Chronic Stress in Dogs Subjected to Social and Spatial Restriction. II. Hormonal and Immunological Responses

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BEERDA, B., M. B. H. SCHILDER, W. BERNADINA, J. A. R. A. M. VAN HOOFF, H. W. DE VRIES AND J. A. MOL. Chronic stress in dogs subjected to social and spatial restriction. II. Hormonal and immunological responses. PHYSIOL BEHAV 66(2) 243–254, 1999.—Two groups of beagles, accustomed to spacious group housing, were subjected to social and spatial restriction and studied for manifestations of chronic stress with a time interval of 7 weeks between the groups. The change from outside group housing (the control period) to individual housing in small indoor kennels resulted in sustained decreases in urinary adrenaline/creatinine and noradrenaline/creatinine ratios for the total group. Urinary dopamine/creatinine and noradrenaline/adrenaline ratios were statistically unaffected. Socially and spatially restricted dogs that had experienced pleasant weather during the control period showed (a) increased salivary and urinary cortisol concentrations, (b) a diminished responsiveness of the pituitary–adrenal axis to a sudden sound blast or exogenous CRH, (c) intact plasma ACTH and cortisol suppressions after dexamethasone administration, and (d) increased concanavalin A induced lymphocyte proliferations. When social and spatial restriction was preceded by a control period during which the weather was bad, these physiological responses were either augmented (lymphocyte proliferation), or offset (salivary and urinary cortisol), or directed oppositely (CRH-induced ACTH and cortisol responses). Together with the previously presented behavioral observations, these data suggest that bad weather conditions during spacious outdoor group housing induced early stress that attenuated the negative appraisal of the subsequent period of social and spatial restriction. In comparison to male dogs, bitches showed increased HPA responses to a sound blast or exogenous CRH. Their increased attenuations of the ACTH and cortisol responses to CRH after 5 weeks of restricted housing indicates that bitches are not only more susceptible to acute stress, but also to chronic housing stress. It is concluded that the quality of circumstances preceding a period of affected well-being determines the magnitude and even the direction of the behavioral and physiological stress responses. Basal salivary and urinary cortisol measurements are useful for the assessment of chronic stress, and of poor welfare in dogs. The use of urinary catecholamine, peripheral leucocyte, and lymphocyte proliferation measures requires further investigation. © 1999 Elsevier Science Inc.

Chronic Stress Dogs Physiology Social and spatial restriction

BEFORE manifestations of chronic stress can be used to establish welfare problems in dogs, parameters have to be identified that validly indicate chronic stress in these species. For the assessment of chronic stress, a wide variety of possible parameters, derived from acute stress responses, are available. In dogs, among other species, acute stress is accompanied by increased activity of the sympathetic–adrenal–medullary (SAM) system (1,19,22,23,44,52,54), and the hypothalamic–pituitary–adrenal (HPA) axis (3,8,12,17,28,29,30,39,53,58).

As in situations of acute stress, chronic stress may lead to an increased central stimulation of the HPA axis, resulting in increased cortisol (the primary glucocorticoid in dogs) secretion that may or may not be sustained. On the one hand, high levels of glucocorticoids will exert inhibitory effects at the central (20) and pituitary level of the HPA axis (38), and the pituitary may become increasingly resistant to the adenocorticotropic hormone (ACTH) releasing hormones vasopressin (40) and corticotropin-releasing hormone (CRH) (63). On the
other hand, there are mechanisms that facilitate stress-induced glucocorticoid secretion. Concomitant with an increased central CRH drive, the adrenal cortices of depressed humans may hyperrespond to ACTH stimulation, or their HPA system may become less sensitive to glucocorticoid negative feedback at the hypothalamic and pituitary level (37).

Stress modulates the immunocompetence, which, subsequently, may lead to increased disease susceptibility of individuals (45). It is, therefore, that altered immune responses may indicate an early state of stress that may readily turn into a state of demonstrable bad welfare, namely disease. Hence, immunological parameters may serve as indicators of welfare. Peripheral leucocyte responses during stress typically entail neutrophilia and lymphopenia, and occur similarly in chronically stressed humans (47), squirrel monkeys (13), silver foxes (35), mink (31), and chickens (27). In dogs, a general leucocytosis, neutrophilia, lymphopenia, and eosinopenia occurs during acute stress (41,66), but whether the same is true during chronic stress is yet unknown. A suppressed mitogen-induced blood lymphocyte proliferation response has been reported in chronically stressed humans (9,33,60), lambs (14,50), mice (61), and rats (55), but not in chronically stressed dogs.

In the present study, healthy beagles that were accustomed to spacious group housing were monitored for both basal activity of the SAM system and the HPA axis during the induction of chronic stress by social and spatial restriction. In addition, stimulation and inhibition tests of the HPA axis were included. Noninvasive sampling procedures were applied to minimally disturb the subjects under study and to investigate noninvasive stress measurements for their future use outside of an experimental setting. The effect of stress on the immune system was tested by measuring lymphocyte proliferative responses and peripheral leucocyte concentrations.

In summary, through the assessment of a set of parameters, we wanted to know which hormonal and immunological parameters indicate chronic housing stress in dogs, and are useful to identify poor welfare in this species. Also, we investigated correlations between cortisol measures, as established indicators of stress, and the remainder of hormonal, immunological, and behavioral (7) parameters.

**METHODS**

**Animals and Treatments**

Fifteen Beagles (mean age 1.6 ± 0.2 year) were obtained from a spacious group housing facility. The first group (2♂♂, 6♀♀) was transported into the research facilities and, for 7 weeks, kept by outdoor group housing (GH) of three or two individuals per lawn of 36 m². Shelters were available for protection against bad weather. Next, the dogs were individually housed (IH) for 6 weeks in indoor kennels of 1.7 m², without the possibility of visual or physical contact between animals. With an interval of 7 weeks a second group (3♂♂, 4♀♀) underwent the same protocol. Food was provided at 07.30 h, whereas water was available ad lib.

**Sampling Procedures: Hormonal Parameters**

Spontaneous voided urine samples were collected two or three times per week, between 0830 and 1200 h, from the third week of GH. For this purpose, the dogs were placed in a recently cleansed concrete surfaced spacious enclosure, and allowed to urinate. Urine samples were collected from the floor by using a syringe. IH facilities were equipped with urine collection trays that were installed two or three times per week from 1800 until 0700 h, and from 0830 until 1200 h. Urine samples were stored at −20°C until analysis. Out of 418 urine samples that were assayed for cortisol and creatinine, 148 were also assayed for catecholamines. The latter samples were adjusted to pH 3–4 using formic acid before storage.

Saliva samples were collected after 4 weeks of GH and after 5 weeks of IH. Every hour, from 0830 to 1730 h, saliva was collected following the procedure as described elsewhere (5). On days 2, 4, 8, 10, 15, 17 and 24 of IH, samples were taken at 0900, 1030, 1200, 1330, and 1500 h. Acute salivary cortisol responses to a sudden sound blast were established during the sixth week of GH and IH. Samples were taken at −15, 5, 10, 15, 20, and 30 min from the start of the stressor. Finally, saliva was collected at −15, 20, 80, 140, and 200 min from the onset of the stresser. Saliva samples were stored at −20°C until analysis.

During the sixth week of GH and IH, the HPA axis was tested by the intravenous administration of 1 μg oCRH/kg body weight (Peninsula Laboratories, St Helens, Merseyside, UK). Blood samples were taken by venipuncture at −30, −1, 10, 20, 30, 45, 75, 105, and 180 min from CRH injection, and collected in prechilled EDTA-coated tubes. After centrifugation for 10 min at 1000 × g, plasma samples were stored at −20°C until analysis for cortisol and ACTH. One week after CRH stimulation, the dogs received an intravenous injection with 0.01 mg dexamethasone (DEX) per kg body weight (Dexadreson, Intervet International, Boxmeer, The Netherlands). Blood samples were taken at −0.5, 0, 2, 4, 6, 8, 11, and 24 h from dexamethasone injection, and processed as for the CRH stimulation test.

**Sampling Procedures: Immunological Parameters**

Blood samples (1 mL) for the determination of leucocyte numbers, were obtained after 1, 3, 7, 11, 14, and 18 days of IH. Blood samples for peripheral leucocyte counts and lymphocyte proliferation studies were taken in the seventh week of GH, on Day 4 of IH, and in the seventh week of IH. At 1200 h, 10 mL blood was taken by jugular venipuncture and distributed over EDTA (1 mL) and heparin coated tubes (9 mL). EDTA blood was immediately assayed for leucocyte concentrations. From the heparinized blood, peripheral blood mononuclear cells (PBMC) were isolated by centrifugation through Ficoll (density 1.078) for 25 min at 800 × g. Cells were washed twice with 40 mL RPMI 1640 medium supplemented with 3% fetal calf serum (FCS, Sebak, Aidenbach, Germany), 2 mM L-glutamine (Biochrom KG, Berlin, Germany), 100 IU/mL penicillin (Kombi Vet, Etten-leur, The Netherlands), 100 μg/mL streptomycin (Alfasan, Woerden, The Netherlands), and 10 μM 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO). For the first washing step 0.2% heparin (Pharmaceutical products, Weesp, The Netherlands) was added. Washed cells were suspended and adjusted to a concentration of 6 × 10⁸ cells/mL. Medium supplemented with 20% FCS and 10% DMSO (Merck, Darmstadt, Germany) and stored at −80°C. To prevent unwanted stress responses to substantial bleeding, lymphocyte proliferation studies were only performed in dogs that weighed more than 11 kg (2♂♂ and 9♀♀).

**Determinations**

Urinary cortisol and creatinine concentrations were determined as described elsewhere (56). Urinary catecholamine concentrations were measured after solid-phase extraction by HPLC and radiochemical detection as validated for the dog (6). Urine samples with a creatinine concentration below 1
mmol/liter were considered too diluted for a valid assessment of hormones, and omitted from analysis. Plasma and salivary cortisol concentrations were determined in a commercially available coated tube assay (Diagnostic Products Corporation, Los Angeles, CA). Plasma ACTH concentrations were determined by radioimmunoassay according to the procedure described elsewhere (2).

Leucocyte concentrations were established using a Helios 5 Diff cell counter (ABX, Hoffmann–La Roche, Montpellier, France). Differential cell counts were performed manually by experienced laboratory technicians.

For the lymphocyte proliferation assay, frozen cell preparations were thawed, they were washed extensively and they were adjusted to a concentration of 1.5 × 10^6 cells per mL (RPMI) medium supplemented with 10% FCS. The cell preparations were then dispensed in 200 μL aliquots into triplicate wells of flat-bottomed 96-well microtiter plates (Costar, Cambridge, MA), and cultured in the presence of 0, 1, 2, and 4 μg/mL concanavalin A (ConA) (Sigma Chemical Co., St. Louis, MO) for 5 days in a humidified atmosphere at 37°C and 5% CO₂. Eighteen hours prior to harvesting the cells were pulsed with 0.4 μCi of [³H]thymidine (1.0 Ci/mmol; Amersham, Bucks, UK). [³H]Thymidine uptake was measured using a Beckman scintillation counter. Results were obtained as counts per minute (cpm), and were expressed as stimulation index (SI), this being the median cpm of wells containing ConA divided by the median cpm of wells containing medium.

Data Processing and Statistical Analysis

The results that were obtained during the various stages of the IH period were separately compared to the mean results for the GH period. Urinary cortisol measurements were calculated as mean values per week of IH. Urinary catecholamine measurements were expressed as mean values for the first week of IH, and as mean values from the third until the sixth week of IH. Except for the PBMC proliferative responses, which were tested by the Wilcoxon matched-pair signed-rank test, all data were analyzed by an analysis of variance (ANOVA) for repeated measurements. Multiple salivary cortisol measurements within 1 day were treated as separate values. Differences between GH and the separate weeks of IH were tested by using the F-statistic. Gender and weather conditions were treated as independent variables when running ANOVAs. During the last month of GH, the dogs in the first experimental group had been subjected to a mean temperature of 20.6°C, on average 9.6 h of sun per day, a mean wind velocity of 2.9 m/s, and a mean daily rainfall of 0.6 mm (data provided by the KNMI, De Bilt, The Netherlands). These animals were designated as the pleasant weather (PW) group. The bad weather (BW) group consisted of the remainder of the animals that had experienced 15.4°C, 4.1 h of sun, 3.6 m/s, and 3.8 mm daily rainfall.

To achieve normality, data expressed as ratios were logarithmically transformed. Univariate F-statistics were corrected with the Huynh–Feldt epsilon adjusted degrees of freedom, when data sets deviated from the sphericity assumption. Results are presented as the mean ± SEM.

Pearson correlations were calculated for basal cortisol measures against the remainder of hormonal, immunological, and

![FIG. 1](image1.png)

**FIG. 1.** The effects of the transfer of dogs from spacious outdoor group housing (GH) to restricted individual indoor housing (IH) on urinary dopamine (white bars), noradrenaline (dotted bars), and adrenaline (filled bars) levels. Mean catecholamine levels ± SEM (n = 15 dogs) are expressed relative to creatinine concentrations × 10⁻⁶. Natural voided urine samples were collected during GH, during the first week of IH (IH1), and during the third to sixth week of IH (IH3–6). Significant (p < 0.05) differences from the levels that were measured during GH are indicated by *.

![FIG. 2](image2.png)

**FIG. 2.** Cortisol/creatinine ratios in the morning urine of dogs that were maintained in a spacious social enriched environment (GH), and that, subsequently, were subjected to social and spatial restriction (IH). Mean values × 10⁻⁶ ± SEM are presented for the period of GH and for the 6 weeks of IH. Data on seven dogs that had experienced pleasant weather during GH (white bars) are presented separately from the data on seven dogs that had been subjected to bad weather (filled bars). Statistics were performed on pooled data of 14 dogs. Significant (p < 0.05) differences from the mean level that was measured during GH are indicated by *.
behavioral (7) parameters. The level of significance was set at a comparison-wise error rate <0.05. Error rates were corrected as by Bonferroni for multiple testing, but not for the number of parameters that were studied.

RESULTS

Urinary Catecholamines

The transfer from spacious group housing (GH) to restricted individual housing (IH) was associated with unchanged dopamine/creatinine ratios (DC), and with significant reductions in the urinary noradrenaline/creatinine (NC) and adrenaline/creatinine (AC) ratios when measurements were performed in morning urine (Fig. 1). The ratio of noradrenaline to adrenaline (NA) was decreased, albeit not significantly, from 2.3 ± 0.3 during GH, to 1.6 ± 0.2 during the first week of IH and to 1.7 ± 0.4 hereafter. Urinary catecholamine levels were the same for the pleasant weather (PW) and the bad weather (BW) groups, and for bitches and male dogs. Catecholamine concentrations in urine that was voided during the night equaled those in urine voided in the late morning.

Urinary Cortisol

Throughout the period of GH, one PW male had urinary cortisol/creatinine (CC) ratios that were five times higher than the mean group ratio, and was, therefore, excluded from further analysis. During GH, morning CC ratios in the PW and BW group were similar. After the transfer from GH to IH, CC ratios in morning urine tended to be elevated, which was significant for the sixth week of IH (Fig. 2). IH-induced changes in the CC ratio differed significantly between the BW and the PW group, with the individuals in the latter group containing lower CC ratios than morning urine, as measured during the IH period. Differences between nocturnal and morning CC ratios were more (p < 0.05) pronounced in the PW than in the BW group. PW individuals had a nocturnal CC ratio that was 37.0 ± 6.2% of the ratio in the morning, whereas in BW individuals the nocturnal CC ratio was only 3.3 ± 6.2% lower than the morning ratio. Nocturnal CC ratios decreased significantly from 6.2 ± 0.5 × 10^{-6} during the first week of IH to 4.9 ± 0.4 × 10^{-6} hereafter.

Salivary Cortisol

During GH, basal salivary cortisol levels were 2.1 ± 0.4 nmol/liter, with no differences between PW and BW individuals. At 20 min after the transfer from GH to IH, salivary cortisol concentrations had significantly increased from 1.9 ± 0.3 nmol/liter to 7.6 ± 1.2 nmol/liter. Salivary cortisol levels returned to baseline levels within 80 min from the transfer of the dogs. Salivary cortisol measurements that were performed on days 2, 4, 8, 10, 15, 17, and 24 of IH, indicated that, in comparison to GH levels, salivary cortisol concentrations tended to be increased in the PW group and decreased in the BW group (a significant group effect in the IH induced salivary cortisol responses). Relative to control levels of 2.1 ± 0.4 nmol/liter, salivary cortisol levels in the sixth week of IH were significantly increased to 3.4 ± 0.5 nmol/liter in the PW group, and nonsignificantly decreased to 1.6 ± 0.4 nmol/liter in the BW group (Fig. 3). Salivary cortisol concentrations declined over the day, the mean level found before 1000 h being significantly higher than the mean level found during the rest of the day (Fig. 4). This daily fluctuation was most pronounced when dogs were subjected to social and spatial restriction.

In response to a sudden sound blast, BW individuals showed a mean salivary cortisol response, expressed as net area under the response curve (nAUC), of 3.1 times the mean response of the PW group (Fig. 5). The same multiplication factor applied on the more pronounced cortisol responses in bitches versus male dogs. Differences between sexes were significant during both GH and IH. Cortisol response differences between the BW and the PW group were significant during GH. After 5 weeks of IH, noise-induced salivary cortisol responses decreased significantly to 13.6 ± 10.7% of the mean response found during GH. This hyporesponsiveness was a common feature for all animals.

ACTH and Cortisol Responses after CRH Stimulation and DEX Suppression

After 5 weeks of IH, CRH-induced ACTH and cortisol responses, as calculated by nAUC, were changed oppositely in the PW and BW group. In comparison to GH, a significant increase of the CRH-induced ACTH release was found in the BW group, whereas a significant decrease occurred in the PW group (Fig. 6). As a result of social and spatial restriction, PW individuals had significantly higher plasma cortisol concentrations immediately before CRH administration. Subsequently, they showed significantly lower CRH-induced cortisol responses during IH than during GH (Fig. 7).

Following the transfer of PW individuals from GH to IH, the mean ratio of CRH-induced ACTH to cortisol responses (expressed as the ratio of the nAUCs) was significantly decreased (−37.2 ± 10.5%).
Throughout the experiment, bitches showed CRH-induced ACTH and cortisol responses that were 39 and 55% higher (p < 0.05), respectively, than the corresponding responses in male dogs. Cortisol levels before and after CRH administration changed differently (p < 0.05) in individually housed bitches and male dogs. In this, bitches responded like PW individuals and showed lower CRH-induced cortisol responses during IH than during GH.

During restricted housing the sensitivity of the pituitary–adrenal axis to inhibitory effects of dexametahasone remained intact. Lowest suppressed ACTH concentrations and time-dependent recovery of the ACTH suppressions between GH and IH, or between PW and BW individuals, were statistically not different (Fig. 8). Similarly, no differences were noticed for the suppressed cortisol levels after DEX administration (Fig. 9).

**Leucocytes**

During GH, BW individuals differed from PW individuals in that they showed increased (p < 0.05) numbers of neutrophils (a mean 11.0 ± 0.9 and 8.9 ± 0.6 × 10⁹/liter in BW and PW individuals, respectively). Total numbers of leucocytes (13.8 ± 0.6 × 10⁹/liter), neutrophil to lymphocyte (NL) ratios (4.1 ± 0.4 × 10⁹/liter), and concentrations of lymphocytes (2.7 ± 0.2 × 10⁹/liter), monocytes (0.6 ± 0.1 × 10⁹/liter) and eosinophils (0.6 ± 0.1 × 10⁹/liter) were similar in both groups. With regard to leucocyte counts PW and BW individuals responded differently to social and spatial restriction (indicated by significant treatment X group interactions). Response differences between the PW and BW group were not consistent for the various stages of IH that we compared to GH.

The transfer of GH to IH was associated with a number of significant changes in the leucocyte levels of both PW and BW individuals, but none endured.
Lymphocyte Proliferation Responses

Basal $^3$H-thymidine incorporation by cultured PBMC was unaffected by housing conditions; GH: 58 ± 18, day 3 of IH: 54 ± 9 and week 6 of IH: 45 ± 4 cpm $^3$H-thymidine incorporation. Compared to GH, ConA-induced proliferative responses tended to be increased after 3 days of IH. Increments were significant after 6 weeks of IH (Fig. 10), and they appeared to be most pronounced in the BW group (not statistically tested). During GH, after 3 days of IH and after 6 weeks of IH, ConA (2 μg/mL)-induced proliferative responses in the BW group had mean SI values of 14.4 ± 4.1, 119.4 ± 74.7, and 409.4 ± 270.9, respectively. In the same order of presentation, the results for the PW group were 19.4 ± 7.3, 38.6 ± 11.6, and 81.5 ± 55.2.

Correlations Between Physiological and Behavioral Data

From the assumption that increased activity of the pituitary–adrenocortical system (as measured by the integrative measurement in urine) is a prominent characteristic of chronic stress, the correlations for responses in the CC ratio were calculated with the remainder of response parameters. With regard to hormonal measures, IH-induced CC responses were positively correlated with changes in basal saliva cortisol concentrations ($r = 0.83, p < 0.001$), and negatively correlated...
with changes in the CRH-induced plasma ACTH ($r = -0.83$, $p < 0.001$), plasma cortisol ($r = -0.74$, $p = 0.003$), and ACTH/cortisol responses ($r = -0.59$, $p = 0.03$).

The investigation of correlations between CC and immunological changes showed a positive correlation with responses in the NL ratio ($r = 0.82$, $p < 0.001$).

Previously, we reported on the behavioral findings of the present experiment (7). Here, we investigated the correlations between the responses in the behavioral parameters and the responses in the urinary CC ratio. A positive correlation was found for CC responses with responses in intentions to change the state of locomotion ($r = 0.65$, $p = 0.01$), while negative correlations were found with digging ($r = -0.63$, $p = 0.02$) and floor licking ($r = -0.61$, $p = 0.02$).

FIG. 8. DEX (0.01 mg/kg)-induced ACTH suppressions in dogs that were kept outdoors in a spacious and social housing system (open markers), and that, subsequently, were socially and spatially restricted for 5 weeks (solid markers). Mean cortisol concentrations in pmol/liter ($\pm$SEM), are presented separately for dogs that had experienced pleasant (PW, $n = 8$) and bad (BW, $n = 7$) weather during the time that they were maintained outdoors.

FIG. 9. DEX (0.01 mg/kg)-induced cortisol suppressions in dogs that were kept outdoors in a spacious and social housing system (open markers), and that, subsequently, were socially and spatially restricted for 5 weeks (solid markers). Mean ACTH concentrations in nmol/liter ($\pm$SEM) are presented separately for dogs that had experienced pleasant (PW, $n = 8$) and bad (BW, $n = 7$) weather during the time that they were maintained outdoors.

DISCUSSION

The main objective of the present study was to find behavioral and physiological markers of chronic stress and, as such, of poor welfare in dogs. To study the behavioral and physiological aspects of chronic stress, two groups of Beagles were moved from a spacious enriched outdoor group housing (GH) system to a socially and spatially restricted indoor environment (IH). Although not intended, during the period of outdoor housing a major difference existed in the weather conditions, which allowed us to create a bad weather (BW) and pleasant weather (PW) group. The meterological differences appeared to have a great impact on the behavioral and physiological changes induced by social and spacial restriction, and,
therefore, presented a unique opportunity to evaluate the circumstances of control periods to an expected stressful treatment (Table 1). Our physiological findings will be discussed first against the background of well-known physiological changes during chronic stress in other species, and secondly in relation to the weather conditions during group housing. Next, a discussion will be held on the relationship with the previously reported behavior of the experimental animals (7). Because bitches may show an increased stress responsiveness in comparison to male dogs (25), response differences between sexes will be discussed as well.

**Urinary Catecholamines**

In acute (19,54) and chronic intermittently stressed dogs (48) an increased activity of the sympathetic–adrenal–medul- lary (SAM) system has been reported, resulting in elevated plasma noradrenaline and adrenaline concentrations. It was, therefore, surprising that in the present study social and spatial restriction was associated with decreases in urinary noradrenaline/creatinine (NC) and adrenaline/creatinine (AC) ratios. Possibly, the activity of the SAM system was not increased because restricted housing reduced the locomotor activity of the dogs (7). Studies in young men have revealed a positive relationship between exercise and urinary catecholamine excretion (32,46).

Relatively high urinary dopamine/creatinine (DC) ratios during the first week of IH, and relatively low urinary noradrenaline/adrenaline (NA) ratios throughout the period of IH were nonsignificant observations. In humans, low NA ratios (51) and increased dopamine excretion have been associated with mental and physical stress (18,21). The present results do not negate that, as in humans, stressed dogs may show increased DC ratios and decreased NA ratios, but the results neither pinpoint DC and NA ratios as useful markers of canine stress.

**Urinary and Salivary Cortisol**

In dogs, an increased level of cortisol is a well-documented indicator of acute stress whether measured in plasma (3,8,12,17,28–30,39,53,58), urine (6,36), or saliva (6,67). From the present cortisol measurements we conclude that increased urinary cortisol/creatinine (CC) ratios and salivary cortisol levels also indicate chronic stress. In comparison to the urinary CC ratios that were found during GH, the ratios tended to be increased during the first 3 weeks of IH, and they were significantly higher after 5 weeks of IH. The increase in cortisol excretion typically occurred in PW individuals, which was

![FIG. 10. In vitro proliferative responses of frozen–thawed canine peripheral blood mononuclear cells. Mean proliferative responses ±SEM (n = 11) are expressed as the ratio of proliferation after stimulation with 1, 2, or 4 μg ConA/mL to the proliferation of medium controls. Cells were collected from dogs when they were maintained outdoors in a spacious enriched social housing system (open markers), after 3 days of social and spatial restriction (solid circular markers), and after 6 weeks of social and spatial restriction (solid triangular markers). Significant (p < 0.05) differences from the proliferative responses during spacious group housing are indicated by *.

### TABLE 1

<table>
<thead>
<tr>
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<th>Individual Housing</th>
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A physiological comparison of dogs that had experienced pleasant (PW) or bad (BW) weather conditions during a period of spacious outdoor group housing, and that, subsequently, were subjected to individual housing in small indoor kennels. A variety of measures were taken under different conditions.
also reflected in higher salivary cortisol levels after 5 weeks of IH (Table 1).

Cortisol concentrations in saliva and urine varied throughout the day, with relatively high levels in the morning. This confirms an earlier report indicating a diurnal variation of cortisol levels in adult Beagles (53). The highest cortisol values occurred within hours of the arrival of the animal caretakers and the provision of food. Thus, it remains unclear to what extent the present variation reflected an endogenous rhythm or merely mirrored excitement and activity. Individually housed PW individuals had higher urinary CC ratios than BW individuals, but only when urine was voided in the morning. This implies that, in response to social and spatial restriction, cortisol excretion was typically increased when the hypothalamic–pituitary–adrenal (HPA) activity was at its peak.

**Hormonal Responses After Noise Exposure, CRH Stimulation, and DEX Suppression**

After 5 weeks of IH, saliva cortisol responses to a sudden sound blast were decreased, whereas the suppression of plasma adrenocorticotrophic hormone (ACTH) and cortisol after dexamethasone (DEX) administration remained unchanged. The ACTH and cortisol responses to corticotropin-releasing hormone (CRH) were attenuated in the PW group and augmented (ACTH only) in the BW group (Table 1).

Behavioral (7) and basal cortisol measurements suggested that socially and spatially restricted PW individuals were more stressed than BW individuals, and, consequently, we consider a HPA hyporesponsiveness as an indication of chronic stress. Assuming that, to some extent, senescence may mimic a prolonged period of stress exposure, this opposes the canine hyperresponsiveness in old dogs (58). Possibly, 5 weeks of spatial and social restriction did not cause sufficient stress to induce a pronounced reduction in the number of limbic mineralocorticoid receptors, a process that has been associated with a HPA hyperreactivity in aged dogs (58). The HPA hyporesponsiveness in our dogs may then have resulted from an upregulation of the number of glucocorticoid receptors in the anterior pituitary (58), and may have been further facilitated by an increased negative feedback due to increased levels of cortisol. The latter is indicated by the negative correlations of IH-induced urinary CC responses with changes in the CRH-induced ACTH and cortisol responses.

The relatively low CRH-induced ACTH and cortisol responses of BW dogs during GH, fits the assumption that these animals experienced bad weather stress before they were socially and spatially restricted. The recovery of the HPA responsiveness during IH is in agreement with the absence of cortisol responses in BW individuals, and suggests that social and spatial restriction may not have been as stressful for the BW group as for the PW group.

In response to social and spatial restriction, the mean ratio of CRH-induced ACTH to cortisol responses decreased significantly in PW individuals (~37%). Apparently, chronic stress sensitizes the adrenal response to ACTH. However, this interpretation may be too straightforward, as the adrenal response has been reported to relate logarithmically to the dose of ACTH (38). When this was taken into account (data not shown), ratios of CRH-induced ACTH to cortisol responses were unaffected in individually housed BW and PW individuals. A negative correlation of IH-induced urinary CC responses with IH-induced changes in ACTH/cortisol response ratios after CRH administration, further supports that in chronically stressed dogs adrenal cortices are sensitized to ACTH.

Throughout the experiment, CRH-induced plasma ACTH and cortisol responses, and noise-induced saliva cortisol responses, were more pronounced in bitches than in male dogs. This confirms earlier findings that bitches may show a relative HPA hyperreactivity to stressors (25). Social and spatial restriction affected the CRH-induced cortisol responses, but not the ACTH responses, differently in bitches and male dogs. In this, the bitches responded like PW individuals, meaning that, in comparison to male dogs, bitches may be more susceptible to enduring environmental stress (25).

Similarly to the situation during senescence (58), ACTH and cortisol responses to DEX administration were unaffected by 5 weeks of social and spatial restriction. The DEX suppression test may not be a useful tool to establish chronic stress in dogs.

**Peripheral Leucocyte Counts**

Differences in peripheral leucocyte counts during GH and IH are difficult to interpret with regard to stress, and, possibly, they mirrored immunological responses to a changed antigen population. Compared to GH, increased numbers of lymphocytes, decreased numbers of neutrophils and/or reduced neutrophil/lymphocyte (NL) ratios were, albeit inconsistently, found during IH. In contrast, in a number of species NL ratios are increased during chronic stress (13,27,31,35,47). IH-induced reductions in NL ratios typically occurred in BW dogs, and may have resulted from the relatively high levels of neutrophils that these animals had during GH. A positive correlation between IH-induced responses in the CC ratio and the NL ratio suggests that, as in other species, in dogs chronic stress is associated with increased NL ratios. Neutrophil and NL measurements strengthened the impression that, in comparison to PW individuals, BW individuals were stressed during GH, but less so during IH.

**PBMC Proliferative Responses**

In different species chronic stress has been associated with reduced lymphocyte proliferative responses (9,14,33,50,60,61), and it is, therefore, remarkable that our dogs had increases in proliferative responses after 3 days and 6 weeks of IH. The present increments may have been related to the freezing of the PBMC. After a maximal storage time of 9 months, the frozen–thawed PBMC were tested simultaneously. Consequently, the cells that had been collected after 3 days and 6 weeks of IH, were cryo-preserved 10 and 50 days, respectively, than the cells that had been obtained during GH. The precise effects of such variable durations of cryo-preservation are obscure. Proliferative capacities of human (62), equine (65) and bovine (42,64) lymphocytes have been reported to either stabilize or decline after freezing, but we were not able to find studies that report on the effects of the duration of cryo-preservation.

Assuming that at least part of the increased lymphocyte proliferative responsiveness was attributable to stress, it is noteworthy that the most pronounced increments were observed in the (BW) individuals that had no cortisol responses. This would suggest that, in dogs, lasting stress is associated with increases in the lymphocyte proliferation capacity, which are attenuated by high levels of cortisol.

**Integration of Physiological and Behavioral Data**

Five weeks of social and spatial restriction induced a number of changes in the spontaneous behavior of the dogs (7),
and these behavioral responses were investigated for correlations with urinary CC responses. There was a positive correlation of CC responses with responses in intentions to change the state of locomotion, a behavior that, as a secondary effect of reduced locomotor activity, the dogs performed less in response to IH. Its relationship with urinary cortisol levels suggests that intentions to changes from one state of locomotion to another are associated with chronic stress.

Floor licking and digging were typically performed by PW individuals during the time that they were housed on lawns (GH). The transfer from GH to IH included a change from grass to concrete as a subsoil. This may explain the decreases in floor licking and digging during IH, and the negative correlations of these changes with urinary CC responses that also typically occurred in the PW group.

The absence of significant correlations between IH-induced urinary cortisol responses and behavioral changes that we associated with chronic housing stress, namely increased autogrooming, copropaghy, low posture, paw lifting, and vocalizing (7) was striking. This indicates that, supposedly, high levels of stress, as measured by cortisol levels, are not necessarily concomitant with pronounced behavioral expressions of stress. For a valid assessment of chronic stress it will, therefore, never suffice to measure only the behavior.

Summarizing Conclusions and Final Points of Discussion

From the above-mentioned findings we conclude that, in addition to the previously reported behavioral changes (7), socially and spatially restricted dogs may show increased cortisol levels and a HPA hyporesponsiveness to acute stimuli. However, such responses may be readily modulated by a preceding episode of (bad weather) stress. Apparently, the appreciation by the dogs of a new housing situation depends on their appreciation of the preceding housing conditions. Cortisol measurements in urine and saliva appear useful tools to identify chronic stress and, of poor welfare in dogs.

The present experiment may have been inadequate to validate immunological and catecholamine parameters as indicators of chronic stress, because it did not sufficiently control for possible side effects of changed antigen populations and locomotor activity, respectively. We tried to prevent unwanted immunological responses to novel antigens after their transition from GH to IH by introducing the dogs into the IH environment repeatedly while they were kept in GH conditions. Possibly these measures were not completely successful. More controlled studies are required to confirm the present immunological findings.

In comparison to male dogs, bitches showed increased behavior (7) and HPA responses to acute stressors. The increased HPA hyporesponsiveness to CRH administration after 5 weeks of restricted housing suggests that bitches are not only more susceptible to acute stress, but also to chronic housing stress.

The statistical analyses that were performed in the present study included corrections for multiple testing of one parameter, but not for the number of parameters that were tested. Corrections for the large number of parameters that were studied would have practically ruled out the possibility of significant treatment effects. In our opinion, the results reflected the reality more precisely when they were rejected less conservatively, and critically evaluated.

We recognize the risk of falsely assuming that social and spatial restriction was stressful to dogs and, subsequently, of misinterpreting the IH-induced changes with regard to stress. However, a number of studies on laboratory, farm and zoo animals emphasize poor housing conditions as a source of stress (4,10,11,16,26,43,49). Furthermore, the aversive character of the IH system is confirmed by the increased cortisol levels in individually housed PW individuals. An extensive amount of data substantiates that dogs show increased cortisol levels in response to short-lasting aversive stimuli (3,5,8,12,17,28,29,30,39,53,58), whereas in a number of species glucocorticoids are known to remain elevated when aversive stimulation endures (15,24,34,57,59). Due to physiological adaptations and stressor-specific responses undisturbed levels of cortisol may not exclude chronic stress, but increased cortisol levels are a strong indication for it.

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