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The hormonal and behavioral response to group formation, seasonal changes, and restraint stress in the highly social Malayan Flying Fox (*Pteropus vampyrus*) and the less social Little Golden-mantled Flying Fox (*Pteropus pumilus*) (Chiroptera: Pteropodidae)

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Abstract

This study examined behavioral and physiological responses (changes in inter-animal spacing, total glucocorticoids, testosterone, and body mass) to the formation of breeding and same-sex groups in two bat species, the socially gregarious Malayan Flying Fox (*Pteropus vampyrus*) and the less social Little Golden-mantled Flying Fox (*Pteropus pumilus*). We hypothesized that social instability, especially in the breeding groups and especially in *P. vampyrus*, would result in elevated glucocorticoids and that social facilitation of breeding and/or male–male competition would result in persistently higher levels of testosterone in breeding males. Seasonal rhythms in all measures were also predicted, and the glucocorticoid stress response was expected to vary by sex, season, and group type. Nearly all animals responded to group formation with elevated glucocorticoids, but, for breeding animals (especially aggressive male *P. vampyrus*), these responses persisted over time. In both species, breeding group formation resulted in elevated testosterone in males. Glucocorticoids, testosterone, testes volume, and body mass generally peaked in the breeding season in males (late summer and early autumn), but the seasonal glucocorticoid peak in females occurred in late winter and early spring. All animals responded to restraint stress with elevations in glucocorticoids that largely did not differ by sex, time of year, reproductive condition, group type, or, in lactating females, the presence of her pup. Changes in both behavior and physiology were more evident in *P. vampyrus* than in *P. pumilus*, and we believe that their underlying social differences influenced their responses to group formation and to the changing seasonal environment.

Keywords: Corticosterone; Cortisol; Group formation; Hypothalamic–pituitary–adrenal axis; Inter-animal spacing; *Pteropus*; Seasonality; Social influences; Stress; Testosterone

Successful reproduction involves a number of factors, including the regulation of various hormonal systems, which often must be accomplished within a changing physical and social environment. Although there are many different neuroendocrine components involved in regulating reproduction and the response to changing environments, the hypothalamic–pituitary–adrenal (HPA) axis and the hypothalamic–pituitary–gonadal (HPG) axis are particularly important (Reeder and Kramer, 2005; Romero, 2002; Wingfield and Sapolsky, 2003). Glucocorticoid hormones (cortisol and corticosterone, the endpoint of the HPA axis) are important regulators of energy balance, and increases in glucocorticoids are considered a hallmark of the stress response (Reeder and Kramer, 2005). The HPA and HPG axes interact with one another in complex ways and serve, in part, to regulate behavior (DeVries, 2002; Handa et al., 1994; Viau, 2002). In turn, behavioral processes can have profound influences on these and other physiological systems (Eherhart et al., 1983; Mendoza and Mason, 1989a). In many species, animals must navigate complex social environments in order to reproduce, often forging social relationships with both known and unknown individuals. Moreover, the natural social and/or mating system in which an animal typically finds itself has profound implications for how an individual approaches and responds

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to these social relationships (Mendoza and Mason, 1989b), both behaviorally and physiologically.

The current study evaluated the behavioral and physiological responses to breeding group formation and to the formation of same-sex groups. We also studied the basic character of the stress response and how this response varies by time of year and reproductive condition. These studies were performed comparatively, using two species of frugivorous flying foxes (Chiroptera: Pteropodidae: *Pteropus*) as model systems. The Malayan Flying Fox (*Pteropus vampyrus*) is a large pteropodid bat, weighing ~1.1 kg with a wingspan of ~1.5 m. *P. vampyrus* is socially gregarious and roosts in groups of tens to thousands throughout Southeast Asia. *P. vampyrus* is a highly seasonal and synchronous breeder, with most females giving birth to a single pup in the spring (Kunz and Jones, 2000). The second species, the Little Golden-mantled Flying Fox (*Pteropus pumilus*) is a relatively small pteropodid bat found in the Philippines, weighing ~200 g with a wingspan of ~0.3 m. Although many species of *Pteropus* are highly social and can be found in groups of up to several hundred or even thousand animals, *P. pumilus* roosts in small aggregations with infrequent social interactions (L.R. Heaney, personal communication; Mickleburgh et al., 1992). They display only mildly seasonal breeding and may give birth to a single pup once or possibly twice per year. Given their different social tendencies, these two species of pteropodid bats are excellent models for exploring social influences on mammalian physiology, as well as the general physiology of the HPA and HPG axes, including seasonal rhythms of these hormones and the response to stress. Both species breed relatively well in captivity, and they have some of the highest plasma glucocorticoid levels ever described in mammals (Reeder and Kramer, 2005; Widmaier and Kunz, 1993; Widmaier et al., 1994).

In general, we hypothesized that the formation of breeding groups would transiently increase glucocorticoid levels in both sexes due to social instability and testosterone levels in males, due to social facilitation of breeding and/or male–male competition relative to the control groups (Capitanio et al., 1998; Schiml et al., 1996; Soto-Gamboa et al., 2005; Wingfield et al., 1990). Additionally, we hypothesized that, after an initial adjustment period, all animals would exhibit clear hormonal seasonal rhythms, as well as changes in body mass and changes in testes volume, that would vary by group type. These physiological changes would be accompanied by behavioral changes, here measured by changes in inter-animal spacing between different types of animals (e.g., between breeding males and females) over time. Changes were expected to be of greater magnitude in *P. vampyrus*, due to the predicted greater frequency of social interactions. Lastly, variability in the ability to mount a response to restraint stress by time of year and reproductive condition was assessed in all subjects. Because the glucocorticoid response to stress is superimposed upon baseline circadian and seasonal glucocorticoid rhythms (Reeder and Kramer, 2005; Romero, 2002), we hypothesized that a greater stress response would be evident in the fall during the mating season (when baseline glucocorticoids were also expected to be high and when groups were predicted to be less stable socially) than during the spring. We further hypothesized that pregnant and lactating females would show a blunted stress response that would be mediated by the presence of pups, as occurs in laboratory rats (Lightman et al., 2001; Stern et al., 1973).

Materials and methods

Animals and animal care

Subjects for this study included 48 *P. vampyrus* (22 males and 26 females) and 32 *P. pumilus* (16 males and 16 females). Animals were housed in captivity at the Lubee Bat Conservancy in Gainesville, Florida, USA. All of the research described in this paper was covered by an IACUC protocol from the Lubee Bat Conservancy (USDA Research Facility #58-R-0131; American Zoo and Aquarium Association Certified Related Facility #RF-4250000). Animals were housed in octagonal, double wire enclosures, measuring approximately 11 m in diameter and 2 m high. Enclosures contained an outdoor portion that encircled a smaller inside roost ("the nighthouse"; 3 m in diameter and temperature-controlled). Enclosures were designed to maximize the ability of bats to feed, rest, and fly freely. Animals were both wild-caught and laboratory-born and were all reproductively mature at the beginning of the study. Each bat was easily identified by a combination of at least three of the following markers: colored thumb bands, numbered thumb bands, ventral and dorsal fur bleaching of unique symbols, color-coded ball-chain necklaces enclosed in surgical tubing (LeBlanc et al., 2002), and uniquely coded transponders (Trovan, Santa Barbara, CA) implanted beneath the skin in the mid-scapular region. Animals were fed a mixture of fresh fruits, vegetables, and monkey chow (Purina) daily at 15:00 h. There was at least one food bowl per subject, and bowls were spaced sufficiently apart to minimize competition. Water was available ad libitum.

Group formation procedures

Subjects in each species were divided into 4 groups: 2 breeding groups (*P. vampyrus*: each had 5 males and 7 females; *P. pumilus*: each had 4 males and 4 females), and 2 same-sex control groups ($N = 12$ for *P. vampyrus* and 8 for *P. pumilus* for each sex). Based upon these groups, final samples sizes for subject type were: breeding females (*P. vampyrus*, $N = 14$; *P. pumilus*, $N = 8$), breeding males (*P. vampyrus*, $N = 10$; *P. pumilus*, $N = 8$), control females (in an all female group; *P. vampyrus*, $N = 12$; *P. pumilus*, $N = 8$), and control males (in an all male group; *P. vampyrus*, $N = 12$; *P. pumilus*, $N = 8$). The different sample size for breeding males and females in *P. vampyrus* was necessitated by the desire to reduce conflict between breeding males in this highly aggressive species.

Procedures for establishing groups varied slightly between the two species due to differences in colony management. In *P. vampyrus*, for the purposes of providing stability prior to the beginning of the study, pre-study same-sex groups containing all 48 subjects were formed in July of 2002 (2 male groups with 11 animals each and 2 female groups with 13 animals each). In mid-October, 2002, breeding and control study groups were created by rapidly moving animals from their pre-study groups into their assigned breeding and control study groups. Assignment of animals to study groups was randomized and balanced for age and size to the best of our ability, but management decisions necessitated the placement of some older (and larger) males into the control group in order to allow some younger, wild-caught males to breed. In *P. vampyrus*, the two breeding pens were adjacent (shared an opaque wire-mesh wall on one side), but the male and female control pens were as far apart as possible and not in visual contact (~40 m apart; on opposite ends of the complex).

In *P. pumilus*, same-sex pre-study groups were formed in early October, 2001, which was approximately 3.5 weeks prior to the formation of experimental groups. Animals were randomly assigned to each group. In this species, the two breeding groups were formed in late October by moving half of the animals from the pre-study same-sex groups into new enclosures. For this smaller species, the large octagonal pens were divided in half by the inclusion of a wire wall with an opaque shade-cloth covering, and the two breeding groups were adjacent to one another, on opposite sides of the wall. The two same-sex control groups were comprised of the animals remaining in the pre-study, same-sex groups (so 'group formation' for control animals in this species was not the formation of new social groups, but rather a social manipulation (the removal of some animals), which

was certainly a disturbance but not an entirely new social group). In this species, the male and female control pens were adjacent to one another, on either side of the opaque wire wall dividing a large octagonal pen. Despite this, however, some subjects within our control groups gained access to the opposite sex and bred (e.g., by digging through the sand under the wall, pushing against the wire at the ends of the dividing walls in order to reach the other side, and by taking advantage of the occasional open door at cleaning time).

Sampling protocol

To assess glucocorticoid and testosterone activity attendant to social group formation, cortisol, corticosterone, and testosterone were measured from samples collected between 13:30 h and 15:30 h on the day of group formation (or for convenience, 1 day prior, called 'day 0') and 1, 4, 15, and 35 days later and then monthly through August (*P. pumilus*) or September (*P. vampyrus*) of the next year. For the collection of baseline blood samples (2–3 ml for *P. vampyrus*; 1 ml or less for *P. pumilus*), bats were quickly hand captured and brought to a centrally located staging area outside of their pen. They were then manually restrained, and samples were collected via venipuncture within 3 min of capture from either a small wing vein or a vein in their forearm. Rapid collection of samples was necessary to avoid measuring the response to handling and sampling itself (Widmaier and Kunz, 1993; Widmaier et al., 1994). Blood was placed into EDTA-containing microtubes in an ice-water bath, and plasma was stored at −20 C until assayed. In this study and subsequent ones, animals only experienced the mild and routine discomfort of handling and blood sampling and mild restraint, thus no analgesics or anesthetics were required. All known methods of anesthesia induce rapid changes in normal physiology (Bazin et al., 2004). Because we were interested in changes in hormones both in basal and experimental conditions, introduction of another variable (anesthesia) would have rendered the results questionable. Additionally, because these bats were in multiple studies, often with repeated sampling in a short period, we wished to avoid measuring the effects of anesthesia rather than the effects of the handling and/or restraint per se. Within each bleeding session, the order in which animals were sampled was randomized to control for order effects.

Concurrent with the collection of baseline blood samples, a variety of morphometric measurements were made for each subject. These included length of the right forearm (measured with calipers at the beginning of the study and used to control for body size), body mass (collected by tightly wrapping a bat in a pillowcase and placing it in a bucket on top of a standard scale; an adjusted body mass measure was obtained by dividing body mass by length of forearm, and variations in body mass over time were assessed), and, for male *P. vampyrus*, testes height and width (measured with calipers through the scrotal sac; testes volume was then calculated by the formula for an ellipsoid ($1/6\pi * x * y * z$; where x, y, and z are the 3-dimensional measurements and where the third dimension (which could not be measured with calipers) was approximated by the height measurement). Total testes volume was calculated by adding the volume calculation for both testes together, this figure was then corrected for body size by dividing by forearm length.

Variations in the response to stress by sex, social group type, and time of year (reproductive condition) were assessed by measuring the response to handling, blood sampling, and restraint in the autumn (November) and in the spring (mid–late April) in all subjects. At each time point, following the regular baseline bleed, each subject was placed into a restraint device consisting of a soft mesh tube with wood plugs at the ends (16 cm in diameter × 57 cm tall for *P. vampyrus* and 10 cm × 24 cm for *P. pumilus*). The restraint tubes were then hung with bats inside of them (head down) in a larger cage within a temperature-controlled environment. A second sample (0.4 ml) was collected 30 min after initial capture and after animals were removed from their restraint tubes. The 'breeding females' (those in a breeding group) in *P. pumilus* in this part of the study that were not pregnant in the spring ($N = 5$ non-pregnant females; the 3 breeding females that were in various stages of pregnancy in the spring were not used in this analysis) and the 'non-breeding' or 'control' females ($N = 7$) at this time of year were used for this analysis, in contrast to *P. vampyrus* females, in which the 12 females that successfully reproduced were pregnant in the spring and are included in our analysis. Samples were analyzed for cortisol, corticosterone, and testosterone.

Finally, variations in the response to the same restraint paradigm by female reproductive status (late pregnancy vs. postpartum/lactating) and postpartum by social condition (whether pups were present with the dam in the restraint tube or removed from them) were assessed. Sample sizes for breeding females were adjusted to include only those whose infants survived ($N = 12$ for *P. vampyrus* and $N = 4$ for *P. pumilus* [3 from "breeding" groups and 1 from the "control" group]). In *P. vampyrus*, pregnancy samples were those collected in mid-late April (above), corresponding to the last month of pregnancy. Two postpartum/lactation samples were collected (in May and June, when pups were between 1 and 2 months of age): one in which a pup remained attached to the dam through sampling and the restraint period or one in which it was removed from her (but still in auditory range). The order in which these "pup present" or "pup absent" samples was collected was randomized and balanced across the study. For *P. pumilus*, which does not breed synchronously, samples were collected throughout the spring and summer, corresponding to late pregnancy and postpartum for each of the four females that successfully reproduced in this time period. Samples were assayed for cortisol and corticosterone.

Hormone assays

As has been the case with other bats studied, both *P. vampyrus* and *P. pumilus* have detectable levels of both glucocorticoid hormones, cortisol, and corticosterone (Reeder et al., 2004a,b; Widmaier and Kunz, 1993; Widmaier et al., 1994). Therefore, each hormone was separately assayed directly in a volume of 10 and 5 μl plasma, respectively, and in duplicate using commercially available radioimmunoassay (RIA) kits (MP Biomedicals, Irvine, CA; formerly ICN), as previously described and validated for these and other species in the genus *Pteropus* (Reeder et al., 2004a; Widmaier and Kunz, 1993; Widmaier et al., 1994). Testosterone was measured in 15 μl of plasma using a commercially available RIA kit (also MP Biomedicals/ICN) as previously described and validated (Reeder et al., 2004a). Least detectable doses were as follows: cortisol—6.6 ng/ml; corticosterone—0.094 ng/ml; testosterone—0.15 ng/ml. All samples were run in 29 assays for cortisol, 29 assays for corticosterone, and 14 assays for testosterone. Intra-assay and inter-assay variabilities were as follows: cortisol—11.68% and 21.06%; corticosterone—11.70% and 21.54%; testosterone—7.5% and 8.78%.

Behavioral observations

Tendencies toward social grouping were assessed by recording the location of all subjects relative to other group members over time. For *P. vampyrus*, home cages were divided into 73 unique locations, based upon an already existent grid on the roof of the cage with 72 different locations and the nighthouse as an additional location (Fig. 1). Bats nearly always hung suspended by their feet from the roof of the flight pen; on the rare occasion when bats were lower than the roof (e.g., on the side of the pen or hanging from ropes within the pen), the nearest roof grid locality was recorded. Instantaneous scan samples were collected in which the locality of each individual in a pen was recorded. From these 73 unique locations, 2701 different combinations of 2 locations were possible (location 1 and location 1 [if animals were in the same unique location]), location 1 and location 2, location 1 and location 3, location 1 and location 4…, location 45 and location 63…, etc.). The midpoint of each location was determined and assigned x and y coordinates. Based upon these coordinates, the distance between the midpoints of any two locations in the pen was calculated and used to estimate the distance between any two animals within the pen. The maximum distance across the pen was 10.92 m; this distance was used for all location combinations in which animals would not have been able to see one another (for example, if 2 animals were in the outside portion of the pen but on either side of the nighthouse and thus out of visual contact, a distance of 10.92 m was assigned rather than the direct distance between the two locations). If animals were in proximity to one another (close enough that one could reach out and touch another with its wings), an inter-animal distance of 0.4 m was assigned (based upon wingspan). A large lookup table was generated in Microsoft Excel from which distances could be calculated in a semi-automated way for each combination of 2 animals within a pen.

A total of 166 maps were recorded per pen (two breeding pens: one all-female pen and one all-male pen) for a total of 664 recordings. Maps were collected at least but often more than 30 min apart, with 4–6 maps generally collected per day. Maps were collected on day 0 (D0), the day the groups were formed, day 1 (D1), 1 day after groups were formed, D2, D3, and D4. Data for

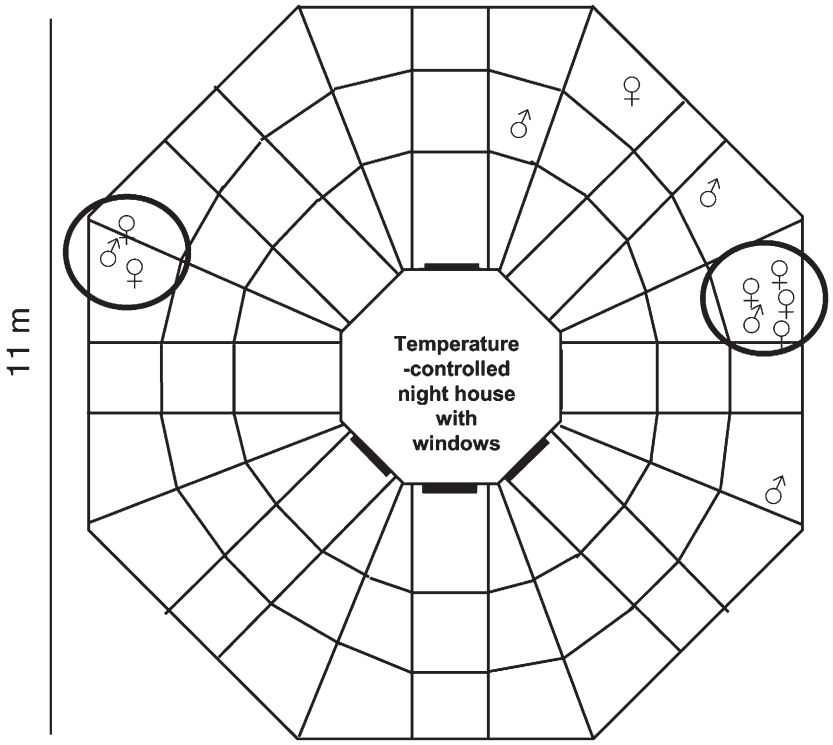

Fig. 1. Schematic of the home cage, showing existing grid structure on roof of cage (which was used for plotting individual animal locations) and representative spacing data for typical *P. vampyrus* breeding group. For the smaller species, *P. pumilus*, the cage was divided in half by the addition of an opaque wall, and two groups were housed within each cage.

D15, D35, and each subsequent month represent averages of 4–6 maps collected per day for 2–3 days (ranging from 9 to 18 maps collected per time period). Within each pen of 12 animals, a total of 66 different dyad combinations were possible (e.g., female 1 with female 2, female 1 with female 3, female 1 with female 4…, female 2 with female 3, etc). In each of the two breeding pens, these 66 dyads broke down into 21 female–female dyads, 10 male–male dyads, and 35 female–male dyads. In order to avoid data pooling errors and to ensure the independence of the data for statistical analyses, the calculated distances for each possible dyad (e.g., female 1 and female 2) were first averaged over the number of maps collected for that period. The numbers shown in Fig. 2A in turn are the averages and standard errors of each of the averaged inter-animal distances per type of dyad (for example, in the control female pen, 66 different female–female dyad combinations were possible; for each dyad combination (e.g., female 1 and female 2), inter-animal distances were first averaged over the total number of maps collected; the control female–control female dyad average shown in the figure is then the average of these averaged inter-animal distances for the 66 dyads). Based upon 664 maps collected, each with inter-animal distances calculated for 66 different dyads, Fig. 2A represents 43,824 individual inter-animal distance calculations.

For *P. pumilus*, in which each group of eight animals was housed in a pen exactly 1/2 the size of the *P. vampyrus* cages (see above), home cages were divided into 40 unique locations. Sampling was performed in the same manner as described above. From these 40 unique locations, 1446 different combinations of 2-locations were possible, and distance between any two locations was calculated as described above. If animals were in proximity to one another, an inter-animal distance of 0.2 m was assigned (based upon wingspan). A total of 208 maps were recorded in each control pen and 240 in each breeding pen for a total of 896 recordings. Maps were collected at least but often more than 30 min apart, with 8 maps generally collected per day. Data for D15, D35, and each subsequent month represent 8 maps collected per day for 3 days. Because of the manner in which control groups were formed in this species (see above), no maps were recorded in the control pens between DO and D2. Within each pen of 8 animals, a total of 28 different dyad combinations were possible. In each of the two breeding pens, these 28 dyads broke down into 6 female–female dyads, 6 male–male dyads, and 16 female–male dyads. For statistical analyses, the calculated distances were averaged and analyzed as described above. Based upon 896 maps collected, each with inter-animal distances calculated for 28 different dyads, Fig. 2B represents 25,088 individual inter-animal distance calculations. Localities and whether or not animals were in proximity with one another were recorded by both DMR and NSK. Inter-observer reliability was calculated for a total of 8 maps, including 89 recordable elements (locations, proximity assessments). Concordance between observers was 90.5%.

Statistical analyses

All data were analyzed parametrically. Inter-animal spacing data were analyzed using repeated measures ANOVA with time and group type as factors. As with all of the analyses in this study, the detailed response to group formation (data collected within the first 35 days) was analyzed separately from the monthly data (seasonal variation). All hormone data and morphometric data (mass and testes volume) were analyzed in the same manner as the spacing data, with repeated measures ANOVA. Significant effects were explored a priori and in limited cases a posteriori with *t* tests; *P* values were corrected for multiple comparisons using the Bonferroni method with layering, corrected *P* values are indicated by "pc." Within each species, autumn vs. spring stress responsiveness was assessed with 2 (baseline vs. stress) × 2 (time of year) × 2 (sex) repeated measures ANOVA. In the study assessing differences in stress responsiveness by reproductive or social condition, for *P. vampyrus*, differences in hormone levels between the 'pup present' and 'pup absent' test were first assessed using 2 (pup present vs. absent) × 2 (baseline vs. stress) repeated measures ANOVA followed by another ANOVA to look for differences in pregnancy vs. lactation. Due to the small sample size in this experiment for *P. pumilus*, ANOVA was not possible, and repeated *t* tests were utilized. In general, cortisol was found to be the major glucocorticoid measured in plasma, and cortisol and corticosterone responses were indistinguishable. Thus, after being separately assayed, cortisol and corticosterone have been combined here for analysis as total glucocorticoids. A notable exception is found in late pregnancy, where the hormones have somewhat different profiles (found in both study species and also in another species of flying fox, *P. hypomelanus*; Reeder et al., 2004a). Thus, separate

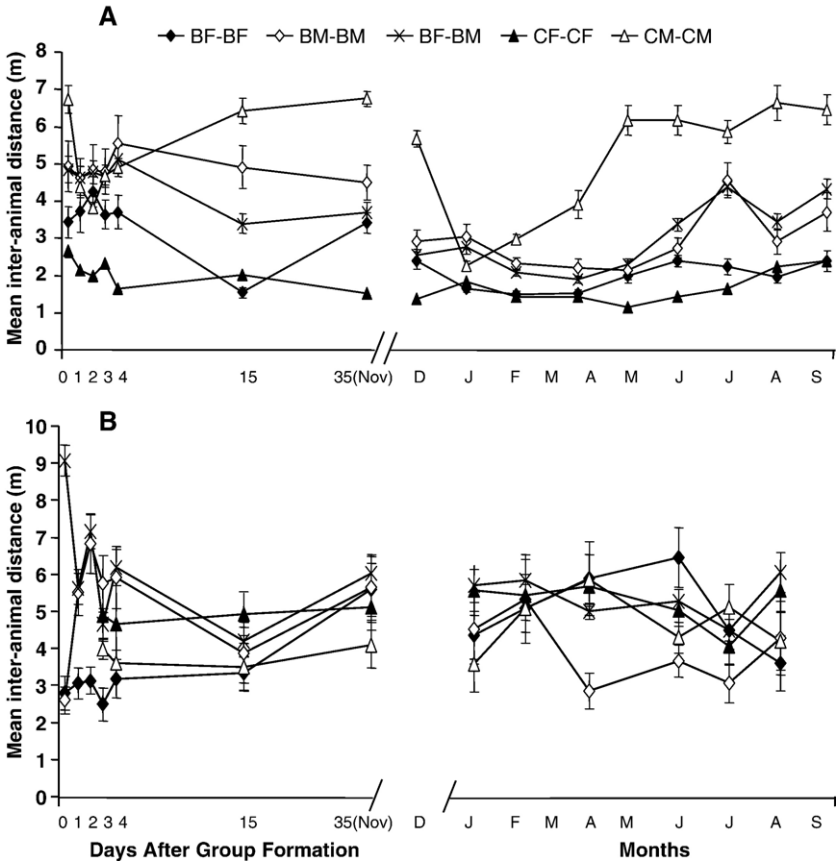

Fig. 2. Inter-animal spacing (mean ± SE) for (A) *P. vampyrus* and (B) *P. pumilus.* Figures on the left are changes over time (in days) for the first 35 days following the formation of new breeding and control (same sex) groups. Figures on the right are changes in time (in months) for the remainder of the year. BF–BF = average inter-animal distance between any 2 breeding females; BM–BM = average inter-animal distance between any 2 breeding males; BF–BM = average inter-animal distance between any pair consisting of a breeding female and a breeding male; CF–CF = average inter-animal distance between any 2 females in the all-female (control) group; CM–CM = average inter-animal distance between any 2 males in the all-male (control) group. See text for sample sizes and calculations.

analyses for cortisol and corticosterone are presented for the late pregnancy stress study only.

Results

Changes in inter-animal spacing in response to group formation and across the season

P. vampyrus

Significant changes in inter-animal distance between *P. vampyrus* individuals of the same and opposite sex were found both during the first 35 days following group formation and for the remainder of the year. That is, whether and to what extent certain types of individuals (e.g., breeding females) preferred to be closer to certain other types of individuals (e.g., breeding males or other breeding females) changed over time in response to group formation and changed over the course of the next year (presumably in response to reproductive events). Average inter-animal distance was calculated for five possible combinations (dyad types) of individuals: breeding females and other breeding females; breeding males and other breeding males; breeding males and breeding females; and within the same-sex control pens: control males with control males, and control females with control females. In general, the pattern of inter-animal spacing, i.e., which groups of animals tended to be the most clustered together and which were spaced the farthest apart, remained relatively stable across time (Fig. 2A). The control female–control female (CF–CF) dyads tended to be the closest of all dyad types over time and were generally clustered together in one area of their pen. Breeding female–breeding female (BF–BF) dyads were the next closest group of animals (also tended to cluster together, but not as tightly as CF–CF dyads). Breeding female–breeding male dyads (BF–BM) were spaced either about the same distance apart as BF–BF dyads or slightly (but significantly) farther apart. The average distance between breeding male–breeding male dyads (BM–BM) was similar to that for BF–BM dyads (except at D15, see below), and the greatest distance between animals was seen in the control male–control male (CM–CM) dyads, which were, except during the winter months, spaced about as far apart as they could be. The stability of these differences in inter-animal spacing between the different dyad types over time is illustrated by the data from both D35 and May (which statistically resemble nearly all of the other time points, with differences discussed in text above), in which significant differences in spacing between dyad types were found (D35, $F_{(4,259)} = 80.513$, $P < 0.0005$; May, $F_{(4,259)} = 70.556$, $P < 0.0005$). At both time points, (1) CF–CF dyads were significantly closer than their

comparison groups, BF–BFs (D35, $t = -8.245$, $df = 106$, pc < 0.001; May, $t = -5.516$, $df = 106$, pc < 0.001) and CM–CMs (D35, $t = -25.606$, $df = 130$, pc < 0.0015; May, $t = -12.36$, $df = 130$, pc < 0.0015), (2) CM–CM dyads were significantly farther away than their comparison groups, BM–BMs (D35, $t = 5.078$, $df = 84$, $P < 0.0005$; May, $t = 5.366$, $df = 84$, $P < 0.0005$) and CF–CF (see above), and (3) no significant differences in inter-animal spacing between dyads in the breeding groups were found.

Despite the stability of the spacing of dyad types relative to one another over time, the inter-animal spacing within each dyad type and the magnitude of differences between the different dyad types varied significantly over time. More specifically, within the first 35 days after group formation, inter-animal spacing changed over time in ways that were specific to the type of dyad (time: $F_{(6,1554)} = 4.599$, $P < 0.0005$; dyad type: $F_{(4,259)} = 55.871$, $P < 0.0005$; time * dyad type interaction: $F_{(24,1554)} = 10.916$, $P < 0.0005$). Within the breeding groups, there were no significant differences within the three dyad types (BF–BF, BM–BM, BF–BM) between D0 and D4. This continued to be the case for BM–BM dyads over the 35-day period, but a significant decrease in inter-animal spacing between D4 and D15 over time (animals got closer together) was found in both BF–BF dyads and BF–BM dyads (BF–BF: $t = 5.502$, $df = 41$, pc < 0.001; BF–BM: $t = 5.438$, $df = 69$, pc < 0.0015). Between D15 and D35, BF–BM remained close together (no significant difference from D15), but the average distance between breeding females (BF–BF) significantly increased ($t = -8.374$, $df = 41$, pc < 0.0015). This may have occurred because breeding animals by D35 were essentially found in small single male–multiple female groups (see example in Fig. 1), which is lost in the averages.

In contrast to the breeding groups, inter-animal distance in the same-sex control groups significantly varied between D0 and D4. For both control females and control males, the average distance significantly decreased between D0 and D1, that is, animals were closer together the day after group formation than the day of group formation (CF–CF: $t = 2.913$, $df = 65$, pc = 0.01; CM–CM: $t = 5.129$, $df = 65$, pc < 0.003). Between D1 and D35, the inter-animal spacing between CF–CF dyads generally decreased, but there were a number of subtle but statistically significant increases and decreases between the sample days (D2 < D3: $t = -3.074$, $df = 65$, pc = 0.009; D3 > D4: $t = 7.290$, $df = 65$, pc < 0.003; D4 < D15: $t = -5.181$, $df = 65$, pc < 0.002; D15 > D35: $t = 6.642$, $df = 65$, pc < 0.0025). The magnitude of differences in inter-animal spacing between time points for CF–CF dyads was miniscule compared to the changes in spacing that occurred between D1 and D35 within the control males, who spread further apart as time progressed (inter-animal spacing increased D2 to D3: $t = -3.452$, $df = 65$, pc = 0.004; and D4 to D15: $t = -3.774$, $df = 65$, pc < 0.0025, then remained high D15 to D35).

Similar differences in inter-animal spacing were found during the rest of the year in *P. vampyrus*, with significant changes over time that varied by dyad type (time: $F_{(9,2232)} = 40.841$, $P < 0.0005$; dyad type: $F_{(4,248)} = 150.585$, $P < 0.0005$; time * dyad type interaction: $F_{(36,2232)} = 11.998$, $P < 0.0005$). Within the breeding groups, significant changes over time were found in all three dyad types (BF–BF: $F_{(9,369)} = 9.805$, $P < 0.0005$; BF–BM: $F_{(9,621)} = 28.479$, $P < 0.0005$; BM–BM: $F_{(9,171)} = 6.525$, $P < 0.0005$). During the period when mating occurred (Nov/Dec), inter-animal spacing was not significantly different between all three breeding group dyad types. After the period when mating primarily occurred, breeding females decreased their average inter-animal distance from each other (Nov > Jan [early pregnancy]: $t = 5.759$, $df = 41$, pc < 0.001). Breeding females maintained this proximity to one another until pups were born in May, when they spaced themselves farther apart and were each in constant contact with their pup; this difference was statistically significant by June ($t = -4.632$, $df = 41$, $P < 0.0005$). During this same time period, BF–BM dyads and BM–BM dyads showed similar changes in inter-animal spacing: animals became closer together by January and became farther apart after pups were born (January, BF–BM: $t = 3.504$, $df = 69$, pc = 0.002; BM–BM: $t = 2.863$, $df = 19$, pc = 0.02; May, BF–BM: $t = 2.053$, $df = 69$, $P = 0.044$, with a further increase in June: $t = -6.145$, $df = 69$, pc < 0.0025; BM–BM: difference not significant). By July, breeding males had significantly increased the distance between themselves and both other breeding males and breeding females, approaching the difference seen between males in the same-sex control group (BM–BM: $t = -3.083$, $df = 19$, pc = 0.018; BF–BM: $t = -4.101$, $df = 69$, pc < 0.002. Distances between breeding males and others then decreased again by August (BM–BM: $t = 3.557$, $df = 19$, pc = 0.008; BF–BM: $t = 3.951$, $df = 69$, pc < 0.015) and increased but were more variable by September.

Significant differences in inter-animal spacing during the year were also observed in the same-sex control groups. As was the case during the first 35 days, CF–CF dyads had small but statistically significant changes in inter-animal spacing during the year ($F_{(9,486)} = 33.638$, $P < 0.0005$). In contrast to BF–BF dyads, control females increased inter-animal spacing between November and January ($t = -3.849$, $df = 65$, $P < 0.0005$) and decreased inter-animal spacing between January and May ($t = 8.661$, $df = 65$, $P < 0.0005$). Between May and September, control females significantly increased the average distance between each other ($t = -10.051$, $df = 65$, pc < 0.0015). Changes in inter-animal spacing within the control males were also significant over the year and again of much greater magnitude than in control females ($F_{(9,585)} = 30.016$, $P < 0.0005$). Essentially, males are significantly closer to one another during the non-breeding (and colder) winter months of January and February (often remaining in the warm nighthouse through late morning; Nov > Jan: $t = 18.912$, $df = 65$, pc < 0.002; Jan< Feb: $t = -4.695$, $df = 65$, pc < 0.001; Feb < April: $t = -2.965$, $df = 65$, $P = 0.004$; April < May (then distances remain large through Sept): $t = -4.949$, $df = 65$, pc < 0.0015).

P. pumilus

Less profound changes in inter-animal distance occurred in *P. pumilus* (Fig. 2B). In the first 35 days after group formation, significant changes in spacing were found within dyads in the breeding groups only (BF–BF: $F_{(6,66)} = 4.412$, $P = 0.001$; BM–

BM: $F_{(6,66)} = 5.189$, $P < 0.0005$; BF–BM: $F_{(6,186)} = 18.069$, $P < 0.0005$). On the day of group formation (D0), *P. pumilus* in the breeding groups grouped together exclusively by sex, such that distance between females and males (BF–BM dyads) was significantly greater than that between animals of the same sex (BF–BM vs. BF–BF: $t = -8.30$, $df = 42$, pc < 0.0001; vs. BM–BM: $t = -8.889$, $df = 42$, pc < 0.0015). By D1, a dramatic shift in social preferences occurred within the breeding pens, such that breeding males were no longer hanging in close proximity to other males ($t = -7.09$, $df = 11$, pc < 0.003) but instead were much closer to females ($t = 6.178$, $df = 31$, pc < 0.0025). This change persisted through D4. Between D4 and D15, inter-animal spacing in both BM–BM dyads and BF–BM dyads decreased, with this difference being significant for BF–BM dyads ($t = 3.627$, $df = 31$, pc = 0.002). This decrease resulted in no significant differences in inter-animal spacing between any group types (including controls) at D15. Subsequently, between D15 and D35, all animals in the breeding pens became more spaced apart, with significant increases in inter-animal spacing found in all three types of dyads (BF–BF: $t = -3.736$, $df = 11$, pc = 0.006; BM–BM: $t = -3.347$, $df = 11$, pc = 0.035; BF–BM: $t = -6.264$, $df = 31$, pc < 0.003); there were no significant differences in inter-animal spacing between any of the dyad types at D35.

Changes in inter-animal distance between and within dyad types over the rest of the year were less evident and were found only within BF–BF ($F_{(6,66)} = 2.667$, $P = 0.022$), BM–BM ($F_{(6,48)} = 2.709$, $P = 0.024$), and CM–CM ($F_{(6,120)} = 2.844$, $P = 0.013$) dyads. Only a few changes over time were found within these groups, and these changes were idiosyncratic and appeared to be random. Differences in inter-animal spacing between dyad types at each time point were only found in April and June; at this time, the inter-animal distance between breeding females was much greater than that between breeding males (April: $t = 2.730$, $df = 22$, $P = 0.012$; June: $t = 3.079$, $df = 22$, pc = 0.03), and breeding males were significantly closer to each other than they were to females (April: $t = -4.815$, $df = 42$, pc < 0.015; June: $t = -2.651$, $df = 42$, pc = 0.055).

P. vampyrus vs. P. pumilus

When comparing data between the species, it is important to note that there is an inherent bias in the data: *P. vampyrus* groups were in a full pen, whereas *P. pumilus* groups were each in half size pens, thus *P. vampyrus* groups had twice as much flight and hanging space as *P. pumilus* but did not have twice the number of animals in each group. Consequently, if animals were attempting to maximize the distance between themselves, the difference would be greater in *P. vampyrus* solely due to differences in pen and group size between the species. Additionally, *P. vampyrus* is approximately three times larger than *P. pumilus* and would therefore, all else being equal, need more space than *P. pumilus.* Despite these biases that would theoretically predispose *P. vampyrus* towards greater inter-animal distances than *P. pumilus*, they were in fact generally and significantly closer together than *P. pumilus* (mean ± SE inter-animal distance in *P. vampyrus* vs. *P. pumilus*: 3.43 ± 0.09 m vs. 4.94 ± 0.17 m; $t = -7.894$, $df = 181.4$ [adjusted for unequal variances], $P < 0.0005$). Within each dyad type, in all but CM–CM dyads, *P. vampyrus* had smaller inter-animal distances than *P. pumilus* (mean ± SE inter-animal distance in *P. vampyrus* vs. *P. pumilus*: BF–BF: 2.60 ± 0.10 m vs. 4.11 ± 0.34 m; $t = -4.223$, $df = 13.1$, $P = 0.001$; BM–BM; 3.79 ± 0.23 m vs. 4.61 ± 0.41 m, but this difference was not significant, $t = -1.926$, $df = 30$, $P = 0.064$; BF–BM: 3.63 ± 0.13 m vs. 5.79 ± 0.18 m, $t = -9.500$, $df = 100$, $P < 0.0005$; CF–CF: 1.82 ± 0.04 m vs. 5.09 ± 0.39 m, $t = -8.391$, $df = 27.5$, $P < 0.0005$). The opposite was true for control males, which were significantly closer to one another in *P. pumilus* than in *P. vampyrus* (4.32 ± 0.40 m vs. 5.24 ± 0.10 m, $t = 2.206$, $df = 30.7$, $P = 0.035$).

Changes in glucocorticoids in response to group formation and across the season

P. vampyrus

A significant change in total glucocorticoid levels (cortisol + corticosterone) was seen in response to group formation and across the year in both species (Fig. 3). For *P. vampyrus*, significant changes over the first 35 days following group formation were seen that varied significantly by sex according to breeding condition (main effect for time: $F_{(4,172)} = 12.777$, $P < 0.0005$; main effect for sex: $F_{(1,43)} = 35.307$, $P < 0.0005$; significant time * sex: $F_{(4,172)} = 4.739$, $P = 0.001$; time * breeding condition: $F_{(4,172)} = 5.892$, $P < 0.0005$, and sex * breeding condition: $F_{(1,43)} = 5.432$, $P = 0.025$, interactions, but no main effect for breeding condition; the datum for one control male was missing for D1; hence, a sample size of 11 rather than 12 was used for this analysis). Males and females in both breeding and control groups responded to the formation of new social groups with an increase in total glucocorticoids by D1, but this difference was only significant for females (breeding females: $t = -4.338$, $df = 13$, pc = 0.005: control females: $t = 3.348$, $df = 11$, pc = 0.036). For breeding females, this increase in measured glucocorticoids was sustained for the 35-day period (D35 > D0, $t = -3.691$, $df = 13$, pc = 0.012). In contrast, in control females, this rise was transient, with glucocorticoid levels by D35 back down to baseline and not distinguishable from those at D0. Breeding males displayed a similar pattern to those of breeding females, in which glucocorticoid levels went up initially (by D1) and stayed up, but there were no significant differences over the 35-day period for breeding males. Control males followed the same pattern as control females, with a transient increase in glucocorticoids from D0 to D1, and in this case with an eventual decline to D35 levels lower than baseline (D0) levels (significant decrease from D1 peak level by D15: $t = 5.813$, $df = 10$, pc < 0.003). Male *P. vampyrus* had significantly higher glucocorticoid levels than females at DO, D1, and D4 (D0: $F_{(1,43)} = 34.404$, $P < 0.0005$; D1: $F_{(1,43)} = 30.299$, $P < 0.0005$; D4: $F_{(1,43)} = 13.171$, $P = 0.001$). Additionally, at D1, when all subjects showed an increase in glucocorticoids in response to group formation, not only were hormone levels higher in males than in females, but they were higher in control animals vs. breeding animals ($F_{(1,43)} = 7.316$, $P = 0.01$), indicating a greater response in control animals. By D15, there are no significant differences

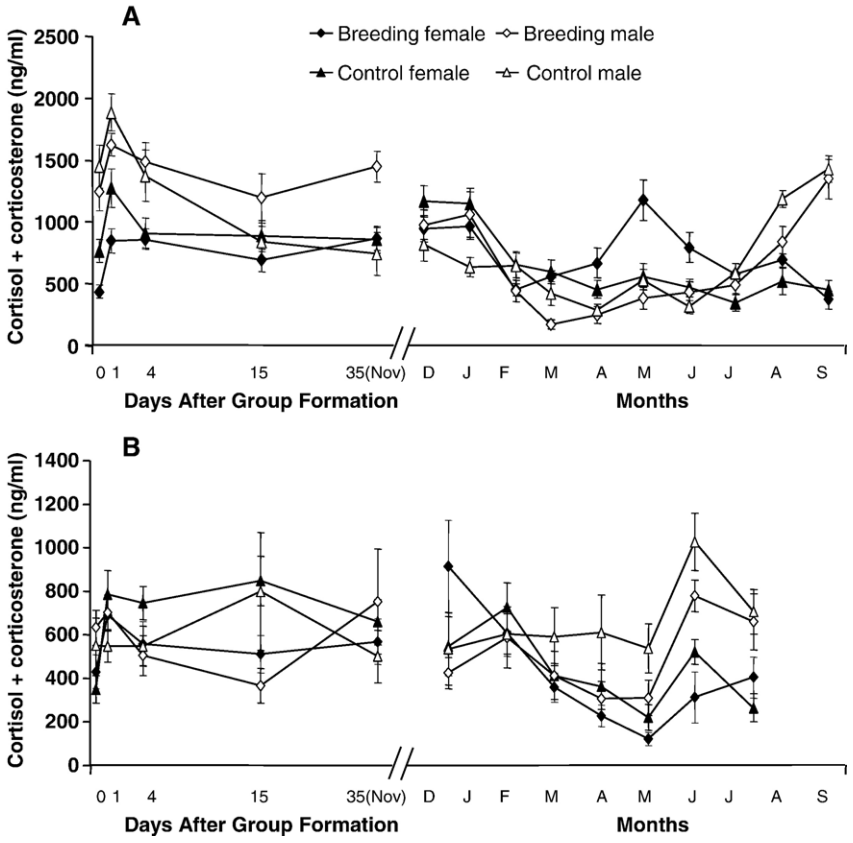

Fig. 3. Total glucocorticoids (cortisol + corticosterone, mean ± SE) levels over time for (A) *P. vampyrus* and (B) *P. pumilus*. Figures on the left are changes over time (in days) for the first 35 days following the formation of new breeding and control (same sex) groups. Figures on the right are changes in time (in months) for the remainder of the year. At each timepoint, samples sizes were as follows: *P. vampyrus*, 14 breeding females, 10 breeding males, 12 control females, 12 control males; *P. pumilus*, 8 breeding females, 8 breeding males, 8 control females, 8 control males.

solely based upon sex or breeding condition, but breeding males had higher glucocorticoid levels than did breeding females and control males (difference was significant for breeding females: $t = -2.490$, $df = 22$, pc = 0.042). This difference was even greater by D35, when breeding males had much greater glucocorticoid levels than did breeding females and control males (vs. breeding females: $t = -3.707$, $df = 22$, pc = 0.002; vs. control males: $t = 3.120$, $df = 19$, $P = 0.006$).

Examination of the monthly data indicates a significant seasonal rhythm in total glucocorticoids that varied by sex and breeding condition (main effect for time: $F_{(11,429)} = 21.368$, $P < 0.0005$; significant interactions for time * sex: $F_{(11,429)} = 18.140$, $P < 0.0005$, time * breeding condition: $F_{(11,429)} = 3.085$, $P = 0.001$, and time * sex * condition: $F_{(11,429)} = 4.784$, $P < 0.0005$; no significant main effect for sex or breeding condition). Breeding and control female *P. vampyrus* showed significant seasonal rhythms in total glucocorticoids ($F_{(11,220)} = 12.414$, $P < 0.0005$) that paralleled one another, with the exception of a peak in glucocorticoids attendant to pregnancy in breeding females (time * breeding condition interaction: $F_{(11,220)} = 4.404$, $P < 0.0005$). Both breeding and control females showed a peak in glucocorticoid levels in December/January followed by a rapid decrease. Breeding and control male *P. vampyrus* showed significant seasonal rhythms (changes over time) in total glucocorticoids ($F_{(11,209)} = 24.09$, $P < 0.0005$) that paralleled one another (with glucocorticoid levels in breeding males either less than or not distinguishable from those of control males, except in January; main effect for breeding condition: $F_{(1,19)} = 3.174$, $P < 0.0005$; time * condition interaction: $F_{(11,209)} = 3.572$, $P < 0.0005$; breeding males vs. control males, January: $t = 2.103$, $df = 20$, $P = 0.048$; March: $t = -2.188$, $df = 20$, $P = 0.041$; August: $t = -2.512$, $df = 19$, $P = 0.021$). Like females, male *P. vampyrus* exhibited a significant seasonal rhythm with a clear peak, but, in this case, the peak occurred in the fall (see D0 values, August and September), and it thus was out of phase with the females' seasonal rhythm. This variation in the seasonal rhythm between males and females resulted in males having greater glucocorticoid levels than females at some times of the year (e.g., as was described above for D0, and also for September: $F_{(1,42)} = 89.591$, $P < 0.0005$) but not at others.

P. pumilus

Data from *P. pumilus* were more variable than those from *P. vampyrus*, and the response to group formation was much less profound. A significant response to group formation, as measured in changes in total glucocorticoids, was only present in females (breeding females: $F_{(4,28)} = 2.797$, $P = 0.045$; control females: $F_{(4,28)} = 7.989$, $P < 0.0005$). In both breeding females

and control females, a significant increase in total glucocorticoids was seen between D0 and D1 (similar to what was seen in all *P. vampyrus* groups; breeding females: $t = -5.288$, $df = 7$, pc = 0.005; control females: $t = -5.963$, $df = 7$, pc = 0.005), and levels remained elevated for the 35-day period. Within each time point, there were no significant differences between groups in total glucocorticoid levels.

Seasonal data for *P. pumilus* were also more variable than those from *P. vampyrus*, and, although there were significant changes over time in all groups, the seasonal peaks were less evident. Because *P. pumilus*, unlike *P. vampyrus*, did not breed synchronously and because two of the control females bred, an obvious glucocorticoid spike attendant to pregnancy is absent in these data. In fact, in this analysis, data from three breeding females and two control females that gave birth at various times in this year were removed (resulting in $N = 5$ for the breeding females and $N = 6$ for control females). In this data set, therefore, the only difference between 'breeding' and 'control' females is the composition of the group in which they were found, rather than breeding status per se. As was the case with female *P. vampyrus*, the glucocorticoid rhythm in female *P. pumilus* peaked in winter (December/January) and then declined. Both breeding and control males had significant changes in total glucocorticoids over the season ($F_{(7,98)} = 3.972$, $P = 0.001$), with a peak in early July (May vs. late June: $t = -5.234$, $df = 15$, $P < 0.0005$), but did not differ from one another (but the power to detect a difference between groups was low: $1 - \beta = 0.173$ for detecting an overall difference between breeding and control males and $1 - \beta = 0.517$ for detecting an interaction between time changes and group type). During and around the time of this peak period for males, glucocorticoid levels were significantly higher in males than in females (May: $F_{(1,23)} = 8.084$, $P = 0.009$; late June: $F_{(1,23)} = 21.198$, $P < 0.0005$; late July: $F_{(1,23)} = 11.309$, $P = 0.003$).

Changes in testosterone and testes volume in response to group formation and across the season

P. vampyrus

Within the first 35 days following group formation, testosterone levels in *P. vampyrus* varied significantly over time in different ways that depended upon breeding condition (Fig. 4A; time main effect: $F_{(4,68)} = 97.645$, $P < 0.0005$; time * condition interaction: $F_{(4,68)} = 3.894$, $P = 0.007$). In both breeding and control males, testosterone decreased over this 35-day period, but this decrease occurred more quickly in control males (D1 > D4, $t = 2.430$, $df = 8$, $P = 0.041$, and D4 > D15, $t = 3.797$, $df = 11$, pc = 0.006, then levels remained low, no difference detected between D15 and D35 levels). In breeding males, testosterone levels at D4 and D15

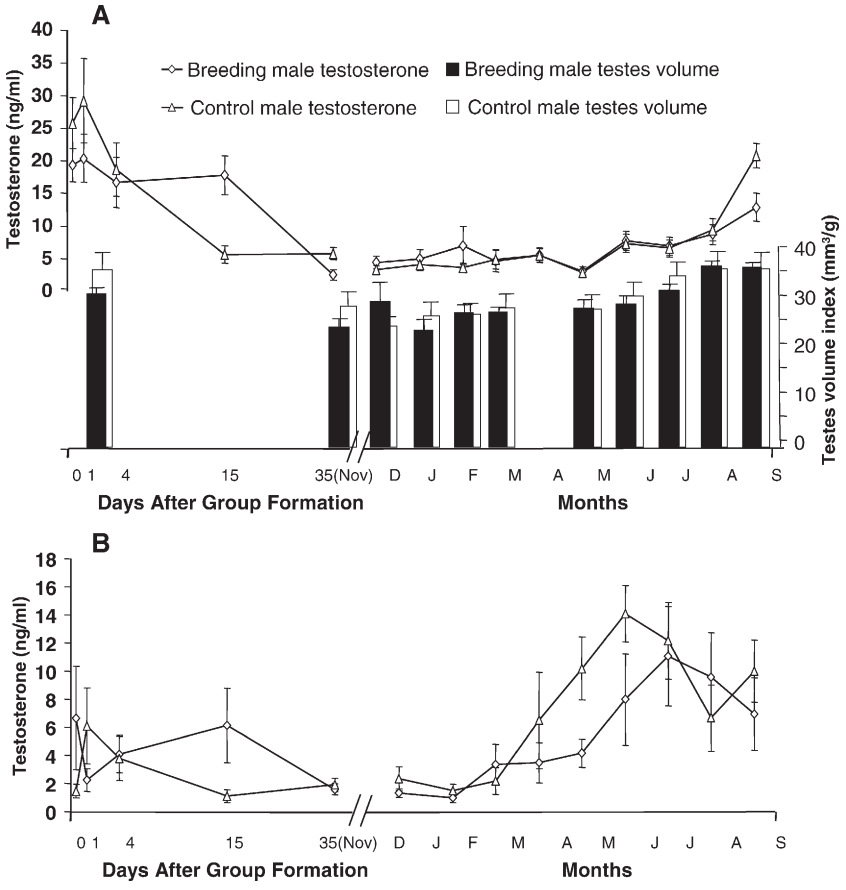

Fig. 4. Testosterone levels (mean ± SE) over time for (A) *P. vampyrus* and (B) *P. pumilus*. Figures on the left are changes over time (in days) for the first 35 days following the formation of new breeding and control (same sex) groups. Figures on the right are changes in time (in months) for the remainder of the year. For *P. vampyrus*, figure A also shows mean ± SE adjusted testes volume (total testes volume/mass in grams to control for body size).

remained indistinguishable from those at DO then significantly decreased between D15 and D35 (t = 4.689, df = 9, P = 0.001). At D15, testosterone was significantly greater in breeding males than in control males (t = 4.005, df = 20, P = 0.001), but by D35, levels dropped so low in breeding males that they were significantly lower than those of control males (t = −2.58, df = 20, P = 0.018). Testosterone levels then remained low for a number of months and began to rise in the summer in both breeding and control males ($F_{(10,190)}$ = 15.251, P < 0.0005). Significant increases between July and September occurred in both breeding males (t = −3.492, df = 9, P = 0.007) and control males (t = −6.274, df = 11, P < 0.0005), and, by September, control males had significantly higher testosterone levels than breeding males (t = −2.759, df = 20, P = 0.012).

Paired testes volume (corrected for body size) changed significantly across the season ($F_{(10,180)}$ = 17.65, P < 0.0005) in a way that varied by breeding condition (time * breeding condition interaction ($F_{(10,180)}$ = 2.581, P = 0.006, but no main effect for breeding condition; Fig. 4A). When only data from January through September were considered, only a significant time effect remained, with paired testes volume increasing in the summer ($F_{(7,126)}$ = 37.105, P < 0.0005; no group main effect or time * breeding condition interaction). There were no significant differences in paired testes volume between breeding and control males in any month, suggesting that it was 'how' each group changed over time that was different. For example, the summer rise in testes volume occurred earlier in control males than in breeding males.

P. pumilus

Testosterone levels in *P. pumilus* during the first 35 days following group formation did not significantly vary (Fig. 4B). In contrast, very marked differences in testosterone levels across the season ($F_{(7,84)}$ = 8.276, P < 0.0005) were seen in *P. pumilus* that varied significantly by breeding condition ($F_{(1,12)}$ = 6.081, P = 0.03). Unlike the profile seen in *P. vampyrus*, where seasonal increases in testosterone were found in late summer, testosterone rose in late spring/early summer in *P. pumilus*. The seasonal rhythm in *P. pumilus* was largely similar in breeding and control groups, except that testosterone rose significantly earlier in control males than in breeding males (in May, testosterone in CM was significantly greater than in breeding males: t = −2.463, df = 14, P = 0.027). Seasonal variations in testes volume were not measurable in *P. pumilus* due to the fact that their testes were often retracted.

Changes in body mass in response to group formation and across the season

P. vampyrus

In *P. vampyrus*, significant changes in body mass that varied by sex and breeding status occurred over time, both in the first 35 days following group formation and across the season (Fig. 5A). During the first 35 days, significant weight changes were found ($F_{(3,126)}$ = 7.029, P < 0.0005) that varied by breeding condition ($F_{(1,42)}$ = 7.16, P = 0.011) and by sex ($F_{(1,42)}$ = 26.021, P < 0.0005). Overall, males were significantly heavier (even after correcting for body size (length of forearm)) than females.

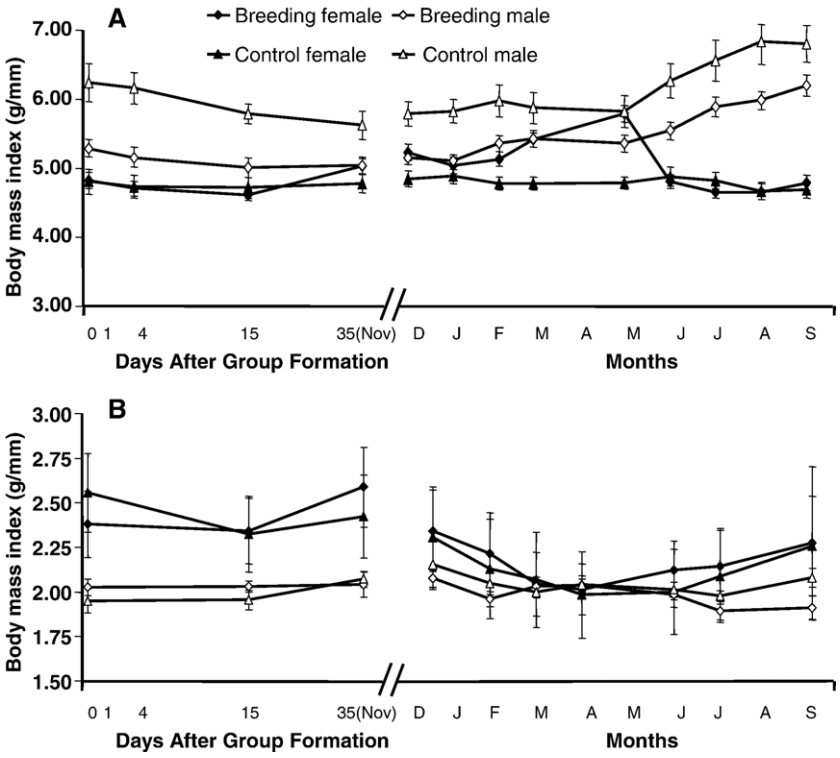

Fig. 5. Adjusted body mass (mean ± SE mass in grams/length of right forearm in mm) over time for (A) *P. vampyrus* and (B) *P. pumilus*. Figures on the left are changes over time (in days) for the first 35 days following the formation of new breeding and control (same sex) groups. Figures on the right are changes in time (in months) for the remainder of the year.

Unfortunately, at D0, a significant size difference existed between breeding and control males ($t = -2.912$, $df = 20$, pc = 0.027) that largely persisted over time. Despite this, breeding and control males had nearly identical (but parallel) profiles of changes in body mass over time. In both breeding and control males, body mass decreased between D0 and D35 (breeding males: $F_{(3,27)} = 6.667$, $P = 0.002$; D0 > D15, $t = 3.187$, $df = 9$; pc = 0.022, D15 not significantly different from D35; control males: $F_{(3,33)} = 4.439$, $P = 0.001$; D0 > 15 (but not significant), D4 > D15, $t = 5.01$, $df = 11$, pc < 0.0025 and D4 > D35, $t = 3.512$, $df = 11$, pc = 0.02). During this same time period, there were no significant differences in body mass in control females. In breeding females, a different response was seen: body mass decreased between D0 and D15 then rose by D35 to a point even higher that that of DO ($F_{(3,33)} = 14.585$, $P < 0.0005$, D0 > D15, $t = 2.424$, $df = 11$; $P = 0.034$; D15 < D35, $t = -6.754$, $df = 11$, pc < 0.001, D0 < D35 (but this was not significant after Bonferroni correction): $t = -2.438$, $df = 11$, pc = 0.066).

Similar trends were found in the monthly seasonal data: significant differences were seen over time in body mass ($F_{(9,351)} = 16.34$, $P < 0.0005$) that varied by sex (main effect: $F_{(1,39)} = 35.919$, $P < 0.0005$; time * sex: $F_{(9,351)} = 57.69$, $P < 0.0005$) and by breeding condition (time * breeding condition: $F_{(9,351)} = 7.838$, $P < 0.0005$; sex * condition: $F_{(1,39)} = 9.579$, $P = 0.004$; and time * sex * breeding condition: $F_{(9,351)} = 3.11$, $P = 0.001$). Body mass fluctuated significantly over time in males ($F_{(9,171)} = 38.372$, $P < 0.0005$). Control males continued to be heavier than breeding males, even after controlling for body size ($F_{(1,19)} = 5.24$, $P = 0.034$), but patterns of body mass change over time did not differ between breeding males and control males (no time * breeding condition interaction for males). Males in both groups began to significantly gain body mass in the summer months, leading up to the next breeding season. Breeding females showed significant changes in body mass ($F_{(9,90)} = 38.419$, $P < 0.0005$) that corresponded directly to pregnancy and lactation. Peak body mass was recorded in late pregnancy (April/May > Dec, $t = -9.227$, $df = 10$; pc < 0.0015; body mass was also significantly greater than control females at this time: $t = 6.864$, $df = 21$, pc < 0.002) followed by a significant drop in body mass immediately after birth (April/May > June, $t = 8.571$, $df = 10$, pc < 0.0010) to levels even lower than those recorded during non-pregnancy (Nov/Dec > July, $t = 6.655$, $df = 11$, $P < 0.0005$) and not distinguishable from those recorded in control females at this time. Control females showed statistically significant changes in body mass over time ($F_{(9,90)} = 3.314$, $P = 0.002$), but these changes were very slight compared to those shown by the other groups. The greatest change in control females was a drop in body mass corresponding to the warmest months of the year (August and September, when females tended to eat less).

P. pumilus

Changes in body mass during the initial period of group formation (D0–D35) and during the rest of the year in *P. pumilus* were less profound than in *P. vampyrus* (Fig. 5B). During the first month following group formation, significant changes in body mass over time were only found in breeding females ($F_{(2,14)} = 9.468$, $P = 0.003$), where levels significantly rose between D15 and D35 ($t = -4.088$, $df = 7$, pc = 0.01). There were no significant differences over time during this first 35 days in control females or any males. In contrast to *P. vampyrus*, female *P. pumilus* were significantly heavier at D0 than males, and this difference persisted through D35 (sex differences: DO, $F_{(1,29)} = 10.337$, $P = 0.003$; D15, $F_{(1,29)} = 5.759$, $P = 0.023$; D35: $F_{(1,29)} = 7.404$, $P = 0.011$). Female but not male *P. pumilus* within this captive colony have consistently had problems with obesity for a number of years. These sex differences in body mass largely disappeared by the second month of the study, and there were no significant differences between the sexes for the rest of the year. Females gradually lost body mass over time, to their lowest point in April (December > April, $t = 5.021$, $df = 10$, $P = 0.001$), then gained again (but not significantly) by late summer. In contrast to *P. vampyrus*, only 3 *P. pumilus* females from the breeding group (and 2 from the 'control' group) bred, and did so at different times of the year. These data were removed from this analysis so that the peak in body mass recorded in pregnant *P. vampyrus* females in Fig. 5A is not represented in Fig. 5B for *P. pumilus*. In contrast to the profound seasonal rhythms in body mass observed in *P. vampyrus* males, there were no significant differences in body mass over time in male *P. pumilus*.

Variations in the stress response by sex, group type, and time of year

In the autumn vs. spring stress reactivity experiment, each group of both species at each time point showed a significant total glucocorticoid stress response (Fig. 6), and ANOVA indicated a number of significant interactions between factors. For *P. vampyrus*, there were significant main effects for condition (baseline vs. stress: $F_{(1,42)} = 135.398$, $P < 0.0005$), breeding condition (breeding vs. non-breeding group: $F_{(1,42)} = 5.492$, $P = 0.024$), and time (autumn vs. spring: $F_{(1,42)} = 65.727$, $P < 0.0005$), but not for sex. Additionally, there were significant interactions for time * sex ($F_{(1,42)} = 13.896$, $P = 0.001$) and time * sex * breeding condition ($F_{(1,42)} = 10.826$, $P = 0.002$). For *P. pumilus*, there were significant main effects for condition (baseline vs. stress: $F_{(1,24)} = 109.211$, $P < 0.0005$) and time (autumn vs. spring: $F_{(1,24)} = 13.978$, $P = 0.001$), but not for breeding condition or sex. There was also a significant interaction between time and sex ($F_{(1,24)} = 6.489$, $P = 0.018$). If one looks not at total glucocorticoid levels but rather at the absolute difference (increase) in hormone levels in response to restraint (subtracting baseline from stress levels), there were no significant differences across time or between group types for either species. A noticeable exception is found in *P. pumilus* males in the spring, where baseline values did not differ from those of females but where stress levels were significantly higher than in females ($t = -2.948$, $df = 26$, $P = 0.007$).

Testosterone levels were variable but nearly always decreased (often significantly) in response to stress in males of both species at both times of the year. For *P. vampyrus*, these differences were significant in breeding males in the spring

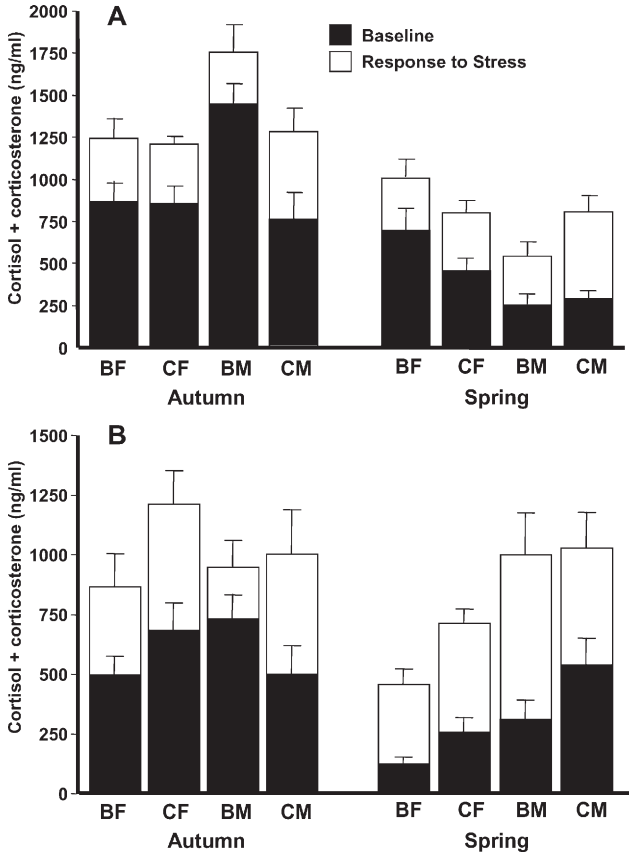

Fig. 6. Total glucocorticoids (cortisol + corticosterone, mean ± SE) at baseline and in response to 30 min of restraint stress examined both in the autumn (November) and in the Spring (late April) for (A) *P. vampyrus* and (B) *P. pumilus*. BF = breeding females; CF = control females; BM = breeding males; CM = control males.

(5.57 ± 1.09 ng/ml vs. 3.93 ± 0.72 ng/ml; $t = 3.448$, $df = 9$, $P = 0.007$) and for control males in the autumn (5.78 ± 0.95 ng/ml vs. 3.05 ± 0.50 ng/ml; $t = 5.784$, $df = 11$, $P < 0.0005$). For *P. pumilus*, these differences were significant for breeding males in the autumn (1.59 ± 0.40 ng/ml vs. 0.94 ± 0.19 ng/ml; $t = 2.808$, $df = 7$, $P = 0.026$), for control males in the autumn (1.91 ± 0.46 ng/ml vs. 1.03 ± 0.16 ng/ml; $t = 2.531$, $df = 7$, $P = 0.039$), and for control males in the spring (10.19 ± 2.24 ng/ml vs. 5.49 ± 1.32 ng/ml; $t = 4.455$, $df = 7$, $P = 0.003$).

Because of differences in the profiles of cortisol and corticosterone in late pregnancy, each hormone was analyzed first separately for the pregnant vs. postpartum restraint stress study. Within each species and for each hormone, potential differences in stress-responsive hormone levels between the social 'pup present' or 'pup absent' conditions were first assessed. For *P. vampyrus*, a significant stress response occurred in both postpartum conditions (Fig. 7), which did not vary by the presence or absence of the pup (significant main effect for stress condition [baseline vs. stress]: $F_{(1,11)} = 35.482$, $P < 0.0005$; no significant main effects for pup condition or interactions for stress condition and pup condition). Because the presence or absence of the pup did not alter the cortisol response, baseline and stress levels from the two postpartum conditions were averaged for comparison to levels in late pregnancy. In this case, there was a significant stress condition effect ($F_{(1,11)} = 44.035$, $P < 0.0005$) and stress condition * reproductive condition interaction ($F_{(1,11)} = 7.893$, $P = 0.017$), but no main effect for reproductive condition. Essentially, baseline cortisol levels are not distinguishable between pregnancy and the postpartum (lactating) period, but cortisol levels in response to stress were higher during the postpartum period than during pregnancy ($t = -3.345$, $df = 11$, $P = 0.007$). Nearly identical results were found with corticosterone (a significant stress response postpartum ($F_{(1,11)} = 24.974$, $P < 0.0005$) but no difference between the two social pup conditions; a significant stress response pre and postpartum ($F_{(1,11)} = 51.092$, $P < 0.0005$) that varied by reproductive condition ($F_{(1,11)} = 10.108$, $P = 0.009$)), except that corticosterone levels in response to stress were higher during pregnancy than during the postpartum period (the opposite of cortisol; $t = 3.113$, $df = 11$, $P = 0.01$). The differences between cortisol and corticosterone canceled out when total glucocorticoid levels were considered as there were no significant differences between pregnancy or the postpartum condition once both hormones were added together. A nearly identical hormone profile was identified in *P. pumilus* (Fig. 7B), but, due to the small sample size ($N = 4$), no significant differences were detected after correction of P values for multiple comparisons.

Discussion

A number of physiological and behavioral changes occurred in both species of bats in response to group formation, restraint stress, and the changing annual season. Both study species are in same genus, *Pteropus*, but, within this large genus (65 species; Simmons, 2005), *P. pumilus* and *P. vampyrus* are not closely related (Bastian et al., 2002). Nevertheless, one would expect to observe similar behavioral and especially physiological tendencies in both species. Not surprisingly, we found that many physiological and some behavioral results were strikingly similar in the two species. We do know, however, that these species vary behaviorally in nature, with *P. pumilus* being found in small infrequently interacting groups (which is unusual in this genus) and *P. vampyrus* being highly gregarious and found in groups up to a thousand times larger than those of *P. pumilus.* These natural behavioral differences were reflected in variations in inter-animal spacing measured in our study. Where physiological differences between the species were evident, we propose that their underlying social differences played a role.

With few exceptions, we found behavioral and physiological changes in both the intensively sampled period of the first 35 days after group formation and in the monthly samples collected over the course of the next year. There is evidence in both species that at least some categories of animals found the formation of breeding groups stressful, or at least, energetically expensive. The transient nature of the elevated glucocorticoids in *P. vampyrus* same-sex groups suggests that social stability was quickly reached. In contrast, the sustained elevations in glucocorticoids in breeding male and female *P. vampyrus* and both breeding and control female *P. pumilus* suggest that these

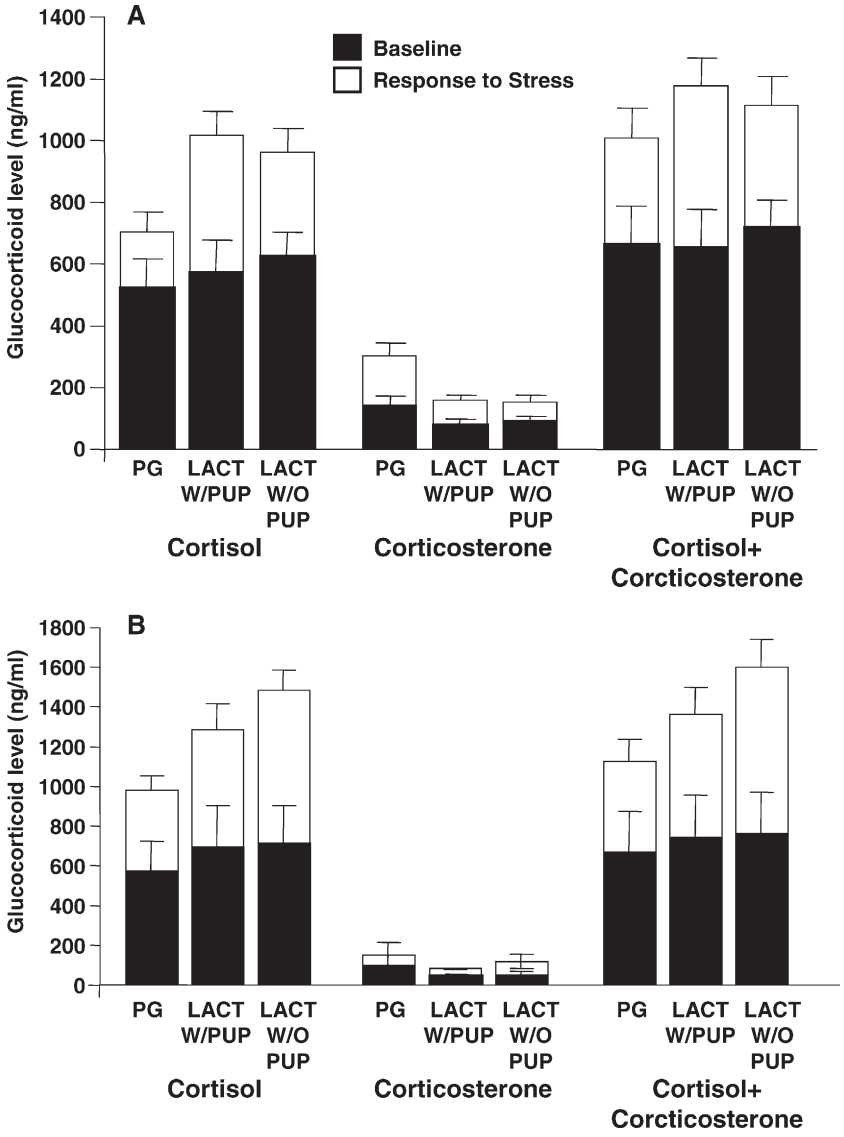

Fig. 7. Cortisol, corticosterone, and total glucocorticoids (cortisol + corticosterone, mean ± SE) at baseline and in response to 30 min of restraint stress in late pregnancy (PG) and during lactation when pups were 1 month old, either with pups attached to mothers for the bleeding and restraint (LACT W/PUP) or with pups separated from dams (LACT W/O PUP) for (A) *P. vampyrus* and (B) *P. pumilus*.

individuals remained in a state of stress (or at least high energetic demand), likely maintained in *P. vampyrus* by the frequent negative social interactions in the breeding pens and in *P. pumilus* by the higher than normal levels of cage disturbance and handling (compared to when these animals were not involved in any studies). The sustained elevations in glucocorticoids were most pronounced in the breeding male *P. vampyrus* who were actively competing between themselves and fighting with females for mating access during this period. The potentially suppressive effects of their sustained glucocorticoids on gonadal function (Viau, 2002; Wingfield and Sapolsky, 2003) may explain why breeding male *P. vampyrus* had significantly lower testosterone levels than control males at D35. If this is in fact the case, this suppression was either not in place or being overridden at the day 15 time point, in which breeding males in both species had higher levels of testosterone than control males (significantly so in *P. vampyrus*) (see Wingfield and Sapolsky, 2003). Based upon the results of our seasonal analysis, it appears that testosterone naturally declines at this time of year (meaning that we formed our groups at the end of the seasonal testosterone peak), but did so more quickly in the control males, who were not allowed regular access to females. This suggests that the formation of mixed-sex groups socially facilitated elevations (or in this case the lack of a decrease) in testosterone in breeding males relative to control males (Schiml et al., 1996). Higher levels of testosterone in breeding versus control males at this point in time may also be due to increased male–male competition in breeding vs. control males (the "challenge hypothesis"; Wingfield et al., 1990). That body mass significantly declined in both control and breeding *P. vampyrus* during this period may be tied to the declining testosterone levels and to male–male competition, but it is unknown whether the decreased body mass was due to fat or muscle loss. Compared to females and to male–female

dyads, male *P. vampyrus* maintained greater inter-animal distances between themselves, suggesting that competition within males may have been high. Although we did not methodologically document dominance hierarchies within male *P. vampyrus*, there were clear dominant and subordinate individuals in each of the breeding pens and in the control groups. While male *P. vampyrus* lost body mass during the initial 35-day period (and male *P. pumilus* did not change body mass during this period), breeding females in both species significantly gained body mass between day 15 and 35 following group formation. This weight gain is likely not attributed to pregnancy as only a few of the *P. vampyrus* females could have been pregnant at this time (based upon when they eventually gave birth) and none of the *P. pumilus* females was pregnant at this time. Increased weight in breeding females may instead be due to the orexogenic properties of glucocorticoids (Dallman et al., 1995; Sapolsky et al., 2000) and/or other factors that stimulated eating during periods of high mating activity in preparation for the energetic demands of pregnancy.

Behaviorally, significant changes in inter-animal spacing during the first 35 days following group formation were found in all *P. vampyrus*; changes in *P. pumilus* only occurred in the breeding groups. In *P. vampyrus*, differences in inter-animal distance between different types of individuals revealed differences in their social preferences. The data from control male and female *P. vampyrus* strongly suggest that, in the absence of potential mates, females prefer to be close to other females (in fact, they form a tight cluster without actually contacting each other). Males, on the other hand, prefer to avoid one another. In fact, the average distance between two males in the all-male control groups approached the maximum average inter-animal distance possible (7.10 ± 0.41 [SE]; Fig. 2A). The decrease in inter-animal spacing within the control male group between days 1 and 4 may be due to the frequent social interactions occurring at this time, after which a dominance hierarchy was apparent. The potential for mating (i.e., when animals of both sexes are found in a group) significantly altered these general sex-specific social preferences. In the breeding groups, females sill preferred to be close to other females and males still preferred to be distanced from other males, but the magnitude of the differences between the sexes was smaller than in the control groups, tempered by the fact that males appear to tolerate being closer to one another if it means greater access to females.

The clear sex differences in social preference evident in *P. vampyrus* were essentially non-existent in the relatively asocial *P. pumilus*. In fact, both male and female *P. pumilus* seemed relatively indifferent to other animals in their pen. Two-hour recordings of social interactions following group formation in this species resulted in blank tapes. When provided with hanging branches and other visual barriers within their enclosure (as both species were), *P. pumilus*, but not *P. vampyrus*, regularly hid themselves. Breeding *P. pumilus* did shift from being closer to same-sex conspecifics to males having spaced themselves further apart to be somewhat closer to females to eventually being evenly distributed in the pen (such that, at day 35, the inter-animal spacing in the *P. pumilus* breeding groups approached the theoretical maximum inter-animal distance for the 8 animals in a pen (6.48 ± 0.63 m)). The lack of changes in inter-animal spacing over time in the control *P. pumilus* groups may indicate greater social stability, or lower levels of social interactions, and/or may be due to the way in which these groups were formed (by removing same-sex conspecifics rather than truly forming new social groups).

Despite the fact that the experimental design and greater body size of *P. vampyrus* theoretically biased this species to be spaced farther apart from one another than *P. pumilus*, for all groups except control males, *P. vampyrus* were closer to one another than *P. pumilus*. These species differences suggest that social relationships (whether agonistic or antagonistic) are generally more important or at least more salient in *P. vampyrus* compared to *P. pumilus.* That control male *P. pumilus* were in greater proximity to each other than their *P. vampyrus* counterparts may imply that competition between males (at least when no opportunity for mating exists) is less in *P. pumilus.* Anecdotal observations of mating behavior in both species support this notion: in *P. pumilus*, breeding males were observed 'waiting in line' to mate with a female; in *P. vampyrus*, males frequently fought each other for access to females.

As would be expected given the species differences in behavior and physiology in the intensely sampled first 35 days following group formation, seasonal differences in *P. vampyrus* were more evident and of greater magnitude than in *P. pumilus*. Clear seasonal changes in inter-animal spacing between both breeding and control *P. vampyrus* were found that could be related to seasonal changes in physiology. Across the season, the same sex-specific and breeding-group-specific patterns of inter-animal spacing described in the first 35 days following group formation were evident, with several notable fluctuations. During the winter months, when testosterone levels, testes volume, and body mass were at the nadir of the seasonal rhythm and therefore when competition was likely significantly lowered, control males were physically closer to one another. The fluctuations in inter-animal spacing in the breeding groups that occurred in the summer were surely related to the synchronous birth of 14 pups in May. Attendant to this event, breeding females, who were each in constant contact with her pup, spaced themselves farther apart from each other and from the males in their pens. Perhaps due to the absence of a postpartum estrous in this species, breeding males became spaced further apart at this time as well (significantly so by July), presumably due to the lack of competition for non-impregnable females. Anecdotal observations of females with pups vigorously rejecting all male advances during this period support this view. Only a handful of seemingly random and idiosyncratic seasonal differences in inter-animal spacing in *P. pumilus* were found, with no consistent sex or group type effects. This may be in part due to the lack of breeding synchrony in this species compared to *P. vampyrus* and to the overall lower level of social interaction (at least as observed during the day).

Despite this behavioral difference between the species, both species showed significant seasonal rhythms in glucocorticoids and testosterone. Although the glucocorticoid seasonal rhythms were much clearer in *P. vampyrus* than in *P. pumilus*, in both species, peak seasonal levels in females occurred during the winter months, whereas the seasonal peak in glucocorticoids in males occurred in August and September in *P. vampyrus* and in June and July in *P. pumilus* (data for August and September were not shown for this species because they were not available in sufficient sample sizes for females, however, glucocorticoid levels continued to be elevated in *P. pumilus* males, with levels in August and September of 816.19 ± 95.32 and 974.62 ± 101.01 for breeding males and 779.98 ± 117.16 and 910.53 ± 206.97 for control males). The earlier seasonal peak in glucocorticoids in male *P. pumilus* relative to male *P. vampyrus* may be related to their significantly earlier seasonal rise in testosterone relative to *P. vampyrus*. Within each species, the earlier rise in testosterone in control males versus breeding males is a bit of an enigma. Perhaps in the absence of females, testosterone rises earlier to facilitate the search for mates or male–male competition may have been higher at this time in the control males than in the breeding males, which had already established relationships with the females in their group.

The out-of-phase seasonal glucocorticoid rhythms between males and females have not, to our knowledge, been previously described for mammals (Reeder and Kramer, 2005; Romero, 2002). In his review of seasonal changes in glucocorticoids in free-ranging vertebrates, Romero (2002) found that seasonal rhythms in mammals were poorly documented and tended to be very species-specific. In most vertebrates, Romero found that glucocorticoid levels were highest during the breeding season relative to pre- and post-breeding periods, and he proposed that this peak occurred for both behavioral and energetic reasons. From an energetic standpoint, seasonal shifts in HPA function may serve to mediate changes in metabolic rate, water metabolism, gonadal function, and immune competence, such that glucocorticoids should be elevated during the most energetically expensive time of the year (Romero, 2002; Wade and Schneider, 1992). From a behavioral standpoint, changes in glucocorticoids may induce rapid behavioral changes. By this logic, seasonal rhythms in glucocorticoid levels occur because animals may or may not need to express glucocorticoid-mediated behaviors (such as flight/migration and eating) at different times of the year (Romero, 2002). Finally, seasonal peaks in glucocorticoids may occur because glucocorticoids prepare other physiological systems for subsequent stressors or other events (Sapolsky et al., 2000). Which of these hypotheses or combination of them explains why our seasonal rhythms vary by sex and species is unclear. In males of both species, elevations in testosterone, glucocorticoids, and body mass occurred roughly simultaneously in late summer and fall, presumably all in preparation and support of mating activity and associated behaviors (such as aggression). Similar changes in testes volume, testosterone levels, body mass, and adrenal mass associated with peak mating activity have been shown in the Australian pteropodid, *P. poliocephalus* (Martin and Bernard, 2000; Martin et al., 1995; McGuckin and Blackshaw, 1991a,b). In females, peak levels occurred in the winter months, perhaps in preparation for the energetic demands of pregnancy. Additionally, the peak seasonal levels of glucocorticoids in males of each species were generally greater than those of females. While it is difficult to interpret the meaning of this atypical mammalian trend in the absence of a knowledge of receptor physiology or of the levels of the glucocorticoid carrier protein (corticosteroid binding globulin; CBG), a similar trend has been noted in *P. hypomelanus* (Reeder et al., 2004a; Widmaier and Kunz, 1993).

Concurrent with our study of seasonal hormonal rhythms was an exploration of the variability in the ability to mount a response to restraint stress by time of year and reproductive condition. Because the glucocorticoid response to stress is superimposed upon baseline circadian and seasonal glucocorticoid rhythms (Reeder and Kramer, 2005; Romero, 2002), we predicted that a greater stress response would be evident in the fall during the mating season (when baseline glucocorticoids were expected to be high and when groups were predicted to be less stable socially) than during the spring. Furthermore, we predicted that pregnant and lactating females would show a blunted stress response that would be mediated by the presence of pups. Stress hyporesponsiveness has been recorded both during pregnancy and lactation (Lightman et al., 2001), but elevations in glucocorticoids in response to stress can be mediated by the presence of another animal to which the subject is emotionally attached or bonded to (Hennessy, 1997). In the case of mothers and pups, separation from a pup induces increases in glucocorticoids in mothers of some species (e.g., squirrel monkeys, *Saimiri sciureus*, see Hennessy, 1997 for review). We found that all animals in all reproductive conditions tested responded to stress with a significant elevation in glucocorticoids and that the magnitude of the increase between stress and baseline levels did not vary across time or between groups. However, given that stress samples were collected after 30 min, this increase in glucocorticoids is likely nearly all in free hormone (unbound by CBG) because CBG does not rise in this timeframe (Breuner and Orchinik, 2001, 2002), thus presumably more readily available for inducing changes (see Breuner and Orchinik, 2002; Romero, 2002 for discussion of the competing views of the role of CBG). Whether or not the roughly equivalent absolute increases in glucocorticoid levels across time and within groups are biologically equivalent would depend upon levels of CBG, glucocorticoid receptor affinity, distribution, and quantity, and even relative levels of the glucocorticoid bio-converting enzymes (11ßHSD1 and 11ßHSD2) in the target tissues (Seckl and Walker, 2001; Sapolsky et al., 2000), all factors that were not examined in this study. That the presence of a pup during restraint stress testing in lactating females did not buffer the stress response (as has been shown in species in which mothers form an attachment relationship with their infant; see Hennessy, 1997) suggests that there is a lack of an emotional attachment between a mother and

her pup in *P. vampyrus* and *P. pumilus*. Deschamps et al. (2003) demonstrated that the presentation of ecologically relevant stressors (e.g., predator simulation) results in the over-riding of stress hyporesponsiveness in lactation, and it would be useful to explore this phenomenon in these species. The increased corticosterone versus cortisol in pregnant female *P. vampyrus* in response to stress is reminiscent of alterations in the baseline cortisol/corticosterone ratio in late pregnancy described in *P. hypomelanus* (Reeder et al., 2004a), which may be related to shifts in aldosteronogenesis or cholesterol metabolism in the adrenal cortex.

Female (and to a lesser extent male) *P. pumilus*, which were much less social than *P. vampyrus*, appeared to be highly susceptible to disturbance (Mickleburgh et al., 1992, suggested the same for wild populations). Even the control females, whose new social groups were simply formed by the removal of some animals, responded to group formation with elevated glucocorticoids. During the first 2 weeks after group formation, *P. pumilus* females in the breeding groups remained significantly closer to one another than at any other time of the year, which perhaps mediated the effects of the disturbance. Additionally, female *P. pumilus*, which started the study in many cases obese, lost body mass and remained thinner for the next year. Moreover, they largely failed to successfully reproduce (in terms of the numbers that gave birth and whose pup survived) during this study, compared to when this colony was not disturbed, and males, but not the females, were reproductively synchronous.

We believe that *P. vampyrus* and *P. pumilus*, with their documented seasonality, high levels of glucocorticoid hormones, and variability in physiological responses that are likely tied to behavioral differences, are excellent models for exploring social influences on physiology, as well as the general physiology of the HPA and HPG axes. Additionally, *P. vampyrus*, along with *P. hypomelanus* (Reeder et al., 2004a), with their exceptionally high and variable glucocorticoid levels may prove to be valuable models for glucocorticoid resistance. Future studies should include quantification of CBG and other metabolic hormones such as insulin. Additional comparative studies between the *P. vampyrus* and *P. pumilus* should be conducted to further explore the importance of social influences on physiological processes.

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