Photoperiod and population density interact to affect reproductive and immune function in male prairie voles

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Nelson, Randy J., Joshua B. Fine, Gregory E. Demas, and Christopher A. Moffatt. Photoperiod and population density interact to affect reproductive and immune function in male prairie voles. *Am. J. Physiol.* 270 (*Regulatory Integrative Comp. Physiol.* 39): R571–R577, 1996.—Seasonal breeding of rodents is often associated with changes in adrenal function; altered adrenal function could account, in part, for seasonal changes in immune function and, ultimately, influence seasonal fluctuations in survival. Animals commonly monitor the annual change in photoperiod to ascertain the time of year and to make appropriate seasonal adjustments in physiology and behavior. Several extrinsic factors affect reproductive responsiveness to photoperiod. The interaction between population density and reproductive and adrenal responsiveness to photoperiod was assessed in the present experiment. Adult male prairie voles (*Microtus ochrogaster*) were maintained individually for 10 wk in long [light:dark (LD) 16:8] or short (LD 8:16) photoperiods in rooms with either high (10.96 animals/m^3) or low (0.18 animals/m^3) population densities. Regardless of population density, short-day voles regressed the size of their reproductive organs; reproductive organ masses were higher in long-day voles housed in high-density compared with low-density rooms. Paired adrenal masses were reduced in short-day voles, but were unaffected by population density; serum corticosterone concentrations were significantly elevated in short-day compared with long-day animals. In both photoperiods, basal blood corticosterone levels were higher in voles from low-density compared with high-density rooms. Splenic masses were unaffected by day length, but were elevated among high-density animals. Similarly, serum immunoglobulin (IgG) levels were elevated among high-density animals. These results suggest that population density per se, in the absence of behavioral interactions, can affect reproductive size, and possibly function, in long-day conditions, and that prairie voles, which are highly social, exhibit higher corticosterone and lower IgG levels in low compared with high densities. These results may be important in understanding arvicoline population fluctuations, as well as improving animal husbandry practices in the lab.

seasonal; arvicoline rodents; immunoglobulin; olfaction; testis; day length

ANIMALS RESIDING in many habitats encounter seasonal changes in ambient energy availability and requirements. Because low energy availability coincides with maximal energy requirements in nontropical habitats, mechanisms have evolved to permit survival during the so-called winter energy "bottleneck" (6, 24, 47). Seasonal breeding is central among the suite of energy-conserving adaptations. Many animals confine energetically expensive breeding activities to specific times of year to maximize survival and reproductive success.

Prairie voles (*Microtus ochrogaster*) inhabit grasslands throughout much of the north midwestern United States and south midwestern Canada (21, 23). The breeding season of prairie voles varies among populations, but generally breeding begins in March and ends in October (28, 36). Although the majority of individuals within a given population stop breeding during the winter, a substantial minority of animals have been observed to breed during the winter in virtually every population studied (reviewed in Ref. 36). Laboratory studies have demonstrated that prairie voles can use the annual cycle of changing day length to time their reproductive efforts (34, 35). However, the existence of winter breeding suggests that other extrinsic and intrinsic factors contribute to the regulation of prairie vole breeding in nature, and these extraphotoperiodic factors may contribute to the significant multiannual population fluctuations often reported for this species (23, 36). Apparently, additional factors must be interacting with photoperiodic responsiveness to permit winter breeding during exposure to short day lengths.

Possible extrinsic factors that could mediate reproductive responsiveness to photoperiod include temperature, food quality and quantity, water availability, and social interactions (6, 22, 34, 37, 45). Although winter breeding often is associated with an increase in population density, exactly how various factors interact to permit occasional winter breeding remains unspecified (6, 36).

A number of studies in rodent species have indicated that overcrowding in laboratory settings or high population densities in the field can affect reproductive function (reviewed in Refs. 31, 44). Initially, it was hypothesized that intraspecific competition and agonistic interactions in high population densities led to the overstimulation of the hypothalamo-pituitary-adrenocortical (HPA) axis (i.e., stress), which resulted in diminished reproductive output, as well as compromised survival (2, 10–14). The vast majority of laboratory studies on the effects of social crowding on reproductive function have maintained animals at extremely high densities within single cages and have reported activation of the HPA axis and subsequent reductions in reproductive and immune function (reviewed in Refs. 1, 30, 31). Although chemosensory cues can enhance the physiological responses to crowding, tactile stimuli have been reported to mediate the adrenal effects of crowding on reproductive and immune function (16). Thus the suppressive effects of crowding on

reproductive and immune function appear to be due to social interactions, primarily of an agonistic nature (44, 48).

The present study was designed to ascertain the effects of population density on reproductive responsiveness to photoperiod in the absence of direct behavioral interaction. We also examined the effects of population density on adrenal size and function and splenic size and circulating blood immunoglobulin G (IgG) levels. Male prairie voles were housed individually in standard mouse cages and maintained either in long or short photoperiod conditions in separate rooms with a high or low population density. It was hypothesized that short-day males maintained in high-density rooms would display compromised immune function, but would not undergo reproductive regression. This latter hypothesis was disproved.

MATERIALS AND METHODS

Animals and housing conditions. Fifty adult (>60 days of age), male prairie voles (*Microtus ochrogaster ochrogaster*) were obtained from our laboratory colony. This colony was established from descendants of animals trapped near Urbana, IL, and was last outbred 3 yr ago. Voles were weaned at 21 days of age and housed with same-sex siblings. Three weeks before the initiation of the experiment, siblings were separated and housed individually in polypropylene cages (27.8 × 7.5 × 13 cm) in colony rooms with 24 h light:dark (LD) 16:8 cycles [lights illuminated at 0700 Eastern Standard Time (EST); light intensities ranged from 180 to 320 lx at cage level with the average intensity at 226.5 lx] at 21 ± 2°C with 50 ± 5% relative humidity. Food (Agway Prolab 1000, Syracuse, NY) and tap water were available at all times before and during the study.

Experimental conditions and autopsy procedures. After 3 wk of individual housing, animals either remained in long-day conditions (LD 16:8) or were moved to short days (LD 8:16; lights illuminated at 1000 EST). Additional experimental groups were formed by assigning animals to different housing conditions. Although all animals remained in their individual cages (27.8 × 7.5 × 13 cm), some animals were maintained in high population-density rooms in which the number of same-sex conspecifics averaged 10.96 animals/m^3, whereas other animals were maintained in low population density rooms in which the number of conspecifics averaged 0.18 animals/m^3 (low density). In all experimental rooms, 15–17 fresh air changes per hour were programmed; air intake and air exhaust did not mingle between rooms. Originally, the low-density groups were housed in another building. To control for an unintentional confounding effect of buildings, two additional low-density groups were maintained in the same laboratory area that previously housed the high-density groups. There were no significant differences between the two iterations of the low-density data, and they were therefore combined for all subsequent comparisons.

At the end of 10 wk, animals were anesthetized with methoxyflurane (Metofane: Pitman Moore, Mundelein, IL) vapors and weighed, and a blood sample was obtained from the retroorbital sinus. All blood samples were obtained within 120 s of first exposure to the anesthesia. The blood samples were allowed to clot at room temperature for 1 h; after clot removal, the samples were centrifuged at 3,500 revolutions/min at 4°C for 1 h. Serum was stored at −80°C until assay for corticosterone. Voles were killed by cervical dislocation, and the testes, epididymides, seminal vesicles, spleens, adrenal glands, epididymal fat pads, and intrascapular brown adipose tissue depots were dissected and weighed. Both absolute and relative (corrected for body mass) organ masses were recorded and analyzed.

Spermatogenic activity was assessed by counting the number of sperm and elongated spermatids in paired testes (capsule removed) and epididymides. Tissues were minced and separately transferred to an Eberbach blender. Testes and epididymides were homogenized for 30 and 45 s, respectively, in 25 and 40 ml, respectively, of 0.15 M sodium chloride containing 0.05% Triton X-100 (Sigma, St. Louis, MO) with 0.15 M thimerosal (Sigma). A sample of the homogenate was removed to determine the number of nuclei with a characteristic shape of mature spermatozoa or elongated spermatids under phase-contrast microscopy. Duplicate determinations were made for each homogenate.

Corticosterone radioimmunoassay. Blood serum corticosterone levels were assayed by radioimmunoassay (RIA) using an ^{125}I kit purchased from ICN Biomedicals (Carson, CA). The vole serum was diluted 1:2,000; all other instructions furnished by ICN were followed. Each sample was assayed in duplicate. This RIA has been completely validated for use in prairie voles (8, 43). The corticosterone assay was highly specific; cross-reactions with other steroid hormones were <0.3%. Serum corticosterone values were determined in a single RIA. The intra-assay coefficient of variation was 4.5%.

IgG antibody assay. IgG levels in the blood samples were determined using a sandwich enzyme-linked immunosorbent assay (ELISA) that was validated for use in prairie voles (3). Serial dilutions of vole serum were run with serial dilutions of mouse serum and mouse IgG. These serial dilutions yielded values in parallel to the standard curves of mouse IgG. Ninety-six-well immunoplates (Nunc, MaxiSorp) were incubated overnight at 4°C with 100 μl/well of a goat polyclonal antibody against mouse IgG (Cappel) diluted 1:3,000 in a carbonate/bicarbonate buffer (0.1 M, pH = 9.6). The following day the plates were washed four times with phosphate-buffered saline (PBS; 0.05 M, pH = 7.4) containing 0.05% Tween 20 and 0.001% NaN_3 using an automatic microplate washer (Bio-Rad, model 1550). A standard curve (upper limit: 10 μg/ml; lower limit: 0.001 μg/ml) was prepared using purified mouse IgG (Sigma) diluted in standard diluent [PBS (0.05 M, pH = 7.4) containing 0.05% Tween 20]. The standards (100 μl/well in triplicate) and samples of vole serum (100 μl/well in duplicate) diluted 1:100 with standard diluent were placed in wells on the plates. The plates were incubated overnight at 4°C. The following day the plates were washed and 100 μl of alkaline phosphatase-conjugated sheep-anti-mouse IgG (Cappel) diluted 1:2,500 in standard diluent were added to each well. The plates were incubated overnight at 4°C. The following day the plates were washed, and 100 μl of substrate buffer [0.1 mM *p*-nitrophenyl phosphate in diethanolamine buffer (0.1 M, pH = 9.5) containing 5 mM $MgCl_2$] was added to each well. The plates were incubated for 30 min, and the optical density of the resulting colored product in each well was measured at 405 nm using a microplate reader (Bio-Rad, model 450). The absolute concentrations of IgG in the samples were determined relative to the standard curve. The intra-assay coefficient of variation was 8.5%. All samples were assayed in a single ELISA.

Statistical analyses. All data were analyzed using two-way between-group analyses of variance (Systat). Additional pairwise analyses of mean values were conducted with Student's *t*-tests. Because all comparisons were planned, no post hoc corrections were used (29). Differences between group means were considered statistically significant if $P < 0.05$.

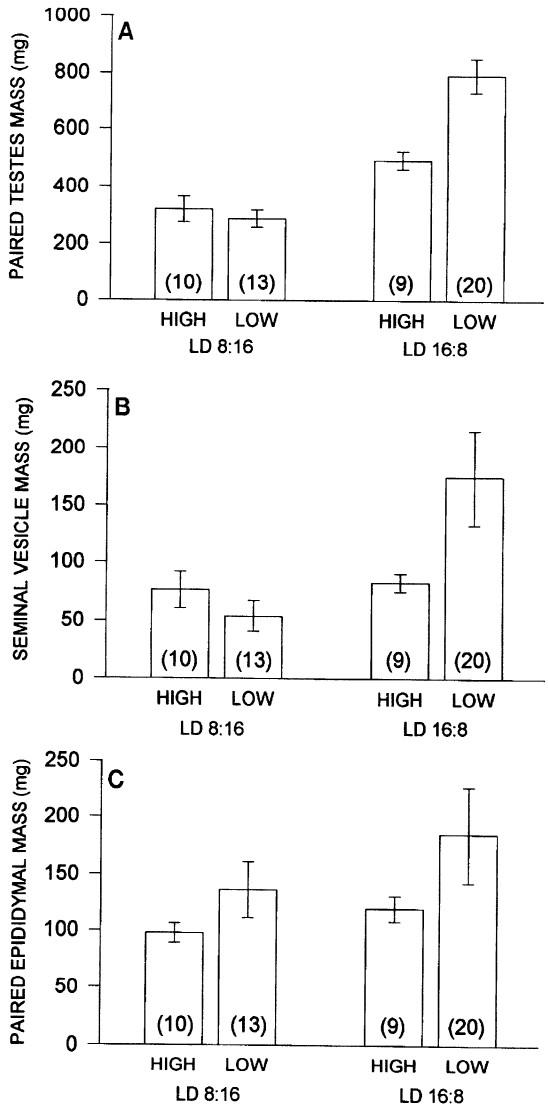

Fig. 1. *A*: mean (±SE) paired testes mass (mg) of male prairie voles housed in rooms with high (10.96 animals/m^3) or low (0.18 animals/m^3) population densities under long (LD 16:8) or short (LD 8:16) days. The sample no. for each treatment group is indicated in parentheses at the base of each bar. *B*: seminal vesicle mass; *C*: paired epididymal mass.

RESULTS

Paired testes, epididymides, and seminal vesicle masses were less in short-day animals than in their long-day cohorts ($P < 0.001$, $P < 0.001$, and $P < 0.05$, respectively) (Fig. 1). Population density did not significantly affect reproductive parameters in short-day voles ($P > 0.05$), but among long-day animals reproductive organ masses were reduced in high- compared with low-density rooms ($P < 0.05$ in each case) (Fig. 1). A similar pattern of results was obtained after organ masses were corrected for body mass (Table 1). Testicular sperm numbers were significantly higher in long-day compared with short-day voles (data not shown) ($P < 0.001$). Voles housed in high population density rooms had significantly lower testicular sperm counts than voles maintained in low population densities ($P < 0.05$), but both long-day groups possessed sperm numbers consistent with fertility (38). The main effects of photoperiod and population density for epididymal sperm counts followed a pattern of results similar to testicular sperm numbers ($P < 0.002$ and 0.01, respectively).

There were no main effects of either day length or population density on body mass ($P > 0.05$) (Table 1), although short-day voles in high-density rooms weighed significantly more than short-day voles housed in low-density rooms ($P < 0.05$) (Table 1). The epididymal fat pads followed the same pattern of results as body mass. Neither photoperiod nor population density exerted an overall significant effect on epididymal fat pad mass ($P > 0.05$ in both cases). However, the epididymal fat pads of short-day voles in high-density rooms weighed significantly more than those of short-day voles housed in low-density rooms ($P < 0.05$) (Table 1). Brown adipose tissue (BAT) was significantly heavier in high-density housing conditions compared with BAT mass of animals maintained in low-density conditions ($P < 0.05$) (Table 1). No significant differences in BAT mass existed between animals maintained in long vs. short days ($P > 0.05$).

Paired adrenal mass was significantly higher in long-day compared with short-day voles ($P < 0.05$), but was unaffected by population density ($P > 0.05$) (Fig. 2;

Table 1. *Mean body mass, intrascapular BAT, epididymal fat pad, and organ masses corrected for body mass of male prairie voles housed in rooms with high or low population densities in long (LD 16:8) or short (LD 8:16) photoperiods*

	LD 8:16		LD 16:8	
	High	Low	High	Low
Body mass, g	48.56 ± 3.32*	39.22 ± 1.37	44.04 ± 2.34†	42.64 ± 1.44†
BAT, mg	371.4 ± 81.2*	231.4 ± 59.7	367.9 ± 62.4*	271.1 ± 63.9
Epididymal fat, mg	1,136.5 ± 289.2*	583.5 ± 156.6	794.2 ± 264.7	682.1 ± 156.5
	Organ masses corrected for body mass, g/g body mass			
Testes	0.0064 ± 0.0005	0.0092 ± 0.0003	0.0112 ± 0.0064*	0.0182 ± 0.0004†
Seminal vesicles	0.0015 ± 0.0003	0.0013 ± 0.0003	0.0019 ± 0.0001*	0.0043 ± 0.0010†
Epididymides	0.0020 ± 0.0002	0.0033 ± 0.0009	0.0027 ± 0.0009*	0.0018 ± 0.0004*
Adrenals	0.0022 ± 0.0002	0.0026 ± 0.0007	0.0028 ± 0.0002*	0.0030 ± 0.0007*
Spleen	0.0109 ± 0.0006*	0.0084 ± 0.0002	0.0080 ± 0.0006	0.0090 ± 0.0002

Values are means ± SE. BAT, brown adipose tissue. Groups lacking or sharing a symbol in each row are statistically equivalent.

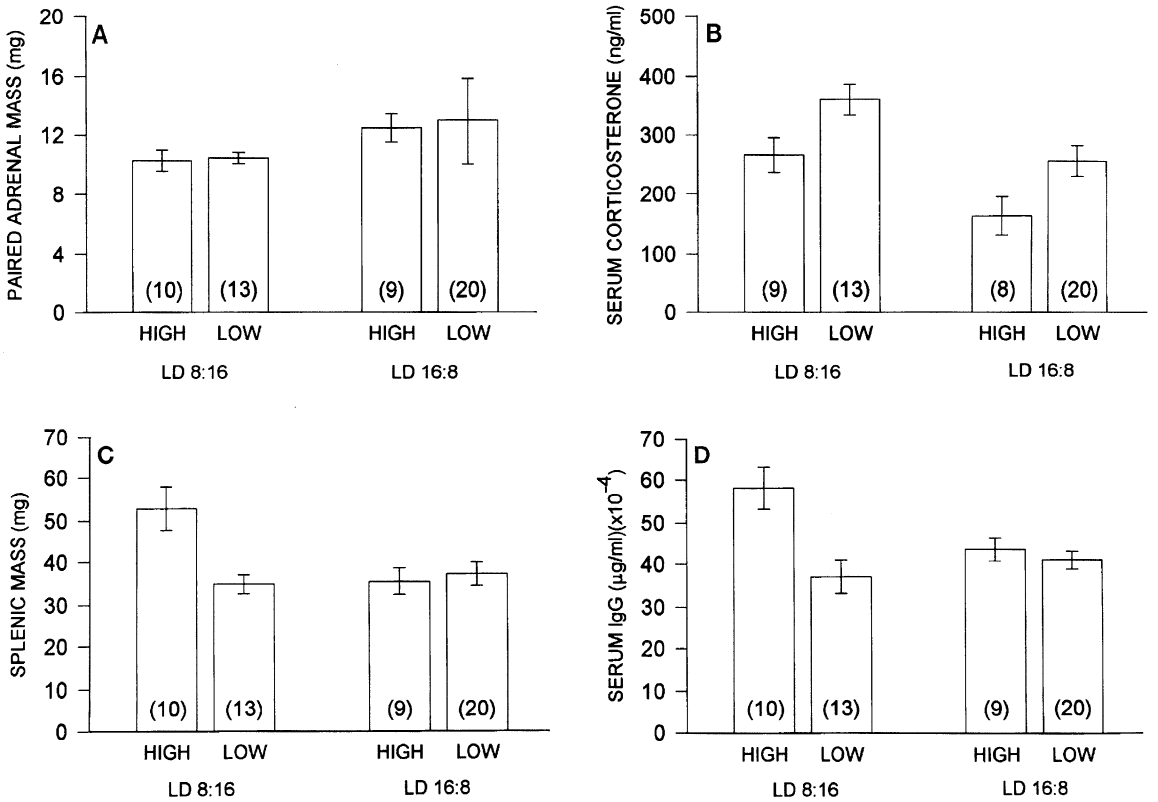

Fig. 2. *A*: mean (±SE) paired adrenal mass (mg) of male prairie voles housed in rooms with high (10.96 animals/m^3) or low (0.18 animals/m^3) population densities under long (LD 16:8) or short (LD 8:16) days. *B*: mean (±SE) serum corticosterone levels (ng/ml) of male voles housed in rooms with high or low population densities under long or short days. *C*: mean (±SE) splenic mass (mg) of male voles housed in rooms with high or low population densities under long or short days. *D*: mean (±SE) serum immunoglobulin G (IgG) titers (μg/ml) of male voles housed in rooms with high or low population densities under long or short days. Other symbols and conventions as in Fig. 1.

Table 1). Serum corticosterone levels were significantly elevated in short- compared with long-day voles ($P < 0.01$) (Fig. 2). Among both long-day and short-day animals, blood corticosterone levels were higher in those maintained in low-density rooms than in high-density rooms ($P < 0.05$) (Fig. 2). Splenic mass was unaffected by day length ($P > 0.05$) (Fig. 2), but splenic mass was significantly higher among high-density animals compared with animals housed in low-density rooms ($P < 0.05$) (Fig. 2). Serum IgG followed the same pattern of results as splenic mass, i.e., there were no photoperiodic main effects of day length ($P > 0.05$) (Fig. 2), but circulating serum IgG levels were higher among high-density animals compared with low-density animals ($P < 0.01$) (Fig. 2). Among short-day voles, IgG levels were higher in the high-density compared with the low-density conditions ($P < 0.05$) (Fig. 2).

DISCUSSION

These results confirm and extend previous observations that reproductive organ masses, as well as adrenal masses, are higher in long days than short days among prairie voles (*M. ochrogaster*) (36; 36a). Short-day voles displayed equivalent reproductive regression regardless of the population densities in the room in which they were housed; long-day voles maintained in low population density rooms exhibited higher reproductive organ masses than long-day voles housed in high population rooms. Fertility data were not obtained. This study also confirmed the prior observation that, despite reduced adrenal masses in short-day voles, serum titers of corticosterone of short-day animals were elevated compared with long-day voles (36a). In contrast to the anticipated outcome of this study, voles maintained individually in rooms with low population densities exhibited higher serum corticosterone levels than voles maintained in rooms with high population densities. Among short-day voles, the relatively low basal levels of circulating corticosterone in animals maintained in rooms with high population densities corresponded with elevated splenic masses and higher circulating levels of serum IgG levels compared with animals in other experimental conditions.

A number of studies have revealed that crowding results in elevated glucocorticoid secretion (31). The elevation in blood levels of glucocorticoids was hypothesized to reflect the stress of increased agonistic encounters (31, 44). Although this high level of stress and subsequent elevation in corticosterone levels was presumed to interfere with reproductive activities (10, 39, 44), the evidence that corticosterone interferes with breeding remains controversial (reviewed in Ref. 31). In the present study, animals did not interact socially and had equal-sized cages; yet, the population density outside the individuals' cages affected reproductive,

adrenal, and immune function. This suggests that tactile stimuli are not necessary for the effects of crowding to be expressed.

The cues that determine reproductive responses to population density have yet to be completely defined. However, in addition to agonistic behaviors and tactile stimuli, chemosensory stimuli are among the cues implicated in the mediation of reproductive responses to high population densities (e.g., Ref. 45). For example, the Lee-Boot and Whitten effects in female rodents represent how chemosensory stimuli associated with different population densities influence reproduction (reviewed in Ref. 45). Thus there is precedence for chemosensory stimuli associated with different population densities to affect physiology.

Chemosensory cues can also potentially affect immune function. A number of studies have indicated that immune function of recipients is altered in the presence of chemosensory stimuli emitted from stressed animals (16, 26, 39). In the present study, serum corticosterone and IgG levels were affected by population density. Because no behavioral interactions were permitted and animals were housed in opaque cages, information about population density must have been communicated by chemosensory or auditory stimuli. It is not apparent that animals in low population conditions should have been providing signals indicating stress to their cohorts; thus our results suggest that information, unrelated to stress, might be communicated to affect immune function in the recipient of the message.

Based on anecdotal evidence in our prairie vole breeding colonies and other vole colonies, it seemed that animals maintained in rooms with high numbers of conspecifics failed to inhibit reproductive function under short day conditions. The combination of these observations and the data suggesting that the stress of high population density numbers disrupt reproductive processes (44) led to the hypothesis that short-day voles maintained in rooms with high population densities would not undergo reproductive regression to the extent exhibited by short-day voles in low-density rooms. This hypothesis was ruled out in the present study. Long-day voles maintained in rooms with low population densities had significantly heavier reproductive organ masses than long-day voles in high-density conditions.

The functional significance of population density on reproductive organ size remains to be tested. Seasonal changes in adrenal mass have been reported for many different species of rodents in the field for years (19, 32, 42). However, the functional consequences of these seasonal changes in adrenal size and function has been an ongoing debate (31). These changes could reflect seasonal variation in stress responses to annual energetic challenges (e.g., Refs. 4, 15, 36, 47). Alternatively, the variation in adrenal mass may reflect reproductive activity (20, 31).

In the present study, the adrenals of short-day voles were smaller than those of long-day animals. However, serum corticosterone levels of short-day voles were higher than long-day voles. Interpretations of blood levels based on a single time point sample must be made with caution. Blood samples were obtained in both photoperiodic conditions 4–8 h before lights off, when previous studies have suggested that blood corticosterone concentrations are basal (8, 43). Thus the mean difference in corticosterone levels obtained at this time probably represents photoperiod-mediated differences in basal levels, rather than different time points on the circadian program of corticosterone secretion.

Chronically elevated adrenocortical hormones, especially the glucocorticoids, compromise immune function in both humans and nonhuman animals (1, 4, 17, 27, 38, 40). Adrenalectomy reverses the effects of sustained glucocorticoid exposure on immune function (17). The exact mechanisms by which the immune system interacts with the HPA axis are unknown, but this interaction probably involves cytokine release rates from activated immunological cells (e.g., Refs. 3, 30, 48). Regardless of mechanism(s), substantial evidence links elevated glucocorticoids with compromised immune function. In the present study, short-day male voles residing in rooms with high population densities displayed low serum corticosterone levels compared with short-day animals housed in low-density conditions; short-day voles housed in rooms with high densities exhibited elevated splenic masses and elevated serum IgG levels compared with the other experimental animals. Voles were not challenged by a specific antigen; only basal antibody concentrations were measured. Thus immune function was not assessed directly. The mammalian spleen is a lymphoid filter for antigenic particles and is important in the phagocytosis of antigens, parasitized blood cells, and immune complexes in the blood (25). The pulp tissue of the spleen includes the so-called red and white pulp tissues. The white pulp is the main lymphatic component of the spleen; lymphocyte recirculation, macrophage development from monocytes, and the final stages of lymphocyte differentiation are mediated in the splenic white pulp of birds and mammals (25). The red pulp tissue is involved in oxygen supply maintenance. Splenic mass was not elevated by photoperiod in the present study, although previous studies have indicated increased splenic mass in short days (5, 46). Elevation of splenic mass in long-day animals maintained at high densities suggests either increased immune function, increased erythropoiesis, or both. Additional research is necessary to determine precisely the effects of high density on splenic function.

One interpretation of these data is that prairie voles respond to low-density housing situation with activation of the HPA axis, a common indicator of stress. Prairie voles are highly social animals that live in extended family groups (21). These animals engage in social bonding and have been reported to display a monogamous social system (9). In addition to the unusual social system, prairie voles display high levels of circulating glucocorticoid concentrations (500–1,000 ng/ml at the circadian peak) relative to other rodent species (8, 43), and these steroid hormones may modu-

late the social interactions. Introduction of isolated prairie voles to another individual of the opposite sex induces substantial reductions in circulating corticosterone levels within minutes of the pairing (18). Adrenalectomy facilitates social pair bonding in females but inhibits it in male prairie voles (18). Thus males exhibit facilitation of social bonding when corticosterone levels are highest. Our data support this contention and suggest that males should form social bonds in nature more readily during short-day lengths, but only when population densities are low. A field study comparing winter vs. summer prairie voles revealed that monogamous social systems break down in prairie voles when the local population densities are at peak levels (33).

Taken together, the results of the present study suggest that animals in low population densities exhibit different reproductive, adrenal, and, possibly, immune functions compared with animals in high population densities in the absence of behavioral interactions. These responses could have significant adaptive value. For example, animals forming communal huddling groups during the winter might accrue benefits in addition to the conservation of heat and moisture. It is possible that grouped prairie voles have reductions in adrenal activity, which could lead to increased immune function during harsh environmental conditions, which could lead to increased winter survival. The effects of population size on BAT may also have important consequences on winter survival. Additional studies are required to ascertain the functional significance of the physiological changes associated with population density.

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