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Trypanosoma cruzi: the development of estrus cycle and parasitemia in female mice maintained with or without male pheromones

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Abstract Female BALB/c or C57Bl/6 mice, kept in small groups of three or five animals with or without male odor, all had a similar progesterone and corticosterone level, mean number of estrus and duration of estrus cycle. However, if males were kept in the same room, the mean duration of the estrus cycle was longer for both strains; and C57Bl/6 females had a significantly higher number of estrus than BALB/c mice and showed a tendency to synchronize the estrus cycle within a group. After infection of females of both mouse strains with vector-derived metacyclic trypomastigotes of *Trypanosoma cruzi*, anestrus with intense phlegm production occurred during the acute phase of infection and this was positively correlated with higher parasitemia. Within individual groups of BALB/c mice, the female with the relatively highest corticosterone and progesterone level had the lowest parasitemia. In groups kept separate from male pheromones, one or two females in each group developed high parasitemias.

Introduction

Various infectious agents seem to affect females less than males (Alexander and Stimson 1988; Roberts et al. 1996). This sexual dimorphism concerning the ability to react to immunological challenges has been noted to be related to the gonadal hormones, which exert an influence on the immune system (Eidinger and Garrett 1972; Ahmed et al. 1985; Grossman 1985; Schuurs and Verheul 1990; Araneo et al. 1991). The principal sex hormones in females, estrogens and progesterone, have been shown to affect immune reactions in various ways. Macrophages have estrogen receptors, which enable them to manifest a wide range of responses when estrogens are present, e.g., an increase of phagocytic capacity, IL-1 release and production of reactive oxygen intermediates and nitrite release, as well as an inhibition of tumor necrosis factor release in postmenopausal women (Hu et al. 1988; Polan et al. 1989; Ralston et al. 1990; Styrt and Sugarman 1991; Chao et al. 1994). Investigations concerning the effects of estrogen on natural killer (NK) cells appear contradictory, in that estrogens have been reported to have either an enhancing or a negative influence on NK cell proliferation (Gabrilovac et al. 1988; Sorachi et al. 1993). Furthermore, estrogens influence the humoral immune response by enhancing B cell maturation, which results in higher immunoglobulin and antibody titers in females, compared to males (Eidinger and Garrett 1972; Paavonen et al. 1981; Ahmed et al. 1985; Nilsson and Carlsten 1994; Gaillard and Spinedi 1998). Estrogens also affect the production of cytokines, e.g., IL-5 and IFN-γ (Grasso and Muscettola 1990; Fox et al. 1991; Wang et al. 1993). Progesterone, apart from its various effects on cytokine production, influences the development of the Th2 immune response by stimulating Th0-type T cells to develop into Th2-type T cells or even by inducing Th1-type T cells to produce IL-4, a typical Th2-response cytokine (Piccinni et al. 1995).

The levels of sex hormones vary during the estrus cycle, which consists of a sequence of physiological events controlled by hormonal secretions of the pituitary gland and the ovaries. In addition, female mice regulate their estrus cycle in relation to their social environment, i.e., according to the presence or absence of males or at least pheromones originating from the urine of males. In the Lee–Boot effect, estrus cycles tend to slow down and eventually stop when groups of female mice are housed together and separated from males (van der Lee and Boot 1955, 1956). After a subsequent exposure to the odor of a male, or at least to its urine, they start cycling again; and their cycles tend to synchronize, in the so-called Whitten effect (Whitten 1959). Although these

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effects should be reflected by changes in the female hormonal state, sex hormone levels have not so far been determined.

In experimental infections with *Trypanosoma cruzi* – the causative agent of Chagas disease – female mice often appear to be less susceptible than males. This effect is due to sex hormones, as orchidectomy in males results in a lower severity of infection and ovariectomy in females causes a higher susceptibility, compared to untreated or sham-operated controls. The female gonadal hormones, estrogen and progesterone, seem to be responsible for the lower susceptibility of females to *T. cruzi*, since the application of estrogen, progesterone or both causes lower parasitemias in ovariectomized females, compared to sham-operated or control animals (Prado et al. 1998). High-dose progesterone or testosterone treatment in female (and in male) mice leads to less severe courses of infection, but testosterone is less effective than progesterone (Tay et al. 1978).

In the present study, we investigated the sex- and stress-hormone levels and the estrus behavior of female mice kept with or without male pheromones/urine. We also examined the supplementary effects of an infection with vector-derived metacyclic trypomastigotes of *T. cruzi* (applied in a natural infection dose) on the estrus cycle and the influence of different housing conditions on the course of parasitemia.

Materials and methods

Parasites

The "Tulahuén" strain of *Trypanosoma cruzi* was kindly provided by Dr. S. Croft (London School of Hygiene and Tropical Medicine, London, UK). Like other *T. cruzi* in our laboratory, this strain was maintained cyclically in mice and bugs or stored frozen at –78 °C (Schaub 1988). To obtain metacyclic trypomastigotes, first instars of *Triatoma infestans* were fed on *Trypanosoma cruzi*-infected mice and the following instars were fed on hens. After feeding the fifth instars, infectious feces was collected. To determine the number of metacyclic trypomastigotes, an aliquot of feces was mixed with human serum and incubated at 37 °C for 30 min, thereby killing all non-metacyclic trypomastigotes by complement lysis (Chao et al. 1985). This mixture was exclusively used for counting the number of metacyclic trypomastigotes in a Neubauer chamber. On average, the feces contained about 10^6 metacyclic trypomastigotes/ml feces. This is consistent with data obtained for another strain of *T. cruzi* and the same vector which, after blood ingestion, deposited a volume of 1–25 µl (mean 10 µl) in the first drop of feces, containing $1–26\times10^3$ metacyclic trypomastigotes (Schaub and Lösch 1988). The concentration in the original sample of feces was then adjusted to 1×10^3 trypomastigotes/ml by diluting about 1:1000 with physiological saline.

Mice

We had chosen two strains of mice, BALB/c and C57Bl/6 mice, because BALB/c mice developed a low parasitemia and survived, whereas C57Bl/6 mice were more susceptible to *T. cruzi* and died. Since the effects in the late phase of infection should also be considered, C57Bl/6 mice were only used for one experiment. The original stocks of BALB/c and C57Bl/6 mice were kindly provided by Dr. H. Mossmann from the Max Planck Institute of Immunobiology, Freiburg. Both strains were bred at our institute. Commercial rodent diet and water were available ad libitum. Juvenile female mice about 5 weeks old were separated from their mothers and kept in small groups of three or five individuals in cages of 26.5×21×14 cm or 43×26.5×15 cm, respectively (the optimal number of mice for cages of these sizes). Social structures are very similar in groups of these sizes (Schuhr 1987). A 16 h/8 h light/dark cycle was chosen, since this rhythm is important for estrus cycle development (Giammanco et al. 1997).

To determine the concentrations of female hormones, seven groups of five BALB/c mice were kept under two different housing conditions, i.e., strictly separated from male odor or in the same room with males. About 20 females exhibiting regular estrus cycles were used at the age of approximately 14 weeks. The remaining 15 animals neither cycled regularly nor exhibited a clear diestrus, but rather showed intermediate states between metestric and diestric, or between diestric and proestric phases during the period of investigation and were returned to our breeding facilities.

To investigate the effects of *T. cruzi*, two and four groups of BALB/c-mice, called series A and B respectively, were maintained in the same room with males; and, when the litter was changed, used litter from males' cages was added to provide the influence of male pheromones to these groups. The other groups were kept in parallel, strictly separated from males. Two groups of C57Bl/6 mice were also kept under the influence of male pheromones. At the age of approximately 12 weeks, all mice were transferred to our security infection laboratory. In series A, this laboratory contained males from another experiment. Therefore, these two groups initially kept separated from males may have been influenced by males thereafter. In series B, the four groups kept separated from males were transferred to a second security infection laboratory without male mice and put into a mouse incubator (Uni Protect, Ehret, Emmendingen, Germany) which contained a charcoal air filter to exclude all effects of pheromones from males in other rooms.

The experiments were performed in accordance with German animal welfare regulations (registration no. 23.8720/20.A.9).

Examination of vaginal smears

To identify females with regular estrus cycle and to monitor infection-derived changes, vaginal smears were taken daily using a disinfected 1-µl inoculation loop (Nunc, Wiesbaden, Germany). After staining with Hema-Diff quick stain (Bioanalytic, Umkirch/Freiburg, Germany), they were evaluated microscopically (magnification 200×).

Starting at the age of about 9 weeks, vaginal smears were taken daily, for 16 days in series A and for 19 days in series B. The control of estrus cycle was first interrupted between the day of infection and the end of the prepatent period and was then interrupted a second time at 65–78 days post infection (dpi). The latter case served to confirm that smear abnormalities had not been caused by daily handling. The smears were classified into different estrus categories, according to the descriptions of Snell (1941): (1) proestrus with predominantly old, cornified, but still nucleus-containing epithelial cells, (2) estrus with exclusively cornified cells without nucleus, (3) metestrus with leucocytes and young round epithelial cells and (4) diestrus with leucocytes, older and irregularly shaped epithelial cells and the first signs of cornification. Extremely prolonged diestrus was considered as anestrus; and cornification appearing like an estric smear but lasting longer than 3 days was considered as an irregularity – persistent cornification – rather than as real estrus (Whitten 1956, 1959). The data for two animals that died of causes unrelated to the infection were omitted.

Determination of concentrations of female and stress hormones

Heart blood was taken during the diestric phase into heparinized syringes and was centrifuged at 400 *g*. This phase was chosen because circadian variation of plasma progesterone is lowest during this phase of the estrus cycle (Corpéchot et al. 1997).

To measure the individual progesterone and corticosterone levels prior to and after infection with *T. cruzi*, in series B about 200 µl blood were collected by puncturing the retroorbital plexus within 3 min of handling and were centrifuged at 400 *g*. Animals having survived the acute phase of infection were killed by cervical dislocation; and blood was taken immediately by heart puncture into heparinized syringes and was centrifuged at 1,000 *g*. To exclude any infection risk during the measurement of hormone concentrations, the resulting plasma was filtered using a 0.22-µl filter (Eppendorf, Köln, Germany).

The sera/plasmas were stored at –80 °C. The concentrations of estradiol, progesterone and corticosterone were determined in the laboratory of Prof. Dr. D. von Holst (University of Bayreuth), using radioimmunoassays (Fenske 1988).

Infection of mice and determination of parasitemia

After anesthetization with an intraperitoneally injected mixture of Rompun (Bayer, Leverkusen, Germany), 10% ketamin (belapharm, Vechta, Germany) and 0.9% NaCl (2:6:17), the mice were infected subcutaneously on the back with about 100 trypomastigotes, i.e., 5 flagellates/g body weight. This is equivalent to the natural infection dose if infectious feces are deposited onto the puncture site of a triatomine's bite (Heide 1999). Starting 16 dpi, the parasitemia was determined every 2 days by examination of 100 microscopic fields of fresh blood preparations (magnification 400×). In previous experiments we had also counted them in Neubauer chambers, thereby correlating 1 flagellate/microscopic field to about 1×10^6 flagellates/ml blood (Schuster and Schaub 2000).

Evaluation of data

In classifying data within a group as high, middle or low level, pairs of data were considered to be not different if the lower value was more than 90% of the higher value.

Statistical comparisons of hormone concentrations, data resulting from the examination of vaginal smears and the comparisons of prepatent periods were performed by using the unpaired *t*-test (Graph Pad Prism; Graph Pad software, San Diego, Calif.). Statistical comparison of the parasite burden was performed by calculating the areas under the individual parasitemia curves, followed by a Mann–Whitney *U*-test (Graph Pad Prism).

Results

Hormones

All estradiol concentrations were below the detection limit of 0.075 ng/ml plasma. In the diestric phase, BALB/c females kept separate from males had a mean concentration of 3.7 ± 3.4 ng progesterone/ml plasma ($n=22$); and those housed under the influence of male pheromones/urine had 5.0 ± 3.9 ng progesterone/ml plasma ($n=20$). The difference between these mean values was not significant (unpaired *t*-test: $P>0.05$). In series B, in which the blood was taken without consideration of estrus phases, females kept separate from males had 7.35 ± 5.76 ng progesterone/ml serum ($n=18$) before and 5.16 ± 3.33 ng progesterone/ml plasma ($n=13$) after the acute phase of infection. In those females kept alongside males, the corresponding concentrations were 4.93 ± 3.56 ng progesterone/ml serum ($n=18$) and 4.74 ± 2.15 ng progesterone/ml plasma ($n=17$).

In series B, the individual corticosterone levels did not differ significantly between the two maintenance conditions, nor between pre- and post-infection. In detail, the mean serum concentration of corticosterone prior to infection was 98.52 ± 46.04 ng/ml serum ($n=18$) in BALB/c mice kept separated from males and 70.99 ± 44.87 ng/ml serum ($n=18$) in the groups kept alongside males (unpaired *t*-test: $P>0.05$). Considering exclusively data from mice which survived the infection, the mean serum or plasma concentration in BALB/c mice kept separated from males was 105.01 ± 47.72 ng/ml serum prior to infection and 73.51 ± 47.72 ng/ml plasma afterward ($n=13$; unpaired *t*-test: $P>0.05$). In females kept alongside males, the concentration of corticosterone was 72.31 ± 45.83 ng/ml serum prior to infection and 103.14 ± 78.17 ng/ml plasma ($n=17$) afterward (unpaired *t*-test: $P>0.05$). For both maintenance conditions, the female within the group with the highest corticosterone level before infection also had the highest plasma corticosterone concentration afterward in five of ten groups; and, in two groups, she had a medium concentration afterward.

Estrus cycles

In uninfected BALB/c mice kept with or without male pheromones, the mean durations of an estrus cycle were 6.7 days and 6.1 days, respectively; and the respective mean numbers of estrus within 16 days were 2.1 and 2.2 (Table 1). Only the duration of estrus was significantly affected, being longer in females housed in the neighborhood of males (2.6 days) than in females separated from them (1.9 days; unpaired *t*-test: $P<0.05$).

Table 1 Estrus cycles in non-infected BALB/c and C57Bl/6 females under different housing conditions. Estrus data were obtained from vaginal smears of the mice in series A. In each column, values followed by the same letter differ significantly (unpaired *t*-test: $P<0.05$). *n* Number of females, *SD* standard deviation

Strain of mice/ housing condition	Mean duration of estrus cycle ± SD (days; with *n* in parentheses)	Mean number of estrus in 16 days ± SD (with *n* in parentheses)	Mean duration of estrus ± SD (days; with *n* in parentheses)
BALB/c mice/without males	6.1 ± 0.9 (8)	2.2 ± 1.1 (10)[a]	1.9 ± 0.6 (9)[c,d]
BALB/c mice/alongside males	6.7 ± 0.9 (7)	2.1 ± 0.9 (8)[b]	2.6 ± 0.7 (7)[c]
C57Bl/6 mice/alongside males	5.7 ± 1.1 (9)	3.3 ± 0.7 (9)[a,b]	2.9 ± 0.4 (8)[d]

In C57Bl/6 females, the mean duration of the estrus cycle did not differ significantly from the corresponding groups of BALB/c females (unpaired *t*-test: $P > 0.05$). However, the mean number of estrus within 16 days was 3.3, differing significantly from the values of both BALB/c groups (unpaired *t*-test: $P < 0.05$) (Table 1). In the C57Bl/6 mice, estrus had a mean duration of 2.9 days. Furthermore, there was a tendency to synchronize the estrus cycles within a group, which did not occur in BALB/c females. The results obtained for BALB/c mice in series A were confirmed in series B.

During the acute phase of infection, the appearance of the smears changed (Fig. 1). Anestrus occurred with simultaneous phlegm production, but so did persistent cornification (examples of the latter are, e.g., mice nos. 7 and 10 among the BALB/c females kept without male odor till the day of infection and mice nos. 5 and 8 kept alongside males). The intensity of anestrus/phlegm production was correlated with the intensity of blood parasite burden, i.e., because of their relatively low parasitemia, BALB/c mice only occasionally manifested anestrus/phlegm production. After the acute phase in BALB/c mice, regular cycling was re-established very slowly.

Fig. 1 Appearance of estrus and cornification in *Trypanosoma cruzi*-infected BALB/c or C57Bl/6 mice kept under different housing conditions (series A). *Circles* indicate days of estrus or persistent cornification which were not considered as regular estrus when lasting longer than three days; and *cross* indicates the day of death. *Between days 65 and 78 post-infection (*d.p.i.*), vaginal smears were not taken. **Number of parasites/100 microscopic fields at the peak parasitemia and (*in brackets*) the d.p.i. of maximum parasitemia

In the C57Bl/6 mice, which are more susceptible to the *Trypanosoma cruzi* strain "Tulahuén" than BALB/c mice, estrus almost completely disappeared and phlegm production was strong (Fig. 1).

Development of infection

In series A, the first parasites could be detected at 22–32 dpi. The mean prepatent period of mice kept separated from males before the infection was shorter but did not differ significantly from the mean value of females which had been kept for the whole time in the neighborhood of males (Table 2). Comparing the individual courses of parasitemia in both groups kept separated from males showed that two of five females possessed high numbers of parasites, while the others were at the level of females kept alongside males (Fig. 2A). While the mean parasitemia peaked at 14.2 flagellates/100 microscopic fields (magnification 400x) in the group of females which had been separated from males before infection, the mean maximum of the mice not separated from males was about 65% lower and did not show a clear peak, but rather developed a plateau at a low level of parasitemia (Fig. 3). The parasite burdens, expressed as the area under the curve of parasitemia, differed significantly between the two experimental groups ($P < 0.0001$; Table 2, Fig. 3).

In series B, in which the mice were kept throughout with or without males even after infection, again the prepatent periods did not differ between the two experimental groups (Table 2). The first parasites appeared in

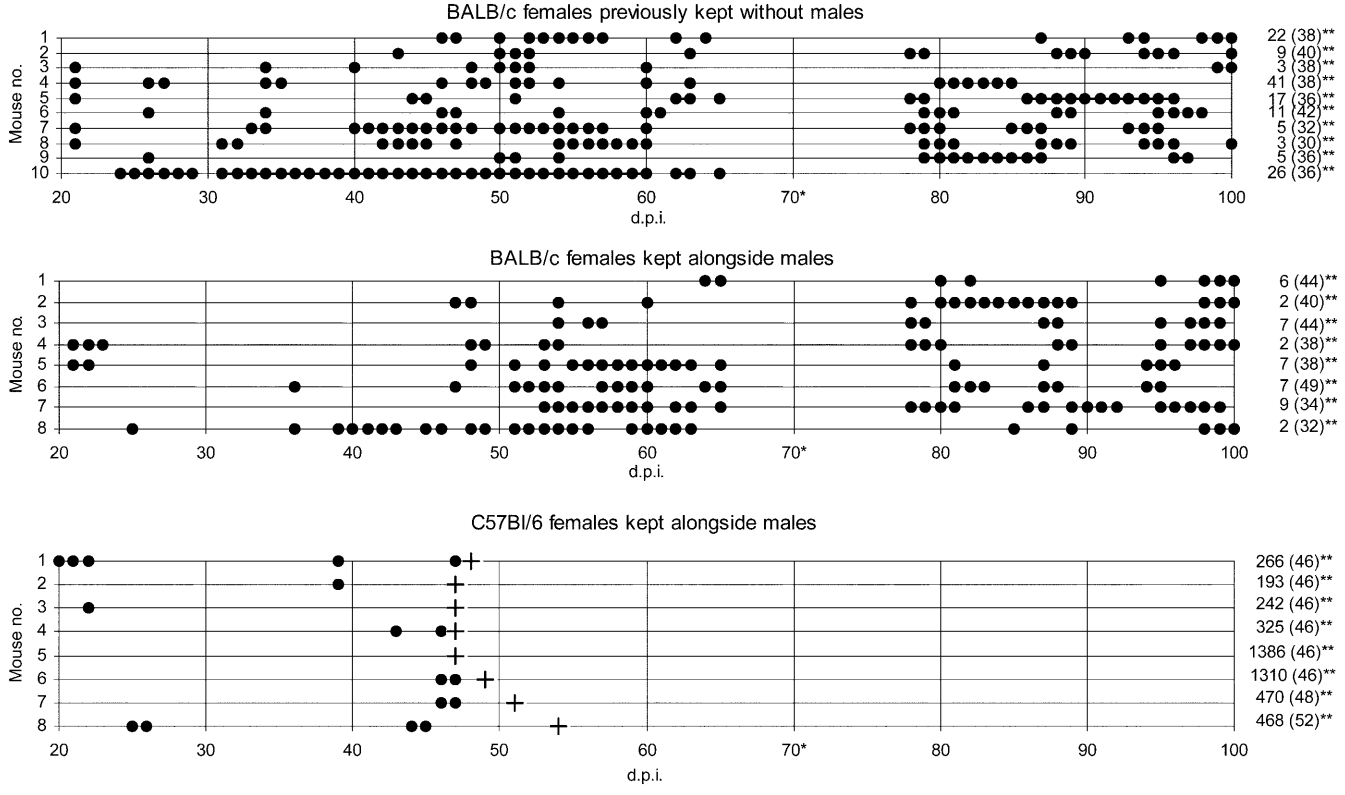

Table 2 Threshold data of parasitemia in female BALB/c mice under different housing conditions. *Series A*: two groups were maintained throughout in the same room as males, two other groups could not smell males (from separation from their mothers until the day of infection). *Series B*: four groups were maintained throughout in the same room as males and another four groups could not smell males at any time. As a measure of the *parasite burden*, the area under the parasitemia curve was calculated; and the unit (mm^2) is the product of [x-axis value (mm) × y-axis value (mm)]. *n* Number of female mice. P_{max} Maximal number of flagellates during the acute phase of infection per 100 microscopic fields (magnification 400×; 1 flagellate/microscopic field≙1×10^6 flagellates/ml blood)

Series	Housing conditions	*n*	Prepatent period (days)		P_{max}		Parasite burden	
			Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
A	Without males	10	26 ± 2.4	22–30	14.2 ± 11.8	3–41	112.5 ± 76.42	22–234
	Alongside males	8	28 ± 2.3	24–32	5.3 ± 2.6	2–9	37.0 ± 19.13	10–56
B	Without males	18	26 ± 2.8	21–31	6.6 ± 4.8	1–17	52.81 ± 42.91	8–159
	Alongside males	17	25.4 ± 3.3	17–33	4.5 ± 2.3	2–11	37.47 ± 21.51	14–58.5

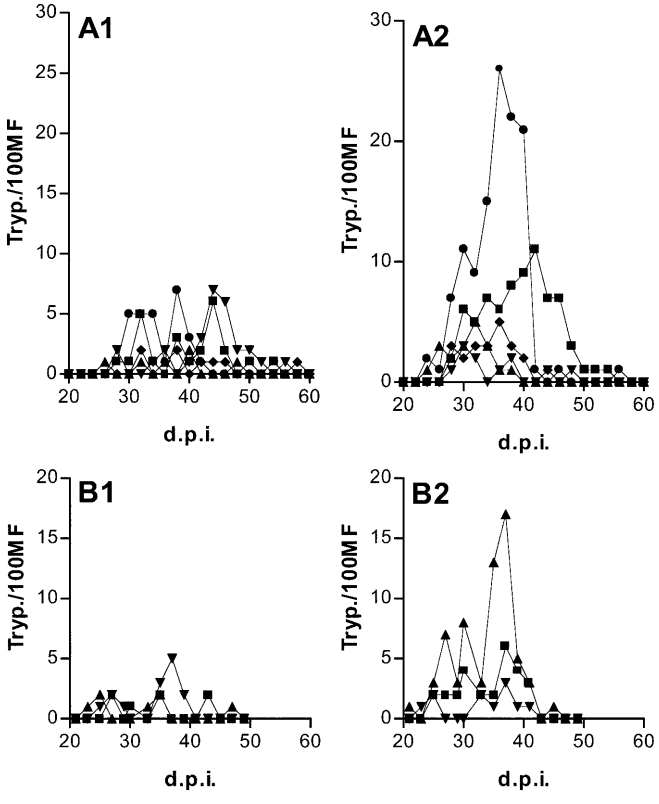

Fig. 2 Individual courses of parasitemia in representative groups. *A1* Females of series A kept alongside males, *A2* females of series A kept without males prior to infection, *B1* females of series B kept alongside males, *B2* females of series B kept without males. *Tryp./100MF* Number of trypanosomes/100 microscopic fields at a magnification of 400× (100 Tryp./100MF are approximately 10^6 trypanosomes/ml blood)

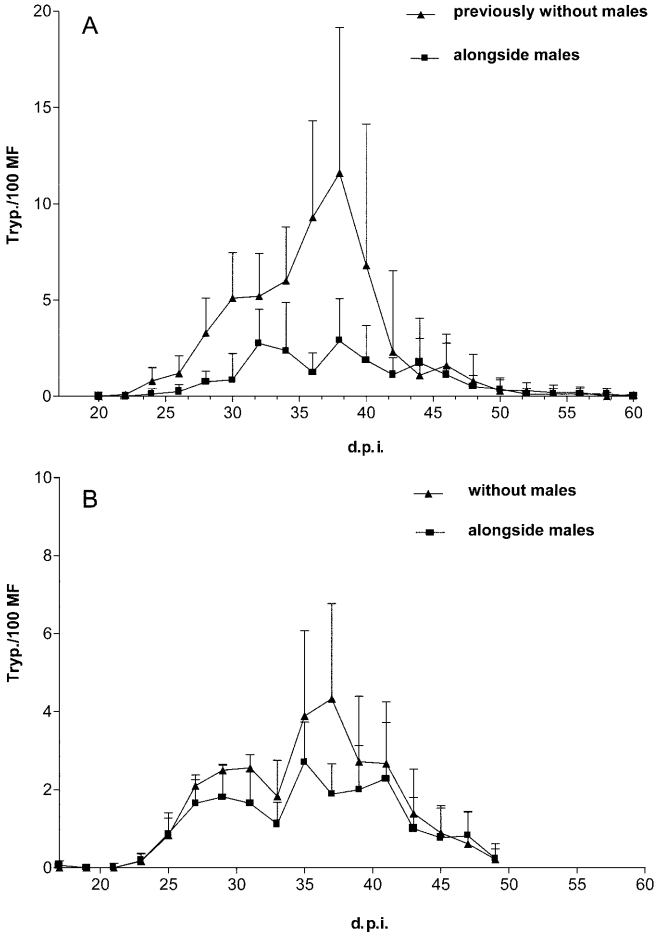

Fig. 3 Mean parasitemia in two series (**A**, **B**) of female BALB/c-mice with or without males (for details, see Table 2). Error bars indicate 95% confidence interval

the blood at 17–33 dpi. The maximum parasitemia reached a mean value of 6.6 flagellates/100 microscopic fields in females separated from males and, in the other group kept alongside males, it reached 4.5 flagellates/100 microscopic fields. The mean parasite burdens did not show significant differences between the two maintenance conditions ($P > 0.05$; Fig. 3). However, in groups of females kept without males in their neighborhood, standard deviations were much higher and usually within a group only one female of three developed a high parasitemia (Fig. 2). The mean parasite burdens of these females were significantly higher than those of the middle or lower classified females (Mann–Whitney *U*-test: $P < 0.05$). In a comparison of groups kept alongside males, these differences were not statistically significantly different ($P > 0.05$). Comparing only the females with the highest parasite burden, the mean for females maintained without males was statistically significantly higher ($P < 0.05$; Table 2).

Where a ranking was possible, i.e., if in pairwise comparisons the lower value was $<90\%$ of the higher one then, in five of twelve groups, the female with the highest progesterone concentration before infection had the lowest parasite burden; and in seven groups there was a middle ranking of parasite burden. The opposite, i.e., a correlation of a low progesterone level with high or middle parasite burden occurred in seven and two groups, respectively, but, in two groups, the females with the lowest progesterone concentrations also had the lowest parasite burdens. In all four groups of females kept separated from males, low parasite burdens were correlated with the highest plasma corticosterone concentrations within the group before infection. The same was found for three of six groups of females kept alongside males. In a comparison for corticosterone concentrations after the acute phase of parasitemia, in five of eight groups a low ranking of the parasite burden was found for the individual with the highest hormone concentration; and a middle rating was found for the other three groups. In only one of these five groups of three females was there a higher ranking corticosterone concentration in a female with a high parasitemia ranking. However, this exception was like the others after the acute phase.

Discussion

When female mice are kept in groups without male odor, their estrus cycles tend to slow down, i.e., the duration of each estrus cycle increases and eventually the cycles stop. This effect, described several times for different strains of mice, including C57Bl and ICR/Albino, is called the Lee–Boot effect (van der Lee and Boot 1955, 1956; Whitten 1959; Ma et al. 1998). In the present investigation, the duration of estrus cycles and the mean number of estrus did not differ between the different experimental groups of BALB/c mice, although the mean duration of estrus was significantly shorter in BALB/c females maintained without males. This shortened period of fertility, which has not been calculated in previous investigations, might be an indication of the Lee–Boot effect. However, the missing suppression of estrus cycles may be due to the small group sizes (three or five animals/cage) chosen during the present investigation, since this effect has been described as being more obvious in large groups (Whitten 1959; Jemiolo et al. 1986).

While we found a slight indication for a Lee–Boot effect, there was no indication in BALB/c females for a Whitten effect, i.e., the synchronization of cycles within a group of female mice kept alongside males. However, when the males and females were close relatives and therefore genetically very similar, estrus acceleration effects after puberty caused by males could not be observed (Lendrem 1985). In addition, close physical familiarity between mice caused a relationship and behavior between the familiar individuals similar to kinship (Kareem and Barnard 1982; Kareem 1983). Although C57Bl/6 is an inbred strain and the animals used are genetically highly homogeneous, as is also the case for BALB/c, the observed tendency of female C57Bl/6 to synchronize the estrus cycle within a group may be explained by the observation that this strain in general appears to exhibit behavioral patterns quite similar to wild-type mice, e.g., higher aggressiveness and more activity than BALB/c mice.

The estrus cycle is not only affected by maintenance conditions, but also by infections with parasites. In Nagana disease, irregular estrus cycles and anestrus have been observed in goats infected with *Trypanosoma congolense* and ewes infected with *T. vivax* (Mutayoba et al. 1988a, b; Elhassan et al. 1994). This effect, however, did not appear when Fresian Holstein heifers were infected with *T. evansi* (Payne et al. 1993). The extent of estrus cycle abnormalities seems to depend on individual susceptibility to the parasite (Mutayoba et al. 1988a; Payne et al. 1993). Also, in the present investigation, estrus abnormalities occurred during the acute phase of *T. cruzi* infection, correlating with the intensity of parasitemia and disappearing after the acute phase. Considering that African trypanosomiasis is completely different from Chagas' disease in the vertebrate host (e.g., *T. cruzi* develops intracellularly dividing amastigotes and non-reproducing blood trypomastigotes, whereas African trypanosomes exist as very rapidly dividing blood trypomastigotes), the occurrence of anestrus seems to be a non-specific reaction to trypanosome infections.

In *T. cruzi* infections, neither the effects of the parasite on the estrus cycle nor the effects of social behavior on the parasitemia have been considered previously. Some publications have dealt either directly or indirectly with the influence of female sex hormones on the course of disease; and there is published evidence that not only does the gender of the vertebrate host have a protective effect, but also female sexual hormones in particular, especially progesterone, have a protective effect, i.e., they reduce the pathological effects of the *T. cruzi* infection (Hauschka 1947; Tay et al. 1978; Alexander and Stimson 1988; Rivera et al. 1991; Brabin and Brabin 1992; Prado et al. 1998). Survival of the acute phase of *T. cruzi* infection depends at least in part on the host's ability to produce IFN-γ and – as a consequence of the activity of this cytokine – on nitric oxide production by parasitized macrophages (Hölscher et al. 1998). In uninfected mice, both 17β-estradiol and progesterone modulate IFN-γ production and the release of reactive oxygen intermediates (Grasso and Muscettola 1990; Fox et al. 1991; Chao et al. 1994). Although, in the present study, there was no significant difference between the progesterone levels in the uninfected females under different housing conditions, in all groups, the females with the highest progesterone concentrations within the group always had low or middle rankings, but never a high ranking as regards the parasite burden.

The increase in parasitemia in female mice kept without any possibility to smell males, compared to females kept in constant olfactory contact with male urine, was evident in series A and B, although in series A the separation from male odor ended with the day of infection. This seems to indicate that the course for immunoregulation by sex hormones is set around puberty – a time at which the two experimental groups had been strictly separated – and is not altered at all or only slightly later. This possibility is supported by investigations with murine malaria (*Plasmodium chabaudi*), in which testosterone regulates the susceptibility. Male mice are more susceptible than females, but females treated with testosterone also become susceptible, an effect persisting even after withdrawal of testosterone, when normal female testosterone levels have been reestablished (Benten et al. 1997). The fact that the mean parasite burdens differed significantly for females maintained with vs without males in series A but not in series B seems to be caused by the group size difference. In the two groups of five females in series A, two females per group (40%) showed a high parasite burden whereas, in series B with six groups of three females, this effect occurred only in one female per group (30%). Thus, in a higher number of groups of three females each, lower mean values should occur. However, those females of series B kept without males and with the highest parasitemias also showed a statistically significant increase in the parasite burden, compared with the other females of the group or those with the possibility to smell males.

Furthermore, housing and individual social experience itself are able to influence the individual's hormonal state and thus affect the function of the immune system. In adult male rats, after losing ranking fights during a confrontation period of 7 days, there was a reduction of CD4 and CD8 T cells, a lowered NK cell activity and an increased number of granulocytes (Stefanski 1998, 2000; Stefanski and Engler 1999). Moreover, in male Guinea pigs, the activity of the complement system was reduced by social stress (Stefanski et al. 1989; Stefanski and Hendrichs 1996). Social stress itself can be measured by stress hormone levels, such as plasma corticosterone concentrations. In female populations of mice, a hierarchical social structure is established similar to the social structure of pure male populations, although in female groups dominance and sub-dominance are not determined by extensive ranking fights. Nevertheless, in single-sex groups of both sexes, dominant animals show lower plasma corticosterone levels than their subdominant cage mates (Schuhr 1987). In the present investigation, the hierarchical structure of the female populations could not be determined, since the groups had been established directly after separating the individuals from their mothers, resulting in an already existing long-term hierarchy at the start of investigation. Nevertheless, differences in individual plasma corticosterone concentrations were detected. Since corticosterone is known to suppress immune functions, the finding of lowest parasitemia in those animals with the highest plasma corticosterone levels indicates that, even in this system, the cause-and-effect relationship between hormones and resistance to parasites is complex and unlikely to be direct (Barnard et al. 1996). The possibility that the hierarchical position affects the parasitemia in females kept without males is supported by the observation of differences in the individual parasite burden within a group. In the two groups of five females (series A), two mice per group developed a high parasitemia; and in the six groups of three females (series B), one female per group developed a high parasitemia.

On the basis of the results of the present investigation, we suggest considering the housing conditions and social environment as part of every experimental design. We especially recommend also keeping experimental animals under socially standardized conditions. Attention should be paid to intersexual influences caused by pheromones of the opposite sex. In parasitological investigations, e.g., vaccination or other immunological experiments, the use of groups of females kept alongside males seems especially to reduce the variation in parasitemia.

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