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Immunity Costs and Behavioural Modulation in Male Laboratory Mice (*Mus musculus*) Exposed to the Odours of Females

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BARNARD, C. J., J. M. BEHNKE, A. R. GAGE, H. BROWN AND P. R. SMITHURST. *Immunity costs and behavioural modulation in male laboratory mice (*Mus musculus*) exposed to the odours of females.* PHYSIOL BEHAV **62**(4) 857–866, 1997.—In a previous study, male laboratory mice experimentally immunodepressed with anti-thymocyte serum (ATS) showed changes in behaviour (aggression, general locomotory activity, and sleeping) and testosterone secretion that are consistent with decision-making being modulated adaptively with respect to immunocompetence. We tested this idea further by repeating the experiment with the addition of female odours (soiled sawdust) to the home cages of males following ATS/control treatment. We predicted that, in the presence of cues suggesting reproductive opportunity, immunodepressed males would trade off potential immunity costs by failing to modulate behaviour. This expectation was borne out in that ATS-treated mice showed no change in aggression, locomotory activity, mounting, or sleeping relative to control animals, and mice overall showed significant differences in behaviour in the expected direction compared with a previous study in which female odours were not presented. However, despite the lack of difference in behaviour between ATS and control treatments, there was still evidence of a degree of behavioural modulation in relation to measures of immunocompetence. © 1997 Elsevier Science Inc.

Immunodepression Mice testosterone Corticosterone Female odours

RECENT ideas and experimental evidence suggest that current immunocompetence may act as a constraint on behavioural and physiological decisions where these risk imposing an additional burden on immune function (5,6,9,11,26). In particular, feedback mechanisms modulating the secretion of potentially immunodepressive hormones, such as some sex steroids and glucocorticoids (1,12,13), have been viewed as elements in the adaptive trade-off of immune function within the organism's life history strategy (4,11,26,28).

In a series of experiments with male laboratory mice of the CFLP strain, we have shown that aggressive behaviour and serum hormone (testosterone and corticosterone) and total IgG concentrations covary in ways that are consistent with the adaptive modulation hypothesis and influence resistance to an experimental infection of the piroplasmid protozoan *Babesia microti* (3–5). Moreover, interrelationships differ between males of different social status and between social/sexual contexts in accordance with rank-related modulation of hormone secretion and an adaptive trade-off between immunocompetence and apparent reproductive opportunity (4,26).

A consistent finding of our earlier work has been that, while increased aggressive behaviour and testosterone concentration were both associated with reduced immunocompetence and resistance to *B. microti*, they are not themselves significantly correlated (4,5). This suggests that hormonal and behavioural changes may provide independent avenues of response to variation in immune status rather than behaviour reflecting changes in underlying hormonal causal mechanisms [e.g., (30)]. This idea was tested in the context of overall time budgeting and the secretion of testosterone and corticosterone by treating adult male CFLP mice with anti-thymocyte serum (ATS) to depress thymus-mediated immune function (6). In comparison with mice given a vehicle (naive rabbit serum (NRS)) control, ATS-treated mice showed a reduction in serum testosterone concentration, aggressive behaviour, and general activity, but maintained time spent sleeping, relative to pre-treatment levels. Behaviours that differed between treatments correlated with measures of immunodepression, but relationships with behavioural changes were independent of those with testosterone.

If immune function is traded off adaptively against other components of life history, however, we might expect a reduced tendency to modulate potentially immunodepressive decisions if their effects are offset by perceived short-term reproductive gain. In this paper, we report an experiment in which the previously described (6) manipulation of immunocompetence using ATS treatment was repeated but with the addition of female odours (soiled sawdust) to the cages of experimental groups after treat-

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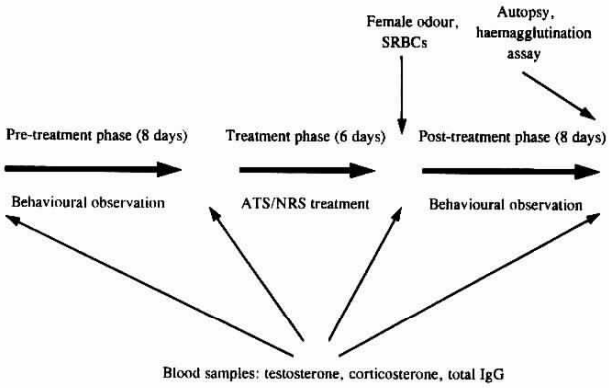


FIG. 1. Flow diagram of the time sequence of the experiment.

ment. The study was conducted as a separate experiment, rather than in a simultaneous design with the previous one (6), because it was important to barrier the "no female odour" treatment from ambient female odours. Several studies have shown effects of even brief or remote exposure to conspecific odours on physiological and behavioural responses among mice and other rodents that are likely to compromise differences in the relationships being sought here [e.g., (10,14,17,18,21,24,25,27,29)]. Of course, separation of odour conditions automatically introduces a potential batch effect, but chance differences between batches can be controlled for in analysis (5). In fact, a significant difference arose in only two variables and was easily taken into account in each case (see Results). Moreover, the differences were less marked than those arising *within* these and other related experiments (6,26).[1] It was found earlier that exposing single male CFLP mice to the soiled sawdust of unfamiliar males and/or females reduced immunocompetence and resistance to *B. microti,* with high-ranking males showing a greater reduction in resistance than low rankers, especially when female odours were combined with those of an unfamiliar male (26). The results reported in (26) can be interpreted in terms of an adaptive redistribution of metabolic resources geared to the likelihood of imminent mating opportunity and aggressive intrasexual activity, and diverting resources away from immune function (23,28). In the context of the present experiment, we expected that the tendency for immunodepressed (ATS-treated) mice to modulate behaviour in relation to controls—in particular reduce aggression and levels of general activity but maintain time spent sleeping—would be reduced, and hence levels of behaviour to be closer to that of control animals than in our earlier experiment. Since female odours were introduced to group cages only after treatment (vide infra), we did not expect any reduction in the tendency to modulate testosterone concentration during the period of treatment itself [see (6)]. However, on the basis of previous work with male mice exposed to female odours [footnote 1 and (7,26)], we expected an increase in testosterone levels over the post-treatment period of grouping, both within this experiment and relative to the previous study (6).

METHODS

The methods followed exactly those previously described (6) except for the addition of soiled sawdust from the cages of female mice during the post-treatment period of grouping (see later and Fig. 1). Mice were from the same breeding stock as those in the previous experiment (6) and were used at exactly the same age.

Pre-experimental Procedure

The subjects were 64 male laboratory mice of the randomly bred CFLP strain [see (2)] purchased from Bantin and King Ltd, Hull, UK. Twenty-three female CFLP mice of the same age as the males were also purchased from Bantin and King Ltd at the same time and immediately established in groups of 7–8 in large polypropylene cages (48 × 28 × 13 cm). At 42 days of age, groups of four males were established in polypropylene cages (12.5 × 45 × 14 cm) for 3 weeks to acclimatize to laboratory conditions. From purchase and throughout the experiment all animals were maintained on a 12 h–12 h reversed light–dark cycle (lights on at 2000 hours, lights off at 0800 hours) and males and females were housed in separate rooms. Food (Harlan Tetrad TRM rat/mouse diet) and water were provided ad lib. At 60 days of age, males were weighed, given individually distinctive fur marks using Clairol "Nice 'n' Easy, Natural Black" hair dye (Bristol Myers Ltd, Uxbridge, UK) and an 88-μL sample of blood was taken from each animal (Fig. 1) using the tail-sampling procedure previously described [footnote 1 and (26)].

Pre-treatment Groups

Three days after the pre-experimental blood samples were taken, the males were reallocated arbitrarily to 16 groups of four individuals (cage dimensions as for pre-experimental groups) ensuring that no individuals within a group had previously encountered each other. Mice remained in their pre-treatment groups for 8 days (Fig. 1) during which the amount of time spent in different behaviours was recorded in two ways following the methods described in (15) and previously established behaviour categories (16,20). First, all social and nonsocial behaviours (Table 1) performed by each individual were recorded using instantaneous spot-checks. Observations of groups (totalling 21 h) were randomized through the 12-h dark phase and carried out under dim red illumination (2). Each mouse was observed as a focal animal for a total of 160 spot-checks over the 8 days during which the behaviour being performed at the moment of observation was recorded on a check sheet. Since social behaviours in particular tend to be brief, they are likely to be underrepresented in spot-check samples (15). In addition, therefore, groups were observed continuously for two 5-min periods per day each (a total of 16 h of observation, again randomized through the dark phase) to record the social behaviours initiated and received by each individual and the identity of the other mice with which it interacted. In the case of each aggressive interaction, the degree of escalation involved was recorded on an arbitrary scale ascending from 1 (Threat, usually with no physical contact) to, rarely, 5 (escalated aggression involving Biting and Chasing) (see Table 1).

Treatment Procedure

At the end of the 8-day pre-treatment period, males were weighed again and a second 88-μL blood sample was taken from the tail (Fig. 1). Pre-treatment groups were then allocated randomly to one of two treatment batches (eight groups each) and mice were separated and housed singly for 6 days in the same sized cages as pre-treatment groups.

[1] Smith, F. V. Behaviour and immune function in laboratory mice (*Mus musculus*). Nottingham: University of Nottingham; 1996 (Unpublished Ph.D Thesis).

TABLE 1

BEHAVIOUR CATEGORIES RECORDED DURING THE EXPERIMENT

Social investigation: A composite category combining the olfactory investigatory behaviours Sniff, Nose, and Body (sensu 14,16)
Aggression: A composite category combining Offensive upright, Offensive sideways, Threat, Circling, Bite, and Chase (sensu 14,16)
Defensive behaviours: A composite category combining Freeze, Flee, Defensive sideways, and Defensive upright (sensu 14,16)
Allogroom: A mouths and licks the fur of B, mostly on the back and nape
Mount: A mounts, or attempts to mount, B from the rear
Eat: Consumes food from the food hopper or elsewhere in the cage
Drink: Drinks from the water hopper
Sleep: Lying or sitting unalert and eyes observed or presumed (when head of focal mouse obscured or in huddle or under food hopper) to be closed
Groom: Grooms genitalia or other body parts, including washing of the face with forepaws
Mobile: Movement around the cage, including running and ambling
Sniff sawdust: Sniffs the sawdust on the floor of the cage
Dig sawdust: Digs in the sawdust with forepaws or kicks the sawdust with hind legs
Crouch: Crouches on hind quarters
Climb: Climbs up the side of the cage or on the wire lid of the cage with all feet off the ground
Sniff air: Sniffs the air, not directed towards any other animal or part of the cage
Sniff towards observer: Sniffs the air in direction of the observer, normally whilst climbing against the side of the cage
Upright scan: Stands upright on back legs, usually sniffs the air
Investigate cage: Sniffs the plastic sides of the cage, usually mobile
Jerk: Uncontrolled sudden movement from a standing position, sometimes flipping over completely or followed by fast running.

On the day following separation, eight groups (32 mice) were injected i.p. with 0.5 mL of ATS (ATS-treated mice) and the remaining eight groups were injected with 0.5 mL of NRS (NRS control mice). These injections were repeated on Days 2 and 4 of separation, except that the volume of serum given was halved on Day 2 (0.25 mL). Two further blood samples were taken. A 20-μL sample was taken from the tail 1 day after the first ATS/NRS injection for white blood cell counts. A 50-μL retro-orbital sample [cf. (2)] was then taken on Day 5, i.e., 1 day after the third injection of ATS/NRS. On the final day of treatment, animals were again weighed and all individuals were injected with 0.2 mL of a sheep erythrocyte suspension (SRBC) containing 25×10^7 SRBC/mL (each mouse receiving 5×10^7 SRBC).

Preparation and Preliminary Evaluation of Anti-thymocyte Serum

Anti-mouse thymocyte serum was prepared by a modification of a previously reported procedure (19). Adult female New Zealand white rabbits were injected with a thymocyte cell suspension prepared from eleven 4-week-old male C57BL/10 mice [ca. $2–5 \times 10^8$ thymus cells/rabbit], using aseptic techniques as described for lymphocytes (8), differing only in that cell suspensions were prepared in RPMI medium without foetal calf serum. Rabbits were injected intravenously into the lateral ear vein with the required number of cells in a volume not exceeding 0.9 mL. This procedure was repeated 2–3 weeks later and the rabbits were exsanguinated after a further 7 days. The serum was separated after clotting at 4°C, heat inactivated at 56°C for 45 min, aliquotted, and stored at −80°C until required. Note that thymocytes were obtained from a different mouse strain to the CFLPs used in the experiment to minimize cross-reactivity with tissue antigens other than those of T-lymphocytes. NRS was obtained from female New Zealand white rabbits of approximately the same age.

As in the previous experiment (6), the efficacy of the ATS preparation in reducing thymocytes was confirmed through tests carried out prior to its use in the experiment. Clumping and lysis of target thymocytes were demonstrated in vitro in the presence of complement (1/200 final dilution of ATS caused 61% reduction in cells in the presence of 1/40 complement and 4.4×10^7 thymocytes from 4-week-old male CFLP mice).

In a pre-experimental trial evaluation of the in vivo consequences of injecting our ATS preparation, a group of three CFLP males were injected i.p. either with ATS or the same volume of NRS (0.125, 0.25, and 0.5 mL, respectively). Peripheral white blood cell concentrations were measured a day before and after injection using standard techniques. Injection with ATS at all volumes caused a reduction in the concentration of peripheral white blood cells averaging 26.1%, whereas injection of NRS to three control males resulted in a mean increase of 11.2% [cf. an average decrease of 49.4% and increase of 18.0%, respectively, in (6)].

Post-treatment Procedure

Forty-eight hours prior to males being reestablished in their groups after treatment, the 23 females were separated, housed singly in smaller cages ($12.5 \times 45 \times 14$ cm), and maintained on a substrate of 144 cm^3 of sawdust. The day after the males were injected with SRBCs, a mixture of 72 cm^3 of soiled sawdust from the cage of two of the females and 144 cm^3 of clean sawdust (making the same total volume as in the pre-treatment phase) was introduced into the cages in which the pre-experimental groups of males were then immediately reestablished. Behavioural observations of the males were then repeated as for pre-treatment groups for a further 8 days (Fig. 1). Every 48 h throughout the 8 days, each group of males received a fresh mixture of soiled and clean sawdust from its donor females as above, thus receiving five samples in total over the post-treatment period. After each donation, females were placed in new cages with clean sawdust so that males always received samples of the same age. To balance the design, one cage of ATS-treated males and one of NRS controls received sawdust from the same two females.

Organ Weights and Blood Assays

At the end of the post-treatment observation period, males were weighed for the final time, killed using chloroform, and exsanguinated. The kidneys, adrenal glands, spleen, thymus gland, testes, preputial glands, seminal vesicles, heart, and mesenteric lymph nodes of each individual were carefully dissected out and weighed.

Haematology and Haemagglutination Assays

The packed cell volume (PCV) of blood samples was determined with a standard PCV reader after centrifugation of blood samples in capillary tubes in a haematocrit centrifuge.

Sheep erythrocytes (SRBC) were obtained fresh in Alsever's solution and washed three times in phosphate-buffered saline (PBS). The concentration of cells was determined by standard haemocytometry and the cells were resuspended to the required concentration in sterile PBS. For determination of the antibody response to SRBC, heat-inactivated (56°C for 45 min) mouse sera were titrated out by serial doubling dilution on plastic microtitre

TABLE 2

MEAN ± SE SERUM CONCENTRATIONS OF TOTAL IgG, TESTOSTERONE, AND CORTICOSTERONE FOR ATS-TREATED AND NRS CONTROL MICE AT EACH SAMPLING POINT DURING THE EXPERIMENT*

		ATS	NRS
Total IgG			
(mg/L)	Pre-experiment	1721 ± 116 (12)	1736 ± 107 (16)
	Pre-treatment	3110 ± 261 (12)	3075 ± 242 (16)
	Post-treatment	3267 ± 280 (12)	4066 ± 334 (16)
	Termination	10025 ± 885 (12)	11542 ± 713 (16)
Testosterone			
(ng/mL)	Pre-experiment	8.6 ± 2.5 (12)	11.9 ± 2.4 (16)
	Pre-treatment	5.5 ± 1.6 (12)	5.1 ± 1.5 (16)
	Post-treatment	5.4 ± 1.9 (12)	7.6 ± 2.0 (16)
	Termination	2.3 ± 0.9 (12)	1.7 ± 0.6 (16)
Corticosterone			
(ng/mL)	Pre-experiment	36.9 ± 3.3 (12)	42.9 ± 3.8 (16)
	Pre-treatment	39.7 ± 3.5 (12)	54.1 ± 5.3 (16)
	Post-treatment	38.3 ± 5.4 (12)	39.8 ± 4.7 (16)
	Termination	181.9 ± 26.8 (12)	161.1 ± 19.9 (16)

* Means based on average values for each rank category per cage (see Methods). Sample sizes in parentheses.

plates, the first well in each titration containing 40 μL of neat serum in PBS. A 3% (v/v) washed SRBC suspension in a volume of 20 μL was added to each well, the plates were left to stand at 37°C for 2 h, and the endpoint for haemagglutination was read by visual inspection.

Measurement of Serum Hormone and IgG Concentrations

Pre-experimental, pre-treatment, post-treatment, and terminal blood samples were assayed for serum concentrations of testosterone, corticosterone, and total IgG following previously reported procedures (2–5).

Testosterone. The concentration of testosterone (ng/mL) was measured using a Coat-a-Count solid-phase ^{125}I total testosterone kit (Diagnostic Products Corporation, Los Angeles, USA) using 25-μL samples of undiluted serum for experimental and calibration assays. Testosterone concentrations were calculated by reference to the calibration curves.

Corticosterone. The concentration of corticosterone (ng/mL) was measured using 6-μL samples of undiluted serum and a Gamma-B ^{125}I-corticosterone kit (Immunodiagnostic Systems Ltd., Los Angeles, USA) based on double antibody radioimmunoassay as advised by the manufacturers. Corticosterone concentrations were calculated by reference to standards provided with the kit.

Total IgG. Serum total IgG concentration (mg/L) was determined by a previously reported method (22) using radial immunodiffusion kits (The Binding Site, Birmingham, UK). Ring diameters were measured in two directions at 90° and the mean was used to calculate the concentration of immunoglobulins from a calibration curve obtained using appropriate standards.

In a small number of cases, limited serum volumes meant it was not possible to obtain a reliable estimate of all three serum factors from a particular sample. As a result, sample sizes in some subsequent analyses vary [see also (2–5)].

Statistical Analyses

All analyses were carried out using Statgraphics Plus version 7 (Manugistics Ltd., Rockville, MD, USA). Parametric analyses were used throughout (data were $\log_{10}$ or square root transformed as necessary and tested for normality using a Kolmogorov–Smirnov one-sample test). Wherever there were a priori reasons for expecting trends or differences in a particular direction, probabilities associated with significance tests are indicated as one-tailed.

RESULTS

As in our previous experiments [e.g., (4–6)], high- and low-rank categories were defined on the ratio of attacks initiated and received by each male during the pre-treatment period of grouping. Once again, high-ranking males were identifiable as having high initiation:receipt ratios and initiating significantly more attacks over the pre-treatment period (mean ± SE no. initiated by high rankers = 6.00 ± 1.07; by low rankers = 0.80 ± 0.21; $F(1, 26) = 22.83$, $p < 0.001$). Four groups subsequently given ATS treatment were omitted retrospectively from further analysis because the incidence of pre-treatment aggression turned out to be too low to rank males reliably. All other groups comprised one high-ranking and three low-ranking males [see also (6)]. All analyses relating to social rank were based on high- and low-rank categories, and data for low rankers were averaged within cages to control for potential problems of nonindependence (4–6).

Pre-experimental Differences in Physiological Measures and Body Weight

A series of two-way analyses of variance (ANOVA), with pre-experimental PCV and serum concentrations of testosterone, corticosterone, and total IgG as dependent variables and pre-experimental body weight as a covariate, revealed no chance significant differences between males subsequently allocated to different treatments [cf. (6)]. One-way ANOVAs combining data from the present experiment and data from the previous experiment (6) showed no significant differences between experiments in pre-experimental concentrations of testosterone or total IgG. However, mice in the present experiment showed significantly lower concentrations of corticosterone [$F(1, 53) = 13.47$, $p < 0.001$; see Table 2 of the present study

and Table 2 of the previous study (6) for mean values] and slightly, but significantly, greater body weight (mean ± SE for the present experiment = 33.57 ± 0.46 g, for the previous experiment = 31.70 ± 0.24 g, $F(1, 54) = 13.16$, $p < 0.001$). Differences in corticosterone and body weight were thus controlled for in combined analyses of hormonal and immune responses and (body weight only) aggressive behaviour by including corticosterone and body weight measures at the appropriate time points as covariates or independent variables.

Effects of Treatment on Measures of Immunocompetence

As in the earlier study (6), a reduction in immunocompetence following ATS treatment was confirmed by significant differences between ATS-treated mice and NRS controls in terminal haemagglutination responses to SRBCs (mean ± SE titres (1/dilution) for ATS mice = 0.36 ± 0.25, for NRS mice = 45.15 ± 7.72, $F(1, 24) = 24.76$, one-tailed $p < 0.0001$) and the change in total IgG concentration over the period of treatment (i.e., post- minus pre-treatment values) ($F(1, 24) = 3.76$, one-tailed $p < 0.05$, Table 2). As in the earlier study, total IgG concentration increased significantly during pre- and post-treatment periods of grouping in both ATS and NRS mice (paired t-test, $p < 0.001$ in all cases, Table 2). During the period of isolation and treatment, however, total IgG increased significantly only among NRS controls (paired $t(15) = 3.02$, $p < 0.01$, Table 2). There were no significant differences between ATS and NRS animals in the pre-treatment degree of increase in total IgG and no significant main or interaction effects of rank category on any measure of immunocompetence. Three-way ANOVA (experiment × treatment × rank) on combined data from the present experiment and data from the previous experiment (6) showed no significant experiment/treatment interaction for either haemagglutination titre or change in IgG so that the relative effects of ATS/NRS treatment did not differ between the experiments. However, the magnitude of the change in IgG across all mice during the period of treatment within experiments was significantly greater in the first experiment (6) ($F(1, 47) = 9.57$, $p < 0.01$). Although haemagglutination responses and treatment-phase IgG concentrations were damped in ATS mice, there was no significant difference between treatments in thymus weight [cf. (6)]. Further analysis showed that this was due to lower than expected relative thymus weights among NRS controls rather than higher than expected weights among ATS mice. Values for ATS mice in the present experiment did not differ significantly from those for ATS mice in the previous experiment (6) (mean ± SE thymus weight (percent body weight) for ATS mice in the present experiment = 0.097 ± 0.006; in the previous experiment = 0.091 ± 0.008; $F(1, 24) = 0.34$, NS). Values for NRS mice, however, were significantly different at the 10% level (mean ± SE weight for the present experiment = 0.097 ± 0.004; for the previous experiment = 0.139 ± 0.028; $F(1, 24) = 3.04$, $0.1 > p > 0.05$). In addition, the variance in thymus weight was lower for both treatments in the present experiment (coefficient of variation for the present experiment: ATS = 22.18, NRS = 18.16; for the previous experiment: ATS = 36.04, NRS = 69.77).

Effect of Treatment on Serum Hormone Concentrations

As in our previous experiments with CFLP mice [e.g., (4–6)], testosterone concentration tended to decrease during periods of grouping (pre- and post-treatment), while corticosterone concentration tended to increase (Table 2). However, the changes were significant only among NRS animals (pre- and post-treatment decrease) in the case of testosterone, and only for post-treatment increase (both ATS- and NRS-treated) in the case of corticosterone (paired t-test, $p < 0.05$). ANOVA on combined data showed no significant difference between the experiments in the change in testosterone concentration during pre- and post-treatment periods of grouping or in concentrations at the end of each period of grouping. Correlation analysis of relationships between change in testosterone and change in corticosterone concentrations over different phases of the experiment revealed a significant (positive) correlation during the period of treatment ($r(55) = 0.34$, $p < 0.05$), but none over the pre- or post-treatment periods. The association during treatment was therefore taken into account in analyses of relationships between hormone concentrations and immune function.

Previously a significant difference between ATS and NRS mice in the change in testosterone concentration over the period of treatment was found (6), with ATS animals showing a reduction compared with an increase among NRS controls. Table 2 shows a tendency for mean testosterone concentration in the two treatments over the same period to change in the same directions as in the previous experiment, but the difference between treatments was not significant.

The degree of reduction in testosterone concentration among ATS animals in the earlier experiment (6) was associated with the decrease in thymus weight apparently resulting from ATS treatment. These results were interpreted in terms of thymus-mediated modulation of testosterone secretion by immunocompromised ATS mice. Here, however, stepwise partial regression analysis of the relationship between change in testosterone over the period of treatment and immunocompetence measures (change in IgG concentration, thymus weight (as percent terminal body weight), and haemagglutination titre, with change in body weight and corticosterone concentration during treatment as additional independent variables) failed to reveal any significant associations, either overall or when treatments were analyzed separately.

No significant effects of treatment emerged for corticosterone concentration.

Effect of Treatment on Body and Organ Weights

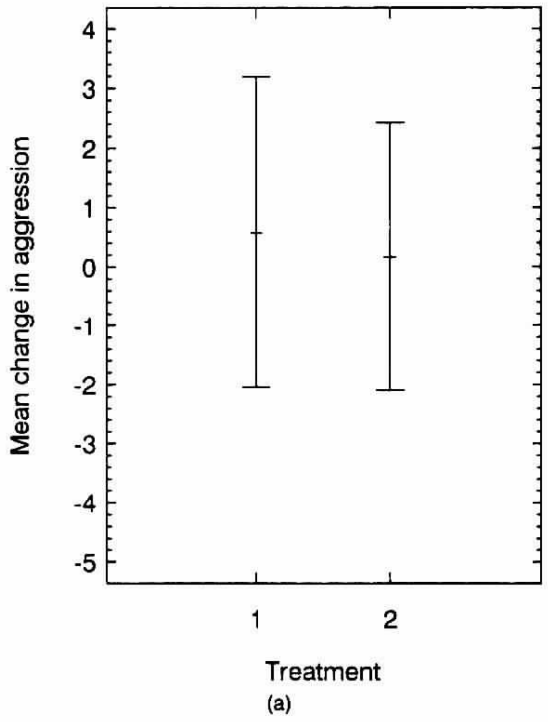
There was no significant difference between treatments in either pre- or post-treatment body weight and no difference in any post-experimental organ weights [cf. (6)].

Effect of Treatment on Behaviour

The pattern of change in behaviour between pre- and post-treatment periods of grouping was very different from that in our previous experiment when no female odour was introduced into cages.

There was now no significant overall reduction in the amount of aggression initiated (paired $t(27) = 0.30$, NS) and no difference in the change (post- minus pre-treatment) between ATS and NRS treatments (Fig. 2a). Despite a significant reduction in immunocompetence measures, and particularly reduced levels of total IgG relative to NRS mice [see (6)], therefore, ATS-treated mice showed no tendency to down-regulate aggressive behaviour. ANOVA on combined data from this experiment and earlier data (6) showed that this resulted in a significant difference between experiments in the change in aggression between the two periods of grouping, as expected from the difference in exposure to female odour ($F(1, 47) = 2.98$, one-tailed $p < 0.05$, Fig. 2a,b), and a significantly higher incidence of aggression in post-treatment groups (treatments combined) ($F(1, 47) = 8.43$, $p < 0.01$). However, there was no significant increase in the degree of escalation (level) of aggression following treatment [cf. (6)].

Previously a significant increase in the amount of time spent sleeping among ATS-treated mice was found (6) (Fig. 3b). When female odour was provided, however, there was a pronounced



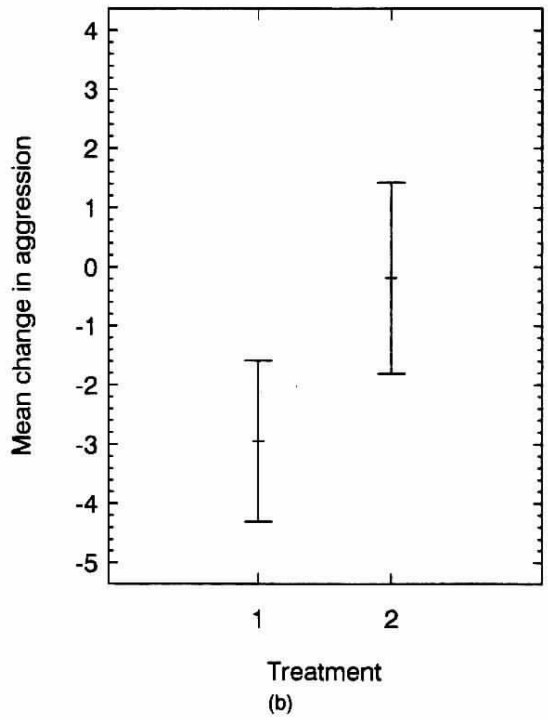
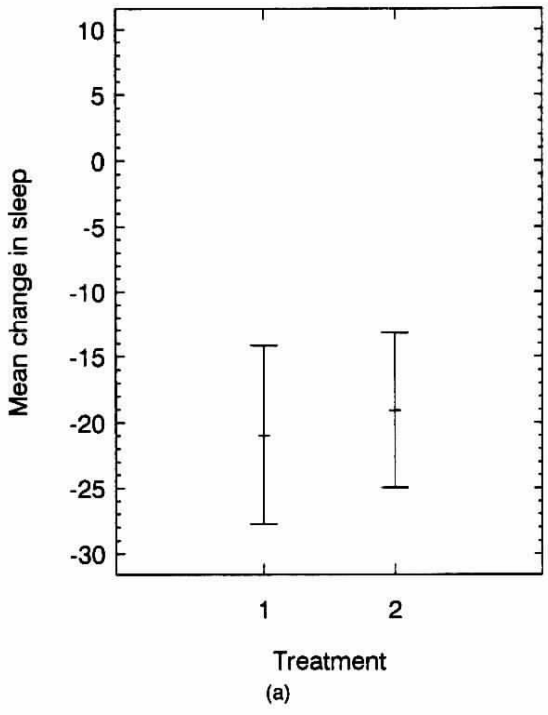


FIG. 2. Least-squares means from ANOVA of the change (post- minus pre-treatment) in the number of aggressive interactions initiated by ATS-treated (Treatment = 1) and NRS-treated (Treatment = 2) mice: (a) in the present experiment; (b) in the previous experiment (6). Bars are least-squares deviations. See text.



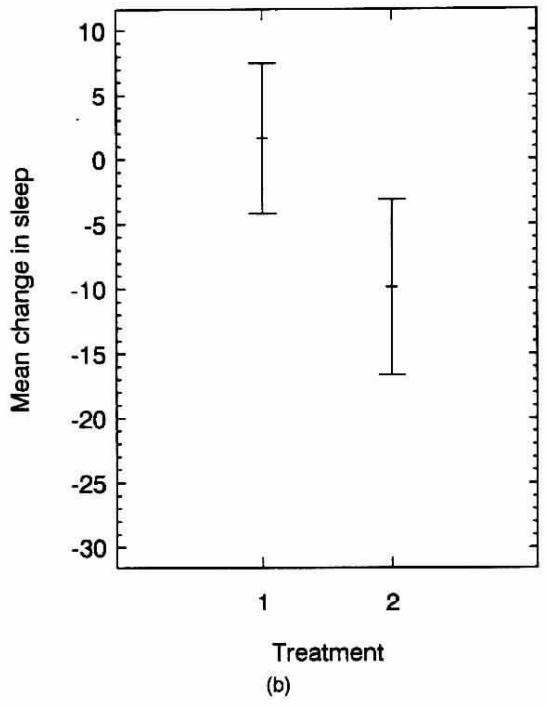


FIG. 3. Least-squares means from ANOVA of the change (post- minus pre-treatment) in the number of times observed sleeping by ATS- and NRS-treated mice: (a) in the present experiment; (b) in the previous experiment (6). Treatments and bars as in Fig. 1. See text.

decline in time spent sleeping (paired $t(27) = 6.64$, $p < 0.0001$) and no difference between ATS and NRS mice (Fig. 3a). ANOVA of combined data from the two experiments showed that the difference in the change in sleep between experiments was significant ($F(1, 47) = 11.47$, one-tailed $p < 0.01$, Fig. 3a,b) and that the amount of sleep in post-treatment groups was significantly lower overall in the present experiment ($F(1, 47) = 7.45$, $p < 0.01$). Associated with the reduction in sleep was a significant increase in general locomotory activity (combined mobile, climb, dig in sawdust, and investigate cage in Table 1, paired $t(27) = 3.95$, $p < 0.0005$), with again no significant difference between treatments. This contrasts with the situation in the previous experiment where general locomotory activity was maintained in NRS controls but declined in ATS-treated animals. A combined ANOVA comparing the change in locomotory activity in the two experiments showed the change overall was significantly greater in the present experiment ($F(1, 47) = 7.67$, $p < 0.01$).

In addition, there was a significant increase in mounting behaviour (paired $t(27) = 5.06$, $p < 0.0001$), with no difference between treatments (Fig. 4a). Earlier experiments (6) showed that there was no change in mounting from pre- to post-treatment periods of grouping, and a combined ANOVA revealed the difference between the two experiments to be significant for both change in mounting ($F(1, 47) = 5.90$, $p < 0.02$, Fig. 4a,b) and overall post-treatment levels ($F(1, 47) = 7.71$, $p < 0.01$).

The only other behaviour showing a significant change from pre- to post-treatment phases was sniffing the sawdust, which increased after treatment (paired $t(27) = 2.51$, $p < 0.02$) but with no difference between ATS and NRS animals.

Relationships Between Immunocompetence and Behaviour

The presence of female odours following treatment resulted in behavioural changes (increased aggression and locomotory activity and decreased sleep) that had previously been shown to be modulated in relation to immunocompetence (6). Furthermore, immunodepressed ATS mice showed similar levels of response to NRS controls. The results are therefore consistent with a trade-off in immunocompetence in the presence of cues relating to apparent mating opportunity. However, it is still possible that mice showed some degree of behavioural modulation in relation to immunocompetence. We tested this with a series of stepwise partial regression analyses.

Regressions for relationships between changes in behaviour (dependent variable) and immunocompetence measures (post- minus pre-treatment change in IgG, haemagglutination titre, and relative thymus weight, with post- minus pre-treatment change in testosterone and body weight as additional independent variables) revealed a strongly significant positive association between change in aggression and change in IgG concentration over the period of treatment ($t(26) = 4.30$, $p < 0.0005$, Fig. 5). Mice that showed an increase in IgG over the period of treatment thus showed a greater elevation in aggression in their post-treatment groups. This was maintained in both treatments when analyzed separately (ATS $t(10) = 3.45$, $p < 0.01$; NRS $t(14) = 3.56$, $p < 0.01$) and thus confirms the previous results (6).

Because mounting behaviour showed no change between pre- and post-treatment periods of grouping in the previous study, the relationship between mounting and immunocompetence was not analyzed. Analysis here, however, revealed a significant positive relationship between the change in mounting and haemagglutination titre both overall ($t(26) = 2.97$, $p < 0.01$) and among NRS controls ($t(14) = 3.65$, $p < 0.01$, Fig. 6) (haemagglutination titres among ATS-treated mice approached zero (vide supra)). Despite the absence of a treatment effect, therefore, there was evidence that

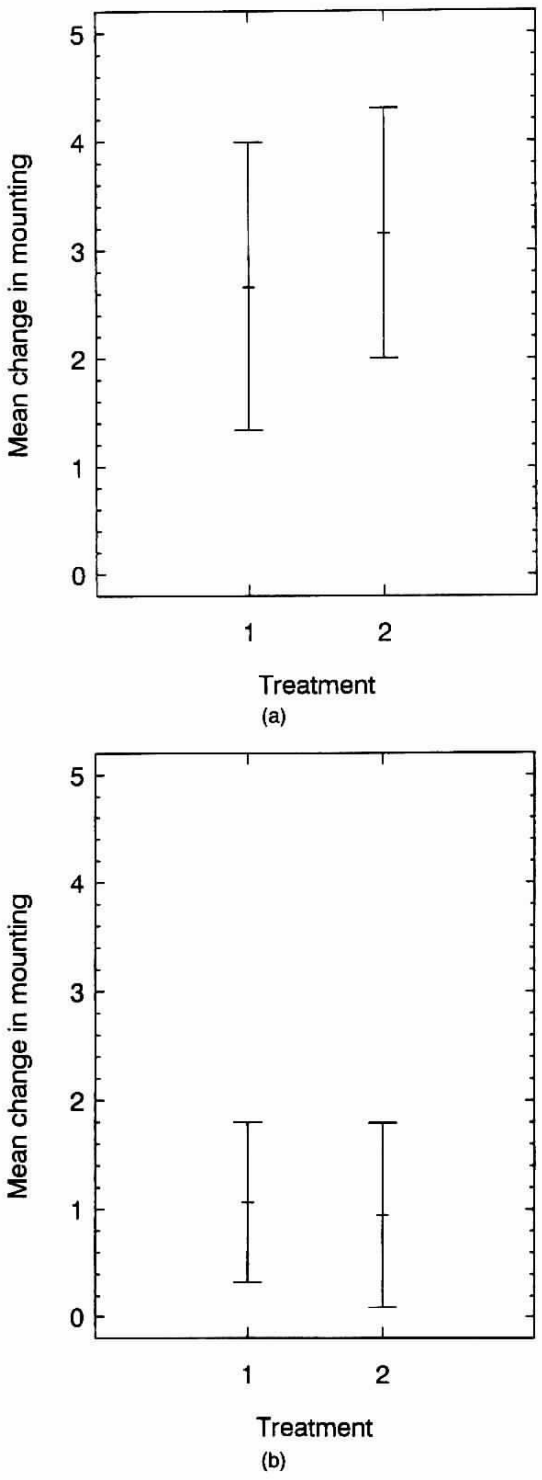


FIG. 4. Least-squares means from ANOVA of the change (post- minus pre-treatment) in the number of mounts initiated by ATS- and NRS-treated mice: (a) in the present experiment; (b) in the previous experiment (6). Treatments and bars as in Fig. 1. See text.

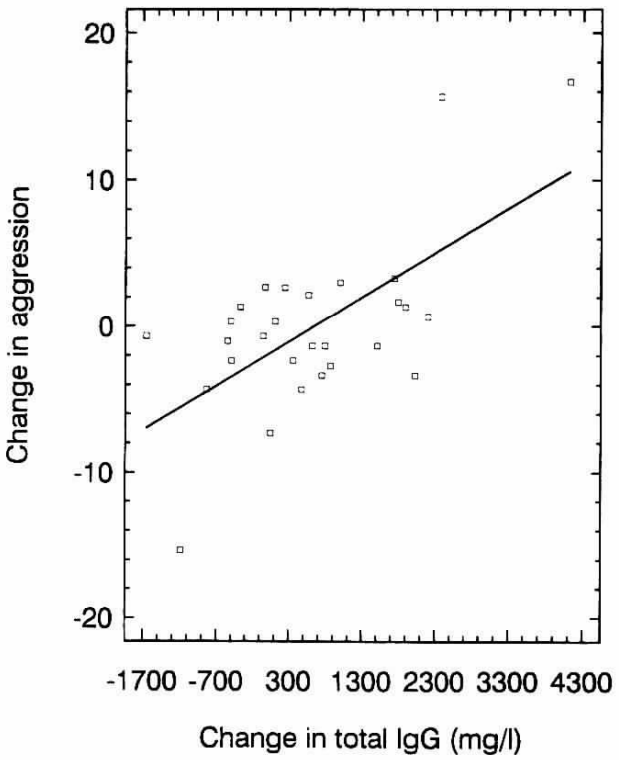


FIG. 5. Component effect from stepwise partial regression analysis of the relationship between post- minus pre-treatment change in total IgG concentration and post- minus pre-treatment change in aggression (treatments combined). Regression equation: $y = -1.59 + 0.003(\text{tigg})$, where tigg = change in IgG concentration. No other independent variables entered the equation. See text.

changes in aggression and mounting were modulated in relation to immunocompetence, with more time being spent in each behaviour after treatment when immunocompetence was greater. No significant relationship emerged for locomotory activity or sniffing the sawdust.

A similar analysis of the change in sleeping failed to reveal any inverse relationships between the degree of reduction in sleep and immunocompetence measures, so there was no evidence that immunodepressed individuals slept more after treatment [cf. (6)]. Instead the results suggested that sleep was associated with enhanced immune function, with significant positive relationships between the (post- minus pre-treatment) change in time spent sleeping and haemagglutination titre (treatments combined $t(26) = 2.39, p < 0.05$; NRS $t(14) = 3.64, p < 0.01$) and between time spent sleeping during the post-treatment phase and the post-treatment increase (terminal minus post-treatment values) in IgG concentration (treatments combined $t(26) = 2.04, p = 0.05$); NRS $t(14) = 2.75, p < 0.02$). The only other variable to enter the equation in the analysis of behavioural effects on post-treatment increase in IgG was mounting, which correlated negatively with change in IgG (treatments combined $t(26) = -2.39, p < 0.05$; NRS $t(14) = -2.86, p < 0.02$). Mice that showed more mounting therefore showed less of an increase in IgG concentration (Table 2). No significant relationships emerged among ATS-treated animals.

DISCUSSION

The results confirmed our expectation that the presence of female odour would reduce the tendency for mice to modulate their behaviour in response to immunodepression. In contrast to the outcome of a previous experiment in which no odour was presented, mice treated with anti-thymocyte serum (ATS) showed no reduction in aggressive behaviour or general locomotory activity and no difference in time spent sleeping relative to control mice. Moreover, in comparison with the earlier experiment, there was a significant increase in post-treatment aggression and general locomotory activity and a significant reduction in sleeping during the post-treatment phase relative to pre-treatment levels. In addition, there was a significant increase in mounting behaviour, again with no difference between ATS and control mice.

Since increases in aggression and locomotory activity, and reduced sleep, had previously been modulated in relation to measures of immunocompetence, the changes here, and the lack of any significant difference between ATS and control mice, appear at first sight to be consistent with a trade-off in immune function. However, the absence of a treatment effect notwithstanding, there was evidence that changes in aggression and mounting at least were modulated to some degree in the present experiment, change in aggression correlating with change in IgG concentration (as in the previous experiment) and change in mounting correlating with haemagglutination titre. There was no evidence of the correlation between sleep and relative thymus weight found previously, pos-

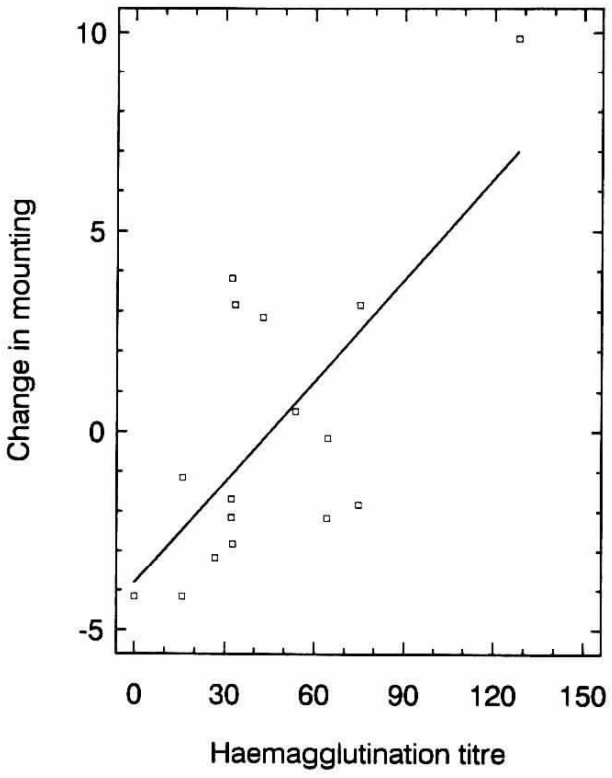


FIG. 6. Component effect from stepwise partial regression analysis of the relationship between haemagglutination titre (1/dilution) and post- minus pre-treatment change in mounting behaviour (NRS controls only). Regression equation: $y = -0.66 + 0.08(\text{ht})$, where ht = haemagglutination titre. No other independent variables entered the equation. See text.

sibly owing to the reduced variance in thymus weight in the present experiment (since the previous relationship held only among ATS-treated mice, it cannot have been affected by the unexpectedly low thymus weights for NRS animals here). Instead, the change in sleep and the amount of sleep in post-treatment groups correlated positively with haemagglutination titre and the change in IgG over the post-treatment phase, respectively, implying a positive causal influence on immunocompetence (see also (13) for similar effects of sleep in rats). Although the change in mounting from pre- to post-treatment phases also correlated positively with haemagglutination titre, the negative relationship between post-treatment mounting and change in IgG suggested a depressing effect of mounting on immunocompetence and strengthened the interpretation of the relationship with haemagglutination titre in terms of modulation. Consideration of post-treatment behaviour and intercurrent measures of immunocompetence thus suggests that different inferences can be drawn from the relationships between haemagglutination titre and pre- to post-treatment changes in sleep and mounting.

Overall, therefore, the results for behaviour imply a degree of modulation following treatment that is consistent with previous findings (6), but some tolerance of immunity cost in that experimentally immunodepressed animals did not differ in their response from controls. This is illustrated clearly by mounting behaviour, which showed a significant increase in the presence of female odour. Mounting appeared to be modulated relative to haemagglutination titre in NRS controls but showed a similar increase from pre- to post-treatment phases among ATS mice in which the average haemagglutination titre was close to zero.

It may be argued that the degree of change in some behaviours (e.g., aggression, mounting), while significant in particular comparisons, is nevertheless very small [Fig. 2, 4; see also (6)]. However, apart from the fact that aggression and mounting are intrinsically brief and rare behaviours, and therefore unlikely to be recorded often, we are not concerned with the absolute magnitude of change (which in any case is 2–3-fold depending on the comparison), but with what significant changes may indicate about the animal's internal state. A single act of aggression, for instance, is likely to affect social relationships between particular individuals, and thus levels of social stress, for a period considerably longer than the act itself. Behavioural changes are thus regarded as indices of longer term change in internal state rather than of direct importance in themselves.

Whereas, with the exception of thymus weight, ATS treatment in the present experiment resulted in similar reductions in immunocompetence relative to control animals to those reported previously (6), the expected change in testosterone concentration over the period of treatment was only weakly in evidence. However, as in the case of sleep, the previous correlation with immunocompetence measures had been with thymus weight, the low mean and variance for which in the present experiment make interpretation of relationships with testosterone response difficult. More surprisingly perhaps, testosterone levels did not increase as a result of exposure to female odours [cf. (7,19,20)], an outcome that may have accounted for the absence of any post-treatment relationship between testosterone and IgG concentrations [cf. (6)]. A possibility that cannot be ruled out, however, and that may account for the lack of increase in testosterone, is that males were responding not to the female quality of the introduced odours but to their novelty (14,21,26). As noted before (26), femaleness and novelty are inextricably conflated where males have not previously encountered female odours on their substrate (14). Even if this was the case, however, the odour treatment still represents a sociosexually salient change in the group olfactory environment (a change in the dominant male's ability to exclude intruders which is likely to increase the incentive for aggressive competition among lower ranking males (14)). The predicted differences in behaviour between our two experiments are thus unaffected.

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