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, glucocorticoids, testosterone, and body mass) in the formation of breeding and non-breeding groups in two bat species, the socially-organized 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 males (especially aggressive male P. vampyrus), these responses persisted over time. In both species, breeding group formation resulted in elevated testosterone in males. Glucocorticoids, testosterone, inter-group spacing, 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 these underlying social differences influenced their responses to group formation and to the changing seasonal environment.

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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 (Reece and Kramer, 2005; Remple, 2002; Wingfield and Sapolsky, 2003). Glucocorticoid hormones (cortisol and...
to three social relationships (Mendoza and Mason, 1989), both behaviors are being studied.

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 causes 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 Malay flying fox (Pteropus vampyrus) is a large, frugivorous 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 social and competitive breeder, with most females giving birth to a single pup in the spring (Kuntz and Jones, 2000). The second species, the little Golden-furred Flying Fox (Pteropus pumilus) is a relatively small frugivorous bat found in the Philippines, weighing 20 g with a wingspan of 0.6 m. Although many species of Pteropus are socially and can be found in groups of up to several hundred or even thousands, P. pumilus roosts in small aggregations with individuals socializing, interacting (L.R. Hardy, personal communication; McElderry et al., 1993). 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 frugivorous bats are excellent models for exploring social influences on mammalian physiology, as well as the general physiological effects of the HPA and HPG axes, including seasonal rhythms of these hormones and the response to stress. Both species breed year-round, and they have some of the highest plasma glucocorticoid levels over described in meerkats (Reeder and Kaufer, 2005; Wisniewski and Kunz, 1997; Wisniewski et al., 2000).

In general, we hypothesized that the formation of breeding groups would transiently increase glucocorticoid levels in both species 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 (Capella et al., 1998; Schondel 1997; Soto-Garza et al., 2005; Wingfield et al., 1999). 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 stress levels, 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 age groups (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 stresses by time of year and reproductive condition was assessed in all subjects. Because the glucocorticoid response to stress is superimposed upon baseline corticoid and seasonal glucocorticoid rhythm (Reeder and Kaufer, 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 socially stable) 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., 1975).

Materials and methods

Animals and animal care

Subjects for this study included 48 female 627 males and 36 females and 32 P. pumilus (66 males and 16 females). Animals were housed in captivity at the Laboratory for Comparative Medicine, Boston, MA, All of the protocols described in this paper were approved by the Animal Care and Use Committee (IACUC) protocol (06-001). American Zoo and Aquarium Association (AZA) and California Association of Zoological Parks and Aquariums (CAZA) membership are required for membership in the following major categories: nonhuman primates, birds, reptiles, and amphibians. All animals were kept in accordance with the guidelines of the American Zoological Association (AZA) and California Association of Zoological Parks and Aquariums (CAZA) and were housed in enclosures that were designed to maximize the ability of bats to live, feed, and breed. Animals were both wild-caught and laboratory-bred and were all fully reproducing males at the beginning of the study. Each animal was identified by a combination of a unique entry in the following categories: (a) species, (b) sex, (c) origin, (d) date of birth, (e) age, (f) sex, (g) date of birth, (h) age, (i) sex, (j) date of birth, (k) age. All animals were kept in a motion-free, vegetable, and monkey-free Stanley (Parry) daily at 15-20 °C.

There were at least two food and water per subject, and food and water were supplied sufficiently to maintain animal well-being. Water was available at all times.

Group formation procedures

Subjects for each sex were divided into 4 groups: 2 breeding groups (P. vampyrus) and 2 same-sex groups (P. pumilus). Each breeding group contained 7 males and 7 females, and 2 same-sex control groups (N = 12) for P. vampyrus and N = 10 for P. pumilus. Groups were housed in enclosures that were designed to maximize the ability of bats to live, feed, and breed. Animals were both wild-caught and laboratory-bred and were all fully reproducing males at the beginning of the study. Each animal was identified by a combination of a unique entry in the following categories: (a) species, (b) sex, (c) origin, (d) date of birth, (e) age, (f) sex, (g) date of birth, (h) age, (i) sex, (j) date of birth, (k) age. All animals were kept in a motion-free, vegetable, and monkey-free Stanley (Parry) daily at 15-20 °C. Water was available at all times. Each animal was given at least two food and water per subject, and food and water were supplied sufficiently to maintain animal well-being. Water was available at all times.
there was a difference in the rate of growth by female reproductive status (low progesterone) vs. postpartum (lactating) and postpartum state conditions (sows were pregnant with the first in the estrous cycle or not). In fact, the estrous cycles of the pregnant sows were significantly shorter than those of the non-pregnant sows. Significant differences were observed in the estrous cycle length and behavior between the pregnant and non-pregnant sows. In addition, the estrous cycle length and behavior were affected by the presence of other sows in the same pen. For example, the estrous cycle length and behavior were significantly shorter in the presence of other sows than in the absence of other sows. In conclusion, the results of this study indicate that the presence of other sows significantly affects the estrous cycle length and behavior in pregnant sows.
Fig. 1. Schematic of the home cage, showing grating structure on one end of cage (which was used for placing individual animal locations) and representative spacing data for defined 8×10 cm grid-burning groups. For the smaller species, P. parvus, the cage was divided initially by the addition of an opaque wall, and two groups were housed within each cage.  

Data were analyzed statistically using Mann-Whitney U tests with multiple comparison using the Bonferroni method with modified, corrected P-values. Unless otherwise stated, all data are presented as means ± standard error of the mean (SEM) and were analyzed with the Statistical Package for Social Sciences (SPSS). Statistical significance was considered to occur at a confidence level of 0.05. 

All data were analyzed parametrically using an ANOVA with post-hoc group comparisons as necessary. As with all of the analyses in this study, the 'group' comparison was not evaluated for significant differences between the groups in the analysis of variance (ANOVA). 

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Fig. 2: Inter-group distancing means ± S.D. for (A) P. vampero and (B) P. pellucida. Figures on the left are changes over time (days) for the first 35 days following the formation of new breeding and control (same sex) groups. Figures on the right are changes in the last 35 days. BF-BM = average inter-sexual distance between any 2 breeding males; BF-BM = average inter-sexual distance between any 2 breeding females; BF-CF = average inter-sexual distance between any 2 females in the all-female control group; CM-CM = average inter-sexual distance between any 2 males in the all-male control group. See text for sample sizes and calculations.

Analyses for sexual and corticosterone responses are presented for the pre-pregnancy term study only.

Results

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

P. vampero

Significant changes in inter-sexual distance between P. vampero 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 in response to extent certain types of individuals (e.g., breeding females) preferred to be closer to certain other types of (e.g., breeding males or other breeding females) changes over time in response to group formation and change over the course of the next year (presumably in response to reproductive events). Average inter-animal distances were calculated for five possible combinations (dyad types) of individuals: breeding females and any breeding females; breeding males and other breeding males; breeding males and breeding females; and within the same-sex control pair: control males and control males, in control females with control females. In general, the pattern of inter-animal spacing, i.e., which groups of animals tended to 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 close to the farthest 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, as 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, P_{dyad} = 0.513, P < 0.0005; May, P_{dyad} = 0.756, P < 0.0005). At both time points, (1) CP-CF dyads were significantly closer than their
BM: F(1,365) = 18.19, P < 0.0005; BF-BM: F(1,365) = 18.06, P < 0.0005. On the day of first conception (D0), females in the breeding groups grouped together exclusively by sex, that is, distance between females and males (BF-BM dyads) was significantly greater than between animals of the sex (BF-BM vs. BF-BF: t = -8.30, dP = 0.42, P < 0.0001; vs. BM-BM: t = -8.89, dP = 0.42, P = 0.015). By D1, a dramatic shift in grouping behavior was evident within the breeding groups, such that breeding males were no longer hanging in close proximity to other males (t = -7.09, dP = 11, P < 0.0005) but instead were much closer to females (t = 6.178, dP = 31, P < 0.0025). This change persisted through D4. Between D4 and D15, inter- animal spacing in both BM-BM dyads and BF-BM dyads increased, with this difference being significant for BF-BM dyads (t = 3.627, dP = 0.015). This decrease resulted in no significant differences in inter-animal spacing between any group type (including control) at D15. Subsequently, between D15 and D35, all animals in the breeding pairs became more spaced apart, with significant increases in inter-animal spacing found in all three types of dyads (BF-BF: t = -3.356, dP = 11, P = 0.006; BM-BM: t = 3.347, dP = 11, P = 0.005; BF-BM: t = -0.264, dP = 31, P = 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 vary the rest of the week were less evident and were found only within BF-BF (F(1,365) = 2.487, P = 0.022), BM-BM (F(1,365) = 2.709, P = 0.024), and CM-CM (F(1,365) = 2.844, P = 0.003) dyads. Only a few changes over time were found within these groups, and these changes were nonsignificant and appear 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, dP = 22, P = 0.012; June: t = 3.979, dP = 22, P = 0.003), and breeding males were significantly closer to each other than they were to females (April: t = -4.815, dP = 42, P < 0.005; June: t = -2.651, dP = 42, P = 0.055).

P. vaysonsii vs. P. pumilus

When comparing data between the species, it is important to note that there is an inherent bias in the data. If vaysonsii groups were in a full pen, whereas pumilus groups were each in half size pens, then P. vaysonsii groups had twice as much flight and hanging space as P. pumilus and 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. vaysonsii solely due to differences in pen and group size between the species. Additionally, P. vaysonsii 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 preclude P. vaysonsii towards greater inter-animal distances than P. pumilus, they were in fact generally and significantly closer together than P. pumilus (means SE inter-animal distance in P. vaysonsii vs. P. pumilus: BF-BF: t = 1.80, dP = 0.41, P = 0.034; t = 4.233, P = 0.0001; BM-BM: t = 1.729, dP = 23, P = 0.041, t = 1.80, dP = 0.41, but this difference was not significant, t = 0.926, dP = 30, P = 0.30; BF-BM: t = 2.63, dP = 11, t = 0.598, dP = 10, P = 0.025; CM-CM: t = 1.182, dP = 23, P = 0.09; t = 0.585, dP = 0.29, P = 0.0003). The opposite was true for control males, which were significantly closer to each other in P. pumilus than in P. vaysonsii (t = 4.22, dP = 0.04 vs. m = 5.32, dP = 10, t = 2.266, dP = 30.7, P = 0.035).

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

P. vaysonsii

A significant change in total glucocorticoid levels (cortisol + corticosterone) was seen in response to group formation and across the year (see the table below, see Fig. 3). For P. vaysonsii, 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(3,172) = 12.77, P = 0.0005; main effect for sex: F(1,172) = 16.37, P = 0.0005; significant time * sex: F(3,172) = 4.739, P = 0.001; time * breeding condition: F(3,172) = 5.892, P = 0.0005, and sex * breeding condition: F(1,172) = 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 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, dP = 13, P = 0.005; control females: t = 3.348, dP = 11, P = 0.036). For breeding females, this increase in measured glucocorticoids was sustained for the 35-day period (D35 > D0; t = -3.691, dP = 13, P = 0.012). In contrast, in control females, this rise was transient, with glucocorticoid levels returning 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 (D35 levels) (significant decrease from D1 peak level by D15: t = 5.813, dP = 10, P < 0.003). Male P. vaysonsii had significantly higher glucocorticoid levels than females at DQ, D1, and D4 (DQ: F(1,172) = 14.404, P < 0.0005; D1: F(1,172) = 30.299, P < 0.0005; D4: F(1,172) = 13.171, P = 0.0005). 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(1,172) = 7.156, P = 0.001), indicating a greater response in control animals. By D15, there are no significant differences
nicely 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, $p = 0.043$). This difference was even greater by D15, when breeding males had much higher glucocorticoid levels than did breeding females and control males (vs. breeding females: $t = -3.707$, df = 22, $p = 0.002$; 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: $F_{(11,241)} = 21.368$, $P < 0.0005$; significant interactions for time * sex: $F_{(11,241)} = 10.184$, $P < 0.0005$; time * breeding condition: $F_{(11,241)} = 3.803$, $P < 0.001$; and time * sex * conditions: $F_{(11,241)} = 4.784$, $P = 0.0005$). This was a significant effect for the sex or breeding condition). Breeding and control female $P. v. v. jundi$ showed significant seasonal rhythms in total glucocorticoids ($F_{(11,241)} = 12.414$, $P = 0.0005$) that paralleled one another, with the exception of peak glucocorticoids attendant to pregnancy in breeding females (time * breeding condition interaction: $F_{(11,241)} = 4.404$, $P = 0.0005$). Both breeding and control females showed a peak in glucocorticoid levels in December/January followed by a rapid decrease. Breeding and control male $P. v. v. jundi$ showed significant seasonal rhythms (changes over time) in total glucocorticoids ($F_{(11,241)} = 24.09$, $P < 0.0005$) that paralleled one another (with glucocorticoid levels in breeding males either than or not distinguishable from those of control males; except in January; main effect for breeding condition: $F_{(11,241)} = 2.714$, $P < 0.0005$; time * condition interaction: $F_{(11,241)} = 3.572$, $P < 0.0005$; 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 $P. v. v. jundi$ exhibited a significant seasonal rhythm with a clear peak, but, in this case, the peak occurred in the fall (see DQ values, August and September), and it thus was not 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 DQ, and also for September: $F_{(11,241)} = 89.591$, $P < 0.0005$) but not at others.

$P. p. umbralis$ Data from $P. p. umbralis$ were more variable than those from $P. v. v. jundi$, 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: $F_{(11,241)} = 2.797$, $P = 0.045$; control females: $F_{(11,241)} = 7.989$, $P = 0.0005$). In both breeding females

Fig. 3. Total glucocorticoids (plasma + corticosterone, mean ± SE) levels over time for (A) P. v. v. jundi and (B) P. p. umbralis. Figures on the left are changes over week in (5 days) for the first 35 days following the formation of (same sex) breeding and control (same sex) groups. Figures on the right are changes in time (in months) for the remainder of the year. On each diagram, sample sizes are as follows: P. v. v. jundi, 14 breeding females, 10 breeding males, 12 control females, 12 control males. P. p. umbralis, 3 breeding females, 4 breeding males, 3 control females, 3 control males.
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: \( t = 5.288, df = 7, p = 0.0055 \); control females: \( t = 5.963, df = 7, p = 0.0053 \)), 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. vampyrus 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. vampyrus, 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 \( N = 3 \) 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 which they were found, rather than breeding status per se. As was the case with female P. vampyrus, the glucocorticoid rhythm in female P. vampyrus peaked in winter (December/January) and then declined. Both breeding and control males had significant changes in total glucocorticoids over the season (\( F_{(3,9,20)} = 3.972, p = 0.001 \)), with a peak in early July (May vs. late June: \( t = 5.334, df = 15, p < 0.0005 \), 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 (day: \( F_{(3,22)} = 8.084, p = 0.009 \); late June: \( F_{(3,22)} = 21.198, p < 0.0005 \); late July: \( F_{(3,22)} = 11.799, p = 0.003 \)).

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 conditions (Fig. 4A); time main effect: \( F_{(4,6,4)} = 97.645, p = 0.0005 \); time * condition interaction: \( F_{(4,6,4)} = 3.894, p = 0.007 \). 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, \( t = 3.797, df = 11, p = 0.002 \), then levels remained low, no difference detected between D15 and D35 levels). In breeding males, testosterone levels at D4 and D15

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remained indistinguishable from those at DO then significantly decreased between D15 and D35 (r = 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(1,190) = 15.251, P = 0.0005). Significant decreases between July and September occurred in both breeding males (t = -3.495, df = 9, P = 0.007) and control males (t = -6.274, df = 11, P < 0.005), and, by September, control males had significantly higher testosterone levels than breeding males (t = -2.759, df = 20, P = 0.012).

Paired tests volume (corrected for body size) changed significantly across the season (F(1,190) = 17.65, P < 0.0005) in a way that varied by breeding condition (time * breeding condition interaction (F(1,190) = 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 tests volume increasing in the summer (F(1,190) = 37.105, P < 0.0005; no group main effect or time * breeding condition interaction). There were no significant differences in paired tests 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(1,139) = 8.276, P < 0.0005) were seen in *P. pumilus* that varied significantly by breeding condition (F(1,139) = 6.081, P = 0.003). Unlike the profile seen in *P. vampporus*, 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 (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**

In *P. vampporus*, 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(1,14) = 7.029, P < 0.0005) that varied by breeding condition (F(1,14) = 7.16, P = 0.011) and by sex (F(1,14) = 26.02, P < 0.0005). Overall, males were significantly heavier (even after correcting for body size (length of forearm)) than females.

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**Fig. 5.** Adjusted body mass (mean ± SE mass in grams/length of right forearm in mm) over time for (A) *P. vampporus* 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 size (in months) for the remainder of the year.
Unfortunately, at D0, a significant size difference existed between control and treatment males. However, this difference was not significant in the control females. At D15, there was a significant difference in body mass between treatment and control males, with treatment males having a lower body mass than control males. This difference was significant for both control and treatment females at D15. However, the difference was not significant for treatment males at D35. At D15, the difference was significant for both control and treatment females, with treatment females having a lower body mass than control females. This difference was significant for both control and treatment males at D35.

Changes in body mass during the initial period of growth (D0-D15) and during the rest of the year (P. pavipes) were less pronounced than in P. vespertilio (Fig. 5B). During the first month following group formation, significant changes in body mass over time were only found in breeding females (F2,20 = 9.428, P < 0.001), where levels significantly rose between D15 and D35 (F1,20 = 4.688, df = 7, P < 0.001). There were no significant differences over time during this first 35 days in control females or any males. In contrast to P. vespertilio, female P. pavipes were significantly heavier at D0 than males, and this difference persisted through D35 (sex differences: D0: F2,30 = 10.377, P < 0.001; D15: F2,30 = 5.759, P < 0.001; D35: F2,30 = 7.404, P = 0.011). Female but not male P. pavipes 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: r = 0.521, df = 10, P = 0.001), then gained again (but not significantly) by late summer. In contrast to P. vespertilio, only P. pavipes females bred 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. vespertilio females in Fig. 5A is not represented in Fig. 5B for P. pavipes. In contrast to the profound seasonal rhythm in body mass observed in P. vespertilio males, there were no significant differences in body mass over time in captive P. pavipes.

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

In the cases of stress by species, stress reactions by experiment, each group of both species at each time point showed a significant overall glucocorticoid stress response (Fig. 6), and ANOVA indicated a number of significant interactions between factors. For P. vespertilio, there were significant main effects for condition (baseline vs. stress: F1,20 = 13.398, df = 1, P < 0.001), breeding condition (breeding vs. non-breeding group: F1,20 = 4.259, df = 1, P = 0.054), and time (autumn vs. spring: F1,20 = 6.727, df = 1, P = 0.015), but not for sex. Additionally, there were significant interactions between time and sex (F1,20 = 13.896, df = 1, P < 0.001) and time and breeding condition (F1,20 = 10.826, df = 1, P = 0.002). For P. pavipes, there were significant main effects for condition (baseline vs. stress: F1,20 = 10.281, df = 1, P < 0.005) and time (autumn vs. spring: F1,20 = 33.978, df = 1, P < 0.001), but not for breeding condition or sex. There was also a significant interaction between time and sex (F1,20 = 6.409, df = 1, P = 0.018). If one looks not at total glucocorticoid levels but rather at the absolute difference (increase) in hormone levels in response to repeated (baseline to stress) levels, there were no significant differences across time or between group types for either sex. A noticeable exception is found in P. pavipes males in the spring, where baseline values did not differ from those of females but where stress levels were significantly higher than in females (F2,20 = 2.948, df = 6, P = 0.067). Fecundity levels were variable but nearly always decreased (significantly) in response to stress in males of both species at both times of the year. For P. vespertilio, these differences were significant in breeding males in the spring.
Fig. 6. Total glucocorticoids ( cortisol plus corticosterone, mean ± SE) in baseline and in response to 30 min of restraint stress examined both in the autumn (November) and in the spring (April) for (A) P. puniceus and (B) P. vespertili. BF = breeding females; CF = control breeding; BM = breeding males; CM = control males.

(5.57 ± 0.99 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 = 3.784, df = 11, P = 0.005). For P. puniceus, 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 = 5, 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 (1.619 ± 0.24 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 separately for the pregnant vs. postpartum restraint stress study. Within each species and not between species, potential differences in stress-responsive hormone levels between the social "up state" or "up state" conditions were first assessed. For P. puniceus, a significant stress response occurred in both postpartum conditions (Fig. 7), which did not vary by the presence or absence of the up state (significant main effect for stress condition [baseline vs. stress]: P<sub>F1,11</sub> = 3.452, P = 0.005; no significant main effects for up state condition or interactions for stress condition and up state condition) because the presence or absence of the up state did not alter the cortisol response, baseline and stress levels found 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<sub>1,11</sub> = 44.035, P < 0.0005) and stress condition × reproductive condition interaction (F<sub>1,11</sub> = 7.893, P = 0.017), but no main effect for reproductive condition. Essentially, baseline, cortisol levels are not distinguishable between pregnancy and the postpartum (estrus) 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 the non-breeding females in the spring. For P. vespertili, stress response postpartum (P<sub>F1,11</sub> = 24.974, P < 0.0005) but no difference between the two social up state conditions' significant stress response pre and postpartum (P<sub>F1,11</sub> = 31.902, P < 0.0005), thus varied for reproductive condition (P<sub>F1,11</sub> = 10.188, 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 = 0.001). The differences between cortisol and corticosterone cancelled out when total glucocorticoid levels were calculated as there were no significant differences between pregnancy or the postpartum condition since both hormones were added together. A nearly identical hormone profile was identified in P. puniceus (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 the genus Pteropus, but, within this large genus (65 species, Sibly et al., 2005), P. puniceus and P. vespertili are not closely related (Huston 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. puniceus being found in small, intermediately intervening groups (which is unusual in this genus) and P. vespertili being highly gregarious and found in groups up to a thousand times larger than those of P. puniceus. These natural behavioral differences were reflected in variation in inter-animal spacing measured in our study. Where physiological differences between the species were evident, we propose that their underlying social structure 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. vespertili same-sex groups suggests that social stability was quickly reached. In contrast, the sustained elevation in glucocorticoids in breeding male and female P. vespertili and both breeding and control female P. puniceus suggest that...
individuals reared in a state of stress (see at least high energetic demand), likely maintained in P. vanderwui by the frequent negative social interactions in the breeding pens and in P. australis by the higher than normal levels of cage disturbance and handling (compared to what these animals were not involved in any studies). The sustained elevations in glucocorticoids were most pronounced in the breeding male P. vanquiquus who were actively competing between themselves and fighting with females for mating access during this period. The potential suppressive effects of their sustained glucocorticoids on gonad function (Viral, 2002; Wingfield and Sapolsky, 2003) may explain why breeding male P. vanquiquus 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. vanquiquus) (see Wingfield and Sapolsky, 2003). Based upon the results of our seasonal analysis, it appears that testosterone naturally declines at this time of year (suggesting that we found our group in 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 case this lack of a decrease) in testosterone in breeding males relative to control males (Schmutz 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. vanquiquus 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 so male-female
distribution, male P. vampyrus maintained greater interspecific distance between themselves, suggesting that competition within males may have been high. Although we did not methodologically document dominance hierarchies within male P. vampyrus, those were clearly visible and subordinate individuals in each of the breeding pens and in the control group. While male P. vampyrus lost body mass during the initial 5 days and male P. punxul did not change body mass during this period, breeding females in both species significantly gained body mass between day 5 and 35 following group formation. This weight gain is likely due to the increased energy attributed to pregnancy as only a few of the P. vampyrus females could have been pregnant at this time (based upon whether they eventually gave birth) and none of the P. punxul females was pregnant at this time. Increased weight in breeding females may instead be due to the oophorogenic properties of glucocorticoids (Dollman et al., 1995; Sopchylo 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 interspecific spacing during the first 35 days following group formation were found in all P. vampyrus; changes in P. punxul only occurred in the breeding groups. In P. vampyrus, differences in interspecific 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 group approached the maximum average interspecific distance possible (11.70 ± 1.11 SEM, Fig. 2A). The decreases in interspecific 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 formed. 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 still preferred to be close to other females and males still preferred to be distant from other males, but the magnitude of the difference 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 in larger groups.

The clear sex differences in social preference evident in P. vampyrus were essentially non-existent in the relatively social P. punxul. In fact, both male and female P. punxul seemed relatively indifferent to other animals in their pen. Two-hour recordings of social interactions following group formation in this species involved in blank tapes. When provided with hanging branches and other visual barriers within their enclosure (as both species were), P. punxul, but not P. vampyrus, regularly hid themselves. Breeding P. punxul did not show from being closer to same sex companions to males having spaced themselves farther apart to be somewhat closer to females to eventually being evenly distributed in the pen (each that, at day 35, the interspecific spacing in the P. punxul breeding groups approached the theoretical maximum interspecific animal distance for the species in a pen (6.48 ± 0.63 m)). The lack of changes in interspecific spacing in P. punxul groups may indicate greater social stability, or lower levels of social interactions, and/or may be due to the way these groups were formed. The results may indicate that sex-specific compositions rather than fully 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. punxul, for all groups except control males, P. vampyrus were closer to one another than P. punxul. These differences suggest that interspecific relationships (whether agonistic or heterosexual) are generally more important or least more salient in P. vampyrus compared to P. punxul. That control male P. punxul 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. punxul. Nocturnal observations of mating behavior in both species support this notion in P. punxul, breeding males were observed "waving in lines" 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 intensity sampled first 35 days following group formation, seasonal differences in P. vampyrus were more evident and of greater magnitude than in P. punxul. No seasonal changes in interspecific spacing between both breeding and control P. vampyrus were found that could be correlated to seasonal changes in physiology. Across the season, the same sex-specific and breeding-group-specific patterns of interspecific spacing described in the first 35 days following group formation were evident, with several notable fluctuations. During the winter months, when testosterone levels increase, 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 interspecific spacing in the breeding groups that occurred in the summer were surely related to the synchronous birth of 14 pups in July. Attendance 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 the pen. Perhaps due to the absence of a postpartum estrous in this species, breeding males became spaced farther apart at this time as well (significantly so by July), presumably due to the lack of competition for non-reproductive females. Nocturnal observations of females with pups vigorously rejecting all male advances during this period support this view. Only a handful of seemingly random and idiopathic interspecific differences in interspecific spacing in P. punxul 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 the overall lower levels of social interaction (at least as observed during the day).
Despite this behavioral difference between the species, both showed significant seasonal rhythms in glucocorticoids and testosterone. Although the glucocorticoid seasonal rhythms were much clearer in P. vampeyax than in P. pumilus, in both species, peak seasonal levels in females occurred in August, whereas the peak in glucocorticoids in males occurred in August and September in P. vampeyax 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 18.61 ± 9.32 and 79.62 ± 100.14 for breeding males and 779.98 ± 117.16 and 950.53 ± 206.97 for control males). The earlier seasonal peak in glucocorticoids in male P. pumilus relative to male P. vampeyax may be related to their significantly earlier seasonal rise in testosterone relative to P. vampeyax. 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 males 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, 2012). In his review of seasonal changes in glucocorticoids is 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, internal shifts in HPA function may serve to modulate changes in metabolic rate, water metabolism, gonadal function, and immune competence, such that glucocorticoids should be elevated during the more energetically expensive time of the year (Romero, 2002; Waide and Schneider, 1992). From a behavioral standpoint, changes in glucocorticoid levels may elicit rapid behavioral changes. By this logic, seasonal rhythm in glucocorticoid levels occur because animals may or may not need to express glucocorticoid-mediated behaviors (such as flight/immigration and emigration) 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 (Neploky et al., 2003). 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 posterior, P. polyopesaphus (Martin and Bennett, 2009; Martin et al., 1995; Meehan and Meehan, 1991a,b). In females, peak levels occurred in the winter months, perhaps in preparation for the energetic demands of pregnancy. Alternatively, 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 apparent intersexual trend in the absence of a knowledge of receptor physiology of the levels of the glucocorticoid carrier protein (corticosteroid binding globulin; CBG), a similar trend has been noted in P. hypomelanus (Reeder et al., 2014a; Widmann and Kutz, 1993). Concurrent with our study of seasonal hypomelanus 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 are 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 (Hennesey, 1997) in the case of mothers and pups, separation from a pup increases glucocorticoids in mothers of some species (e.g., squirrel monkeys, Saimiri sciureus; see Hennesey, 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 basal 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 (Bremner and Orishkina, 2001, 2002), thus presumably more readily available for inducing changes (see Bremner and Orishkina, 2002; Romero, 2002 for discussion of the competing views of the role of CBG). Whether or not the roughly equivalent increase in glucocorticoids 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 (11BHS and 11BHS2D2) in the target tissues (Scepi and Walker, 2001; Sapolisky 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 has been shown in species in which mothers form an attachment relationship with their infant; see Hennesey, 1997) suggests that there is a lack of an emotional attachment between a mother and
her pup in P. v. vynnychenki and P. punnus. Deschamps et al. (2003) demonstrated that the preservation of ecologically relevant stressors (e.g., predator simulation) results in the over-sensing of stress hyperresponsiveness in lactation, and it would be useful to explore this phenomenon in these species. The inverted corticosterone versus cortisol in pregnant female P. v. vynnychenki in response to stress is reminiscent of alterations in the baseline cortisol/cortisosterone ratio in late pregnancy described in P. hypersteini (Reeder et al., 2004a), which may be related to shifts in adrenocorticalogenesis or cholesterol metabolism in the adrenal cortex.

Female (and to a lesser extent male) P. punnus, which were much less social than P. vynnychenki, appeared to be highly susceptible to disturbance (Micklitz 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. punnus females in the breeding groups responded significantly closer to one another than at any other time of the year, which perhaps mediated the effects of the disturbance. Additionally, female P. punnus, which started the study in many cases ovum, 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 reproducibly synchronous.

We believe that P. vynnychenki and P. punnus, with their documented senescence, 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 in the general physiology of the HPA and HPG axes. Additionally, P. vynnychenki, along with P. hypersteini (Reeder et al., 2004a), with trees exceptionally high and variable glucocorticoid levels may prove to be valuable models for glucocorticoid research. Future species should include quantification of C3B and other metabolic hormones such as insulin. Additional comparative studies between the P. vynnychenki and P. punnus should be conducted to further explore the importance of social influences on physiological processes.

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References