 557-563. NY: McGraw-Hill.

Smith, H. J. and Archibal, R. M. (1969). Development of cross immunity in lambs by exposure to bovine gastrointestinal parasites. *Can. Vet. J.* **10**, 286.

Studdert, V. P. and Arundel, J. H. (1988). Dermatitis of the pinnae of cats in Australia associated with the European rabbit flea (*Spillopsyllus cuniculi*). *Vet. Record* **123**, 624-625.

Traub, R. (1980). The zoogeography and evolution of some fleas, lice and mammals. In *Fleas. Proceedings of the International Conference on Fleas, Ashton, England, June 1977* (ed. R. Traub and H. Starcke), pp. 93-172. Rotterdam, Netherlands: A. A. Balkema.

Uller, T. and Olson, M. (2003). Prenatal exposure to testosterone increases ectoparasite susceptibility in the common lizard (*Lacerta vivipara*). *Proc. Roy. Soc. Lond. B* **270**, 1867-1870.

Vatschenok, V. S. (1988). *Fleas – Vectors of Pathogens Causing Diseases in Humans and Animals.* Leningrad, USSR: Nauka Publ. House (in Russian).

Vaughan, J. A., Jerse, A. E. and Azad, A. F. (1989). Rat leucocyte's response to the bites of rat fleas (Siphonaptera: Pulicidae). *J. Med. Entomol.* **26**, 449-453.

Warburg, A., Saraiva, E., Lanzaro, G. C., Titus, R. G. and Neva, F. (1994). Saliva of *Lutzomyia longipalpis* sibling species differs in its composition and capacity to enhance leishmaniasis. *Phil. Trans. R. Soc. Lond. B* **345**, 223-230.

Whiting, M. F., Carpenter, J. C., Wheeler, Q. D. and Wheeler, W. C. (1997). The Strepsiptera problem: phylogeny of the holometabolous insect orders inferred from 18S and 28S ribosomal DNA sequences and morphology. *Syst. Biol.* **46**, 1-68.

Wikel, S. K. (ed) (1996). *The Immunology of Host-Ectoparasitic Arthropod Relationships.* Wallingford, UK: CAB International.

Wikel, S. K., Ramachandra, R. N. and Bergman, D. K. (1996). Arthropod modulation of host immune responses. In *Immunology of Host–Ectoparasitic Arthropod Relationships* (ed. S. K. Wikel), pp. 107-130. Wallingford, UK: CAB International.

Wikel, S. K. and Alacron-Chaidez, F. J. (2001). Progress toward molecular characterization of ectoparasite modulation of host immunity. *Vet. Parasitol.* **101**, 275-287.

Willadsen, P. (2001). The molecular revolution in the development of vaccines against ectoparasites. *Vet. Parasitol.* **101**, 353-367.

Willadsen, P. and Kemp, D. H. (1988). Vaccination with 'concealed' antigens for tick control. *Parasitol. Today* **4**, 96-198.

Willadsen, P., Eisemann, C. H. and Tellam, R. L. (1993). Concealed antigens: expanding the range of immunological targets. *Parasitol. Today* **9**, 132-135.

Zuk, M. and McKean, K. A. (1996). Sex differences in parasite infections: patterns and processes. *Int. J. Parasitol.* **26**, 1009-1024.