

**Neutrophil:Lymphocyte Ratio as a Possible Indicator of
Chronic Anthropogenic Stress in Bats (Mammalia: Chiroptera)**

by

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Abstract

Exposure to chronic stressors has negative impacts on wild animals. Heightened levels of glucocorticoid hormones commonly are measured to test for exposure to stress. Due to the fast-changing nature of circulating levels of glucocorticoids, the neutrophil:lymphocyte ratio, which increases in response to stress more slowly than glucocorticoids, may be a more appropriate indicator of chronic stress. For this investigation, I first tested levels of cortisol and neutrophil:lymphocyte ratios of big brown bats (*Eptesicus fuscus*) and confirmed that cortisol and, in females, neutrophil:lymphocyte ratios increase after exposure to stressors. I then examined neutrophil:lymphocyte ratios and indexes of body condition of *Myotis lucifugus*, *M. septentrionalis*, and *M. sodalis* from two types of sites: those that were and those that were not impacted by anthropogenic disturbances. Neutrophil:lymphocyte ratios were significantly higher in reproductively active *M. septentrionalis* from impacted habitats (impacted \bar{X} = 0.40, unimpacted \bar{X} = 0.18), but did not differ significantly in other groups. Indexes of body condition were significantly lower at impacted sites for female *M. sodalis*. Other females were not significantly different, but indexes of body condition for male *M. septentrionalis* were significantly greater in impacted sites. Although results were inconsistent and sometimes conflicting, significant differences in neutrophil:lymphocyte ratios and indexes of body condition suggest that anthropogenic disturbances have the potential to act as stressors that can affect health of bats, and warrant further investigation into their effects on neutrophil:lymphocyte ratios.

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Glory to God in the highest, and War Eagle!

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INTRODUCTION

Impacts of chronic stress on the physiology and behavior of animals has received considerable attention (Anderson and Keith 1980; Yarmoloy et al. 1988; Knight and Cole 1991), particularly with reference to anthropogenic disturbances. Urbanization, recreational activity, construction of roads, and noise from military activity can cause behavioral changes leading to altered density, dispersal, and home ranges of wildlife (Larkin and Pater 1996; Etter et al. 2002; Prange et al. 2004; Johnson et al. 2005). These and other activities by humans also result in physiological changes, such as reduced reproductive success and immunocompetence in wildlife (Spraker et al. 1984; Giese, 1996; Mullner et al. 2004; Ditchkoff et al. 2006).

A stressor is a force, either intrinsic or extrinsic, that has the capability to shift any of a number of physiological systems from homeostasis (Chrousos 1997). Once an animal has experienced a stressor, it is referred to as being stressed. In response to a stressor, the animal undergoes a highly conserved suite of physiological and behavioral changes that are designed to remove stress from its environment and to re-establish homeostasis, i.e., to adapt (Selye 1951). Some of these changes are rapid, occurring within a few seconds after exposure to a stressor, while others may take several minutes or hours (Sapolsky et al. 2000).

Seconds after experiencing a sudden, or acute, stressor, the sympathetic nervous system of an animal stimulates the adrenal medulla to produce catecholamines (epinephrine and norepinephrine), which generate a body-wide, aroused response, such as heightened alertness, increased heart rate and respiration, and inhibition of feeding. These changes help the animal respond to the immediate, perceived threat from the stressor (Sapolsky et al. 2000).

A few minutes after exposure to the stressor, activation of the hypothalamic-pituitary-adrenal axis leads to increased production of glucocorticoid hormones in the adrenal cortex, which mediate further physiological changes. Stress-induced levels of glucocorticoid hormones alter metabolism by increasing circulating glucose and mobilizing lipids and amino acids. Additionally, glucocorticoid hormones can alter immune function (Tsigos and Chrousos 2002). Increases in levels of glucocorticoid hormones lead to trafficking of white blood cells, enhanced function of T and B cells, and suppression of inflammation (Dhabhar 2002; Webster et al. 2002). Other potential effects include suppressed growth and reproduction, death of neuronal cells, and disruption of second-messenger systems (Wingfield et al. 1997). These deviations from normal physiological states contribute to the ability of an animal to properly respond to the stressor (Sapolsky et al. 2000).

While the response to acute stress generally is beneficial, problems can arise when an animal is chronically exposed to a stressor. Metabolic changes intended to help an animal adapt to a stressor would lead to reduced body condition if prolonged (Wingfield et al. 1997; Kitaysky et al. 1999; McEwan 2004). Immunosuppression can lead to disease in chronically stressed animals (Spraker et al. 1984; Kiecolt-Glaser et al. 1996; Dhabhar and McEwan 1997; Padgett and Glaser 2003; Bradley and Altizer 2007). Effects of suppressed reproduction may not be apparent at first, but could impact performance of individuals and their offspring in disturbed populations for years following exposure (Tilbrook et al. 2000; Romero 2004).

To determine whether an animal has been exposed to a stressor, researchers must have a way to measure exposure or ability of an animal to mount a stress response. The most common and direct method for this is to measure glucocorticoid hormones, i.e., cortisol or corticosterone. These steroids always are present to some degree throughout the body of an animal, but their rate

of secretion from the adrenal cortex increases upon activation of the hypothalamic-pituitary-adrenal axis (Sapolsky et al. 2000). The short term, or acute, stress response is assessed most often by measuring changes in circulating levels of glucocorticoid hormones (Romero 2004; Reeder and Kramer 2005; Wikelski and Cooke 2006). This involves obtaining a baseline measurement from a pre-stress sample of blood, exposing the animal to a stressor, and then taking a second sample of blood to record the increase in levels of glucocorticoid hormones in response to the stressor. Baseline levels of glucocorticoid hormones also have been used to identify exposure to chronic stressors, either from levels of circulating plasma or by measuring deposition of glucocorticoid hormones in feces or urine (Hopkins et al. 1999; Creel et al. 2002).

Despite extensive literature using levels of glucocorticoid hormones to measure stress exposure (reviewed in Romero 2004), there are characteristics of glucocorticoids that may limit their usefulness as indicators of chronic exposure to stress. First, baseline measurements must be taken within the first few minutes of capture in most wild animals due to the rapid change in levels of glucocorticoid hormones after exposure to the acute stress of capture (Reeder and Kramer 2005; Romero and Reed 2005). This requirement can be difficult to meet for field research on many animals, as it necessitates constant vigilance and immediate removal of animals from traps. Second, acclimation of the hypothalamic-pituitary-adrenal axis, resulting in reduced levels and responses of glucocorticoid hormones, occurs in some animals after repeated or continuous exposure to a stressor (Barton et al. 1987; Spencer and McEwan 1990; Harbuz and Lightman 1992; Ruane and Komen 2003; Romero 2004). This phenomenon could lead to misinterpretation of low levels of glucocorticoid hormones as indicative of the absence of chronic stressors when the animal actually has been stressed chronically.

In recent years, some researchers have begun to evaluate the neutrophil:lymphocyte ratio as an indicator of exposure to chronic stressors (Davis et al. 2008). Counts of white blood cells were used to measure stress in animals before hormonal assays were developed (Hoagland et al. 1946; Dhabhar et al. 1996). Neutrophils (heterophils in birds, reptiles, and amphibians; thus, an heterophil:lymphocyte ratio in these vertebrates) and lymphocytes are the two most common types of white blood cells in vertebrates, and a ratio between them can be obtained easily after performing a standard count of white blood cells. Neutrophil:lymphocyte ratios change in response to exposure to a stressor, and they have been used to infer stressfulness of different environments and activities, such as transportation and crowding for poultry, livestock, and wildlife (Onbasilar and Aksoy 2005; Obernier and Baldwin 2006). Kim et al. (2005) observed an increase in both neutrophil:lymphocyte ratio and glucocorticoid hormones in cynomolgus monkeys (*Macaca fascicularis*) that had experienced 15 days of stress from being transported. Engler et al. (2004) observed that neutrophils increased while lymphocytes decreased (thus, an elevated neutrophil:lymphocyte ratio) in mice that experienced repeated social stressors. Dhabhar et al. (1996) demonstrated that, after an animal experiences a stressor, numbers of circulating lymphocytes decrease while neutrophils increase, and that these changes seemed to be the result of trafficking of leukocytes into and out of the blood stream, mediated by stress hormones. Thus, when an animal perceives a stressor, production of these hormones leads to an increase in the neutrophil:lymphocyte ratio (Dhabhar et al. 1996).

There are some characteristics of the neutrophil:lymphocyte ratio that would make it useful for detecting exposure to chronic stressors. One characteristic is that stress-induced increase in neutrophil:lymphocyte ratio tends to lag behind that of the response of glucocorticoid hormones. Levels of glucocorticoid hormones increase rapidly (within minutes) in response to

an acute stressor (Muir and Pfister 1987; Romero and Reed 2005). Conversely, Burguez et al. (1983) administered injections of glucocorticoid hormones to horses (which simulated activation of the hypothalamic-pituitary-adrenal axis) and reported that neutrophil:lymphocyte ratios did not increase significantly until >2 hours post injection. A similar pattern occurred in levels of leukocytes in wild Pyrenean chamois (*Rupicapra pyrenaica*) after capture (Lopez-Olvera et al. 2007). Another characteristic is that stress-induced changes in neutrophil:lymphocyte ratios tend to persist longer than changes in levels of glucocorticoid hormones. Glucocorticoids can return to baseline levels <1 hour after activation of the hypothalamic-pituitary-adrenal axis in birds and mammals (Muir and Pfister 1987; Romero and Reed 2005), but these changes in leukocytes can persist for at least several hours beyond that (Dhabhar et al. 1996; Lopez-Olvera et al. 2007; Davis et al. 2008). Due to these characteristics, as well as the possibility of adaptation of the hypothalamic-pituitary-adrenal axis, several reports have suggested that the neutrophil:lymphocyte ratio could be more useful than circulating levels of glucocorticoid hormones in examining chronic stress in animals. In chickens, changes in heterophil:lymphocyte ratios are more consistent and longer lasting than the response of glucocorticoid hormones, which can make them better indicators of exposure to stressors (Gross and Siegel 1983; McFarlane and Curtis 1989). Vleck et al. (2000) examined glucocorticoid hormones and heterophil:lymphocyte ratios in wild penguins (*Pygoscelis adeliae*) and observed that heterophil:lymphocyte ratios, but not levels of glucocorticoid hormones, were elevated in individuals that had healing wounds from fights compared to individuals that had been fighting recently. They concluded that altered levels of leukocytes may be more useful than glucocorticoid hormones for detecting exposure to chronic environmental stressors in some animals.

Bats (Chiroptera) are the second largest group of mammals after rodents. They are also one of the most ecologically and economically important groups due to their roles as pollinators and as predators of night-flying insects (Jones et al. 2009). Therefore, knowledge of what causes stress in bats, and how they adapt to those stressors, would be valuable in designing conservation strategies to protect populations of bats. Anthropogenic disturbances during hibernation (e.g., noise, lights, and physical contact) can stimulate arousal and cause bats to expend more energy than normal, which can lead to a fatal reduction in stored fat (Speakman et al. 1991; Thomas 1995). Other anthropogenic stressors such as chronic exposure to pesticides affect insectivorous bats, which bioaccumulate pesticides from their prey (Clark 1988; Senthilkumar et al. 2000; O'Shea et al. 2001) and may result in increased mortality (Geluso et al. 1976; Clark and Stafford 1981; Lloyd and McQueen 2000).

There are few studies examining glucocorticoids in captive and wild bats, and there is no study on the relationship between neutrophil:lymphocyte ratio and stress. Levels of glucocorticoid hormones in bats increase in response to stressors as in other mammals, but can vary by sex and reproductive condition (Widmaier et al. 1994; Reeder et al. 2004). Only recently have researchers begun to examine physiological changes associated with exposure to anthropogenic stressors in wild bats (Allen 2009; Allen et al. 2009).

The goal of this investigation was to determine if neutrophil:lymphocyte ratio can serve as a viable indicator of exposure to chronic anthropogenic stressors. First, I needed to ensure that stress-induced changes in concentrations of leukocytes in bats follow a pattern similar to other vertebrates, i.e., that neutrophil:lymphocyte ratios, as well as levels of cortisol, increase after exposure to a stressor. Second, I tested the hypothesis that neutrophil:lymphocyte ratios of bats would vary based on exposure of bats to chronic, anthropogenic sources of stress. I

predicted that bats inhabiting areas subject to high degrees of disturbances by humans would have higher neutrophil:lymphocyte ratios than bats from less-disturbed habitats, taking into account effects of sex and reproductive status. I also examined relationships between neutrophil:lymphocyte ratio and index of body condition, with the prediction that bats experiencing chronic stressors will show a negative correlation between these parameters.

MATERIALS AND METHODS

The first portion of my study examined the response of neutrophil:lymphocyte ratios in big brown bats (*Eptesicus fuscus*) to acute stress of capture and handling to ensure that changes in neutrophil:lymphocyte ratios of bats were similar to other animals. Big brown bats are large, insectivorous, vespertilionid bats that have a wide geographic range in North America. Females form maternity colonies in trees, caves, and man-made structures in spring and summer, while males generally are solitary until they join females at hibernacula in autumn. In this experiment, individuals were captured during early evening on 2 September 2009 from a maternity colony in the attic of a house in Lafayette, Chambers County, Alabama. The first bat was captured at 1735 h CST and the last bat was caught at 1813 h. The time after 1735 h for each bat was used as a covariate in analyses to account for the possibility that presence of humans at the colony affected individuals that had not yet been captured. Because reproduction ceased 2-3 months prior to collection, females were assumed non-reproductively active or juveniles, and males were assumed to be juveniles because adult males are rarely found within maternity roosts, but ages were not verified.

Two samples of blood were taken from each bat: one immediately after capture to ensure that baseline levels of the neutrophil:lymphocyte ratio were recorded and another 60 minutes later. Between the first and second samples, bats were housed individually in well-ventilated, opaque, plastic containers. A 26-gauge needle was used to lance the uropatagial vein and a small amount of blood was collected into a heparinized capillary tube. A blood smear was prepared, fixed with methanol, and stained with a Hema-3 kit (similar to Wright-Giemsa staining method;

Fisher Scientific Company L.L.C., Middletown, VA), coverslipped, and stored for later analysis. A differential count of white blood cells was performed for each individual by identifying 100 different leukocytes with a light microscope (40x or 100x magnification). Neutrophil:lymphocyte ratio was calculated by dividing percentage of neutrophils by percentage of lymphocytes. The remaining blood was centrifuged to obtain plasma for hormonal analysis. Levels of cortisol were measured with a commercially available enzyme-immunoassay kit (Cayman Chemical, Ann Arbor, MI). Levels of corticosterone were measured via radioimmunoassay as detailed in Mendonça et al. (1996). I also calculated an index of body condition for each individual using residuals of a linear regression of mass and length of forearm (Jakob et al. 1996). Two-way and one-way repeated-measures GLMs were used as appropriate, with time after first capture used as a covariate.

The second part of my study focused on three species of bats, *Myotis lucifugus*, *M. septentrionalis*, and *M. sodalis* (little brown myotis, northern myotis, and Indiana myotis, respectively); *M. sodalis* is federally listed as endangered. These are small vespertilionid bats that roost in colonies in trees and human-made structures during summer and hibernate in caves or mines during winter. These also are some of the species that are most threatened by the spread of white-nose syndrome (Blehert et al. 2009). Bats were captured by mist nets throughout Indiana and Kentucky during May-August 2007. Three sites were considered impacted by anthropogenic disturbances: Camp Atterbury, a training base for the Indiana National Guard in Bartholomew, Brown, and Johnson counties, Indiana; Big Oaks National Wildlife Refuge in Jefferson, Jennings, and Ripley counties, Indiana, part of which is used as a bombing range by the United States Air Force; and Fort Knox in Bullitt, Hardin, and Meade counties in Kentucky, home to the United States Army Armor School. These impacted sites tended to contain many

hectares of forests and fields, but experienced regular military-training activities, artillery fire, aircraft flyovers, or a combination of these. Locations of captures were within these military installations, but distances from sources of noise varied. Three additional sites were used as unimpacted sites: Muscatatuck National Wildlife Refuge, Jackson and Jennings counties, Indiana; Brashears Creek, Spencer County, Kentucky, a rural area consisting mostly of farms and forests; and Mammoth Cave National Park, Edmonson County, Kentucky.

Blood-sampling methods were as described above except that each bat was only bled once, <3 minutes after capture. I also calculated indexes of body condition for these bats as described above. Separate regression analyses were calculated for each reproductive status of each sex of each species, although pregnant females were excluded. One-way and two-way GLMs were performed on neutrophil:lymphocyte ratios by sex, species, reproductive status, and type of site, with Fisher's LSD post-hoc tests as appropriate. Regressions were used to examine relationships between neutrophil:lymphocyte ratio and index of body condition. Data were analyzed with Statview (version 5.0.1; SAS Institute Inc., Cary, North Carolina) and JMP (version 6.0.0; SAS Institute Inc., Cary, North Carolina).

RESULTS

Neutrophil:lymphocyte ratio and response of glucocorticoid to an acute stressor

A total of 15 big brown bats were captured; 4 males and 11 females. Blood smears were made for each individual, but I was unable to acquire enough blood for hormonal analysis from several bats.

A repeated-measures, two-way GLM indicated an interaction between sex and time for neutrophil:lymphocyte ratios ($F = 5.6$, $d.f. = 1$, $P = 0.037$), so sexes were analyzed separately. After 60 minutes, neutrophil:lymphocyte ratios were significantly increased in females ($F = 27.5$, $d.f. = 1$, $P < 0.001$; Fig. 1), but not in males ($F = 4.3$, $d.f. = 1$, $P = 0.174$). For females, baseline and 60-minute means of neutrophil:lymphocyte ratio were $0.229 \pm 0.126 SE$ and 0.702 ± 0.272 , respectively. For males, baseline and 60-minute means of neutrophil:lymphocyte ratio were 0.185 ± 0.145 and 0.253 ± 0.096 , respectively. Neutrophil:lymphocyte ratios of males were not significantly different from baseline levels of females. There was no relationship between neutrophil:lymphocyte ratios and index of body condition in males or females ($P > 0.05$).

Neither baseline nor elevated levels of cortisol or corticosterone significantly differed by sex ($P > 0.1$), so hormonal data from males and females were pooled for further analysis. Levels of cortisol significantly increased by 60 minutes post-capture ($F = 5.5$, $d.f. = 1$, $P = 0.046$), although there was a high degree of variance (Fig. 2). For cortisol, mean baseline and 60-minutes post-capture were 13.8 ± 24.3 and 97.8 ± 67.4 ng/ml, respectively. Levels of corticosterone at 60-minutes post-capture ($\bar{X} = 6.6 \pm 4.5$ ng/ml) were not different from baseline ($\bar{X} = 4.9 \pm 3.4$ ng/ml; $F = 0.9$, $d.f. = 1$, $P = 0.33$; Fig. 3).

Neutrophil:lymphocyte ratios of bats from impacted and unimpacted habitats

Success of capture varied among groups of bats. More *M. septentrionalis* were caught than any other species; 122 males and 102 females, *M. sodalis* was second, with 17 males and 89 females, and *M. lucifugus* was the least sampled, with 21 males and 49 females. Blood samples could not be obtained from each individual, and after sorting samples based on species, sex, reproductive status, and type of site, some groups contained insufficient samples for statistical analysis (Table 1). Statistical analyses were conducted only on male and female *M. septentrionalis* and on female *M. lucifugus* and *M. sodalis* where samples were sufficient.

Neutrophil:lymphocyte Ratio.—A two-way GLM of neutrophil:lymphocyte ratio by species and sex demonstrated that sex had an effect on neutrophil:lymphocyte ratios ($F = 5.4$, $d.f. = 1$, $P = 0.021$; Fig. 4). Regardless of species, neutrophil:lymphocyte ratios of males were significantly lower than females (Fisher's LSD; $P < 0.001$). However, this difference was driven by *M. septentrionalis* due to a small sample of males of the two other species. Males were analyzed separately from females because of this effect.

A GLM with only females revealed that species also had an effect on neutrophil:lymphocyte ratio ($F = 7.7$, $d.f. = 2$, $P < 0.001$; Fig. 5). Neutrophil:lymphocyte ratios of female *M. sodalis* ($\bar{X} = 0.36 \pm 0.18$) were significantly greater than for females of *M. lucifugus* ($p < 0.001$, $\bar{X} = 0.19 \pm 0.12$) and *M. septentrionalis* ($P = 0.001$, $\bar{X} = 0.22 \pm 0.16$). Therefore, I analyzed reproductive status and type of site separately for each species.

Among *M. septentrionalis*, the species for which I had the largest sample, neutrophil:lymphocyte ratios of females varied significantly by reproductive status ($F = 5.8$, $d.f. = 2$, $P = 0.006$; Fig. 6). Neutrophil:lymphocyte ratios of female *M. septentrionalis* were higher

during pregnancy and lactation than during post-lactation ($\bar{X} = 0.29 \pm 0.18, 0.29 \pm 0.18, 0.14 \pm 0.08$, respectively). A similar pattern of variation was present in *M. lucifugus* and *M. sodalis*, but it was not significant, likely due to the small sample of post-lactating females in both species. Because of the significant variation in neutrophil:lymphocyte ratios by reproductive status in *M. septentrionalis*, along with the small sample of post-lactating females in the other species, I combined pregnant and lactating females into one reproductively active group for each species, and only used this group of reproductively active females for comparisons of sites.

Overall, a two-way GLM of neutrophil:lymphocyte ratios of reproductively active females by species and site revealed an interaction between species and site ($F = 4.7, d.f. = 2, P = 0.013$). Thus, when analyzing species individually, I observed a significant difference in neutrophil:lymphocyte ratios of *M. septentrionalis* by site. Females from impacted sites had significantly greater neutrophil:lymphocyte ratios than females from unimpacted habitats (impacted $\bar{X} = 0.40 \pm 0.15$, unimpacted $\bar{X} = 0.18 \pm 0.11$; $F = 15.7, d.f. = 1, P = 0.001$; Fig. 7). Neutrophil:lymphocyte ratios of female *M. lucifugus* and *M. sodalis* did not vary significantly by site.

Males were not reproductively active throughout the sampling period. Neutrophil:lymphocyte ratios of male *M. septentrionalis* did not vary by any measure. Notably, average neutrophil:lymphocyte ratio (0.15) for male *M. septentrionalis* was significantly lower than for reproductively active females ($F = 25.0, d.f. = 1, P < 0.001$), and similar to post-lactating females ($F = 0.03, d.f. = 1, P = 0.847$).

Index of Body Condition.—Although samples of blood were not taken from all captured individuals, length of forearm and mass were measured for all bats. Therefore, I had a larger

pool of samples from which to examine index of body condition. Pregnant females were excluded from comparisons due to their variable mass.

An interaction was observed between species and site for index of body condition of female bats, so species were analyzed separately. *M. sodalis* females from impacted sites had lower index of body condition than those from unimpacted sites ($F = 8.83$, $d.f. = 1$, $P = 0.006$; Fig. 8). Indexes of body condition of female *M. lucifugus* and *M. septentrionalis* did not vary significantly by type of site. Indexes of body condition of male *M. septentrionalis* also were affected by type of site, but the effect was opposite that of female *M. sodalis*; indexes of body condition for male *M. septentrionalis* were significantly greater in impacted sites ($F = 8.5$, $d.f. = 1$, $P = 0.005$; Fig. 9). Additionally, indexes of body condition of male *M. septentrionalis* were significantly and negatively correlated with neutrophil:lymphocyte ratios ($F = 5.416$, $R^2 = 0.085$, $P = 0.024$; Fig. 10). Index of body condition did not correlate with neutrophil:lymphocyte ratio in any other group.

DISCUSSION

I documented that neutrophil:lymphocyte ratios in bats can respond to an acute stress during capture as they do in other vertebrates. Ratios for females exhibited a stress-induced increase that is characteristic among vertebrates (Davis et al. 2008). Magnitude of the increase was similar to other animals as well; stressed levels were more than twice the baseline levels (Lance and Elsey 1999; Kannan et al. 2000; Kim et al. 2005; Onbasilar and Aksoy 2005). Unlike other studies, I did not observe a similar significant increase in neutrophil:lymphocyte ratio of males. I sampled a colony known to be a maternity colony in September, shortly after big brown bats would be volant (July in Alabama). It is likely that captured males were juveniles, as well as some of the females. The effect of age on neutrophil:lymphocyte ratios is unknown, but responses to stress in juveniles may be more limited than those of adults. There is an adrenal-hyporesponsive period in young mammals, but this only lasts about 2 weeks in neonatal laboratory rats (Sapolsky and Meaney 1986; Levine 1994), while any juvenile bat that was sampled would have been ≥ 3 months old. Thus, it remains unknown whether the lack of change in males is a developmental effect, or if males were responding differently than females to stress of capture in terms of a response in neutrophil:lymphocyte ratio.

Levels of cortisol also increased as could be expected after activation of the hypothalamic-pituitary-adrenal axis in response to stress of capture and handling. Observed elevated levels (about 175 ng/ml 60-minutes post-capture) were within the range reported for big brown bats and other bats, but were well below the reported average levels of 800 ng/ml 15 and

30 minute post-handling (Reeder et al. 2004; Wada et al. 2010). This could indicate that stress-induced levels of cortisol had begun to return to a normal level by 60 minutes after handling.

Unfortunately, I was only able to evaluate a short segment of changes in the neutrophil:lymphocyte ratio. It remains unclear how long it takes for the neutrophil:lymphocyte ratio to significantly increase after exposure to a stressor or how long the increase persists. However, I did recapture one female 24 hours later, and its neutrophil:lymphocyte ratio was elevated as much as it was at the 60-minute mark, showing that these changes might persist for at least that long. Ultimately, it does appear that profiles of neutrophil:lymphocyte ratios change in response to a stressor and potentially could be used to assess exposure to acute or chronic stress in bats.

I also examined neutrophil:lymphocyte ratios and indexes of body condition in bats from sites that were impacted by military activity and unimpacted sites, with the assumption that bats from impacted sites were experiencing chronic anthropogenic stressors. First, neutrophil:lymphocyte ratios were significantly higher in pregnant and lactating *M. septentrionalis* than in post-lactating females. Elevated levels of glucocorticoid hormones are present in reproductively active male and female bats (Reeder et al. 2004; Klose et al. 2006) and may help to explain this observation. As previously discussed, it is believed that changes in neutrophil:lymphocyte ratios are mediated partly by changes in levels of glucocorticoid hormones (Dhabhar et al. 1996). Several hypotheses have been proposed to explain seasonal elevation in levels of glucocorticoid hormones, including increase in availability of energy by up-regulating metabolism, and the possibility that they help in maintaining a more sensitive risk-avoidance strategy during demanding periods (Klose et al. 2006). Pregnancy and lactation are the most energetically demanding times for bats (Kurta et al. 1989); perhaps, the need to acquire

adequate resources may constitute an underlying chronic stressor that could amplify responses to other stressors, such as anthropogenic disturbances. In my study, neutrophil:lymphocyte ratios of females seemed to return to a lower level after their reproductive period, which was nearly identical to neutrophil:lymphocyte ratios of non-reproductively active males. This could provide support for that theory. Regardless of their source, these seasonal variations should be taken into account for any future work examining neutrophil:lymphocyte ratios.

After accounting for effects of sex and reproductive status on neutrophil:lymphocyte ratio, I was able to observe that pregnant and lactating *M. septentrionalis* (the species with the largest sample) from impacted sites had significantly greater neutrophil:lymphocyte ratios than females from unimpacted sites. This suggests that some factor associated with impacted sites (presumably military activity) potentially had enough of an effect to account for the significantly elevated neutrophil:lymphocyte ratios observed in female *M. septentrionalis*. However, this effect was not present in any female of other species (for which there were relatively small samples).

I also examined index of body condition to determine if type of site had any other measurable effect on condition of bats. Female *M. sodalis* displayed a significantly lower index of body condition at impacted sites, but females of the other two species did not display this effect. In contrast, male *M. septentrionalis* exhibited a significantly higher index of body condition on impacted sites. It is interesting that female *M. septentrionalis* and *M. lucifugus* did not show the same pattern as female *M. sodalis* despite having an adequate sample. This difference in pattern may be attributable to differences in roosting ecology between the two species. *M. sodalis* is a tree-roosting specialist (Humphrey et al. 1977), while *M. septentrionalis* and *M. lucifugus* use caves and human-made structures in addition to trees. It may be that cave-

roosting and house-roosting species are more protected from military activity, such as artillery and bombs. However, this does not correlate with neutrophil:lymphocyte ratios, because female *M. septentrionalis* was the only group with significantly increased neutrophil:lymphocyte ratios on impacted sites. Perhaps, female *M. septentrionalis* were experiencing greater levels of anthropogenic stress (indicated by elevated N:L ratios), but were better able to adapt to those stressors than female *M. sodalis*, which could be indicated by the opposite trend seen in indexes of body condition.

There was no consistently significant relationship between index of body condition and neutrophil:lymphocyte ratios, which would have been predicted if neutrophil:lymphocyte ratios also act as an indicator of condition. Male *M. septentrionalis*, a group that exhibited lower neutrophil:lymphocyte ratios overall, also exhibited significantly elevated index of body condition on impacted sites. This could mean that they generally are better able to adapt to anthropogenic stressors, or that they are better able to take advantage of resources on military bases compared to control sites. Conversely, female *M. sodalis* exhibited generally high neutrophil:lymphocyte ratios while having significantly lower indexes of body condition on impacted sites, suggesting that they are not able to adapt as well as other species to anthropogenic stressors. So the overall pattern is consistent, but samples were not adequate to truly test this trend.

There are some drawbacks to assessing responses to stress with neutrophil:lymphocyte ratios in bats. First, levels of leukocytes can vary widely among and within species, so experiments must be designed to allow for adequate sample size to minimize effect of variation among individuals, even after accounting for sex and reproductive status. However, this also is true for levels of glucocorticoid hormones, and the same consideration for size of samples must

be given when examining these hormones (Vleck et al. 2000). Second, any measurement involving white blood cells is dependent on health of the animal being studied. Animals with infections likely have levels of leukocytes that are different from healthy levels, although a large sample would help dilute the effect of a few sick individuals on the rest of the sample. Third, and possibly the most important, is that little is known about how leukocytes of bats respond to stress. Neutrophilia and lymphopenia seem to be characteristic of exposure to stressors in bats and other vertebrates, with some degree of variation (Davis et al. 2008). Specifics, such as duration and magnitude of changes in neutrophil:lymphocyte ratios, and even normal ranges, largely are unknown in most species of bats. My results suggest that neutrophil:lymphocyte ratios in bats respond as expected (increase in response to stress, change more slowly than levels of glucocorticoid hormones), but more research should be conducted across species, sexes, and reproductive status to examine neutrophil:lymphocyte ratios over varying spans of time and with different degrees of stressful stimuli to refine our understanding of the response of bat neutrophil:lymphocyte ratios to stress.

Significant differences in neutrophil:lymphocyte ratio and index of body condition were identified by site. These results suggest that anthropogenic disturbances have the potential to affect health of bats living in these areas. Conversely, results sometimes were inconsistent, and I cannot conclusively confirm that neutrophil:lymphocyte ratios constitute reliable indicators of chronic exposure to anthropogenic sources of stress. Given larger samples and a more complete understanding of factors affecting responses of neutrophil:lymphocyte ratios, differences that I revealed would justify further examination of the neutrophil:lymphocyte ratio in measuring chronic stress. Complete extent of the physiological impacts of disturbance likely cannot be inferred from neutrophil:lymphocyte ratios alone. However, if the neutrophil:lymphocyte ratio

actually is representative of chronic stress experienced by an animal, it could be a valuable and easily obtained measurement, especially if supported by other measurements such as index of body condition.

TABLE 1.—Samples of bats of the genus *Myotis* from impacted and unimpacted sites by sex and reproductive status. Numbers in parentheses indicate number of individuals with data for neutrophil:lymphocyte ratio.

Sex and type of site	<i>M. lucifugus</i>		<i>M. septentrionalis</i>		<i>M. sodalis</i>	
	Impacted	Unimpacted	Impacted	Unimpacted	Impacted	Unimpacted
Male	4 (2)	17 (1)	27 (15)	64 (44)	1 (1)	4 (0)
Female						
Pregnant	11 (11)	7 (0)	18 (7)	3 (6)	10 (2)	30 (13)
Lactating	9 (7)	8 (7)	12 (7)	6 (5)	13 (2)	17 (4)
Post-lactating	1 (0)	5 (3)	16 (0)	29 (19)	0 (1)	2 (0)

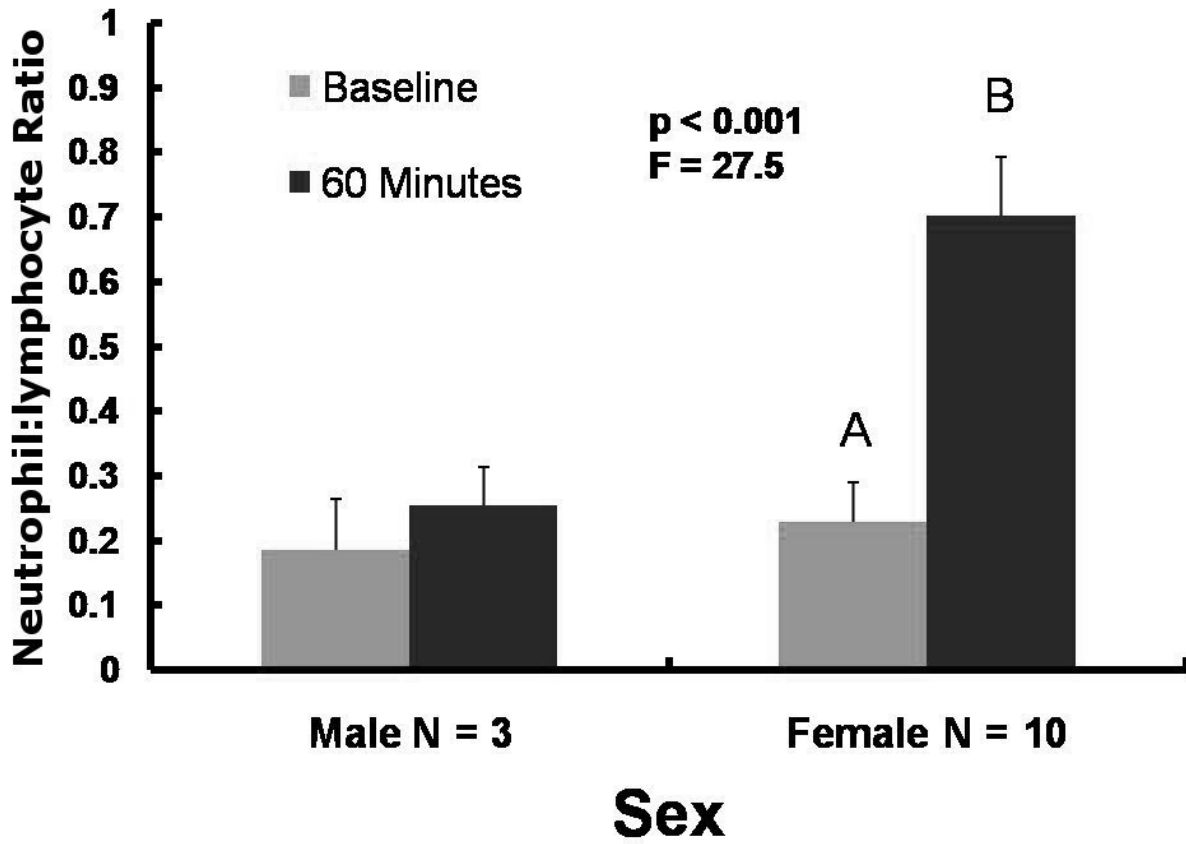


FIG. 1.—Neutrophil:lymphocyte ratios increased in female big brown bats (*Eptesicus fuscus*) in response to capture and handling. Letters (A and B) represent significant differences. Lines represent standard error.

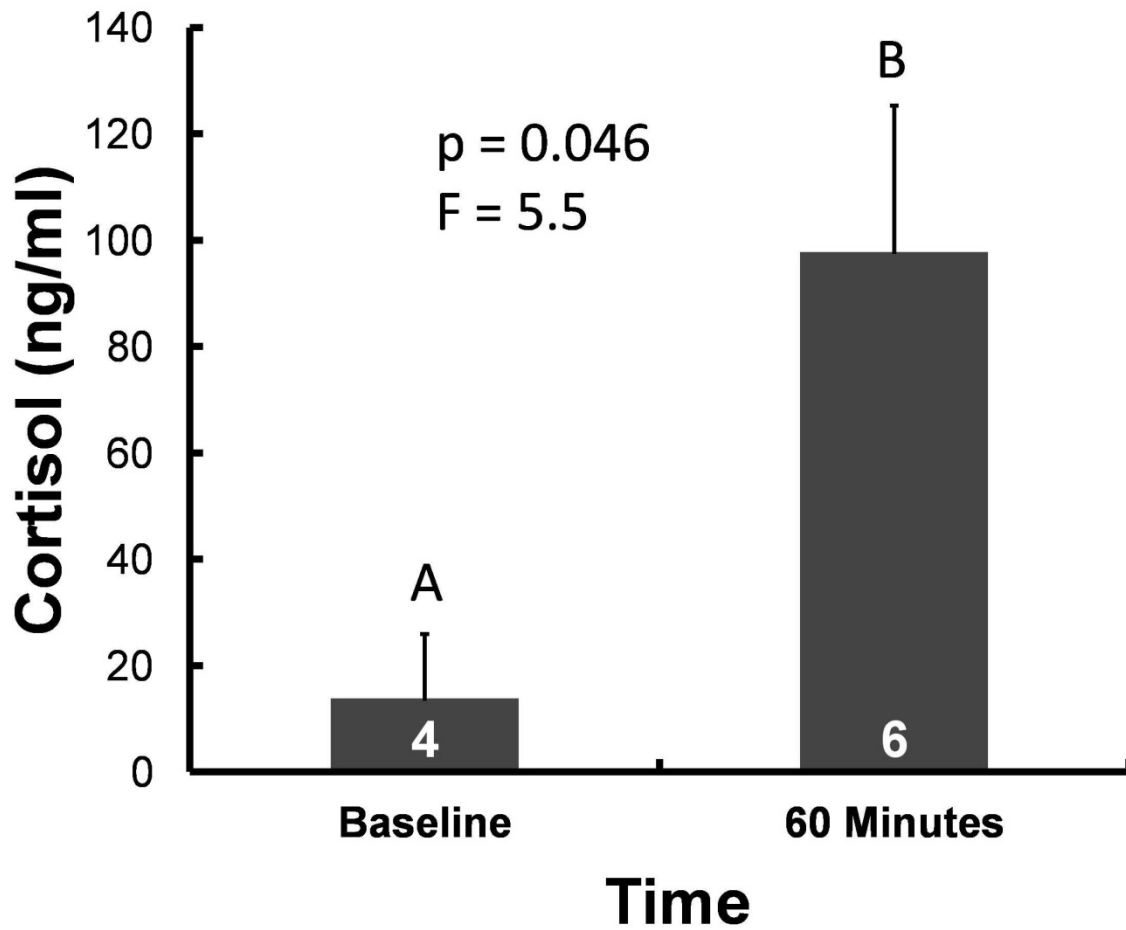


FIG. 2.—Cortisol levels increased in the big brown bat (*Eptesicus fuscus*) in response to capture and handling. Letters (A and B) represent significant differences. Lines represent standard error. Sample sizes are indicated at the base of each bar.

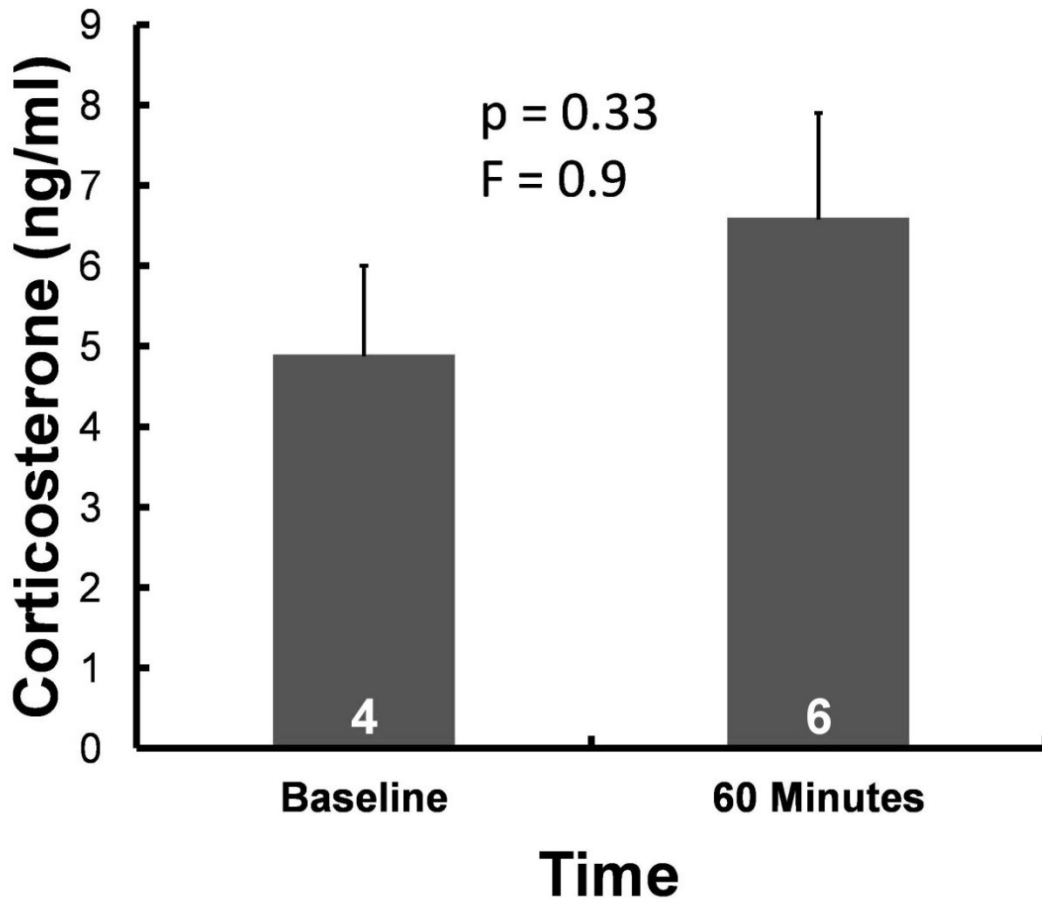


FIG. 3.—Corticosterone levels did not change in the big brown bat (*Eptesicus fuscus*) in response to capture and handling. Lines represent standard error. Sample sizes are indicated at the base of each bar.

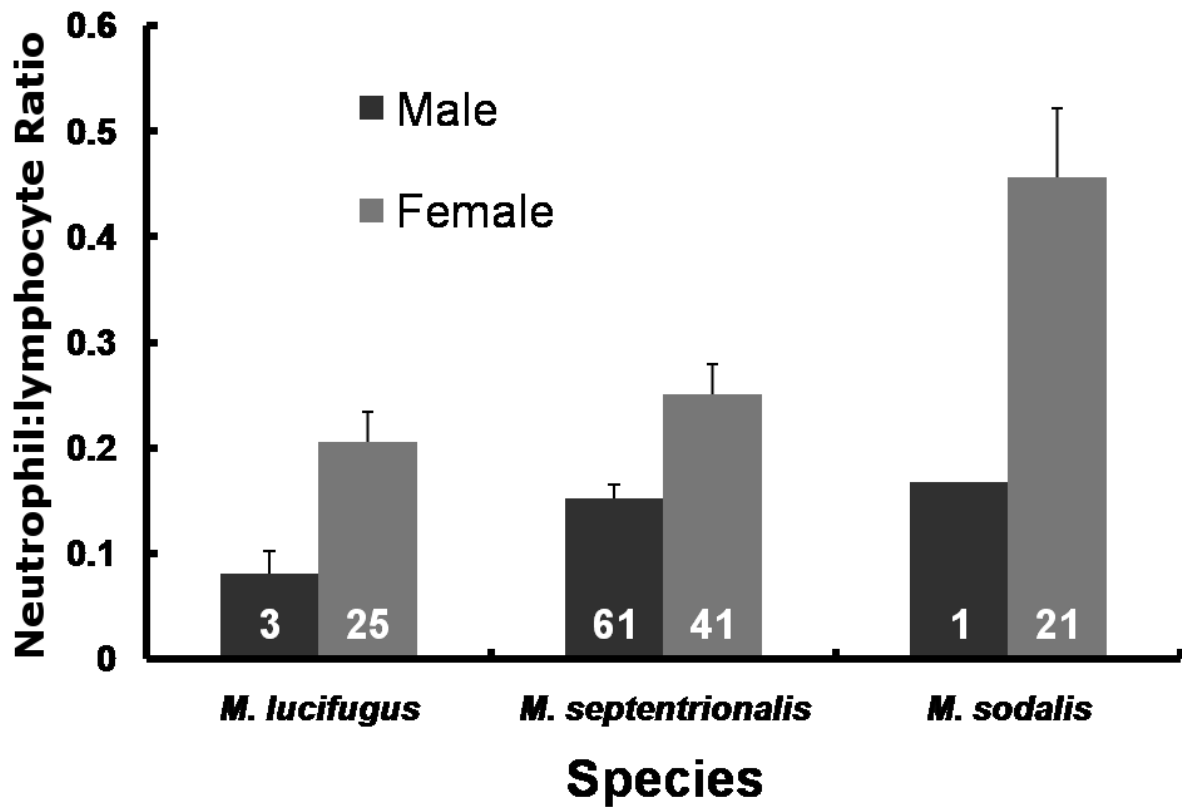


FIG. 4.—Neutrophil:lymphocyte ratios by species and sex of three species of bats in the genus *Myotis*. Lines represent standard error. Sample sizes are indicated at the base of each bar.

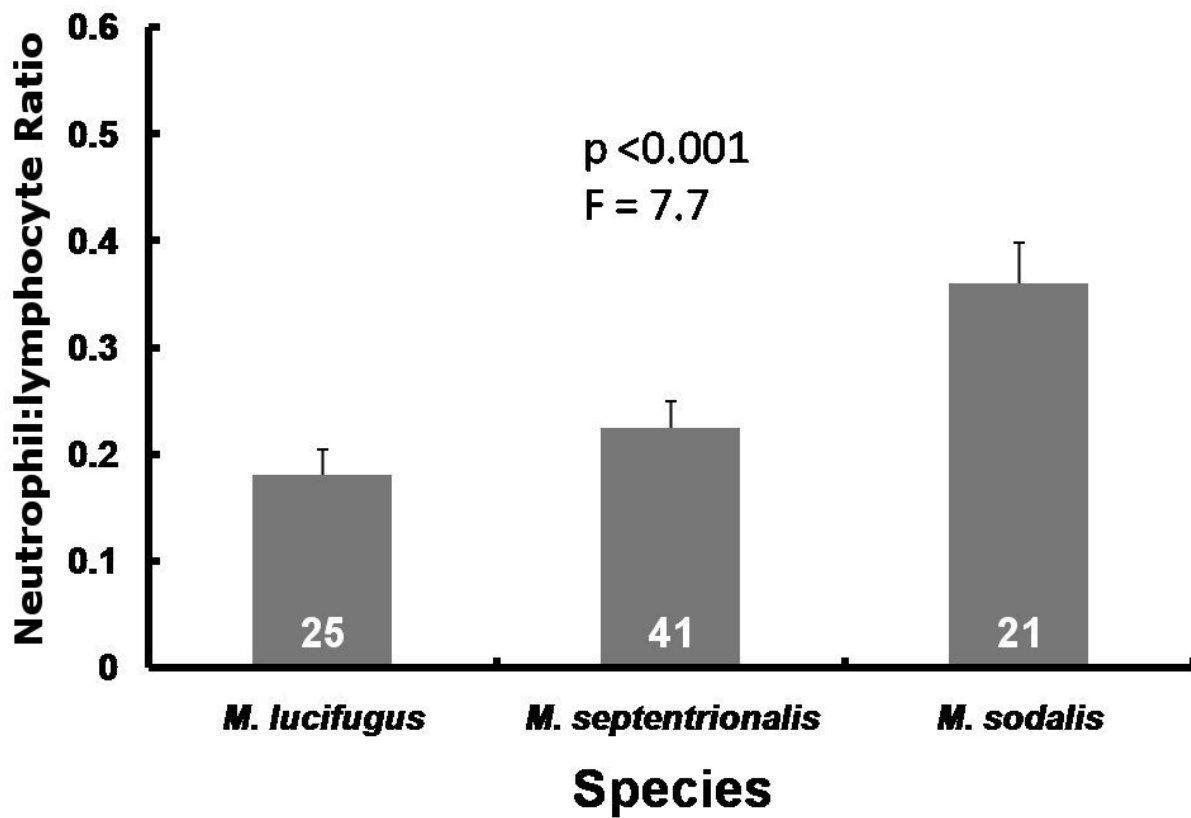


FIG. 5.—Neutrophil:lymphocyte ratios of females of three species of bats in the genus *Myotis*.

Lines represent standard error. Sample sizes are indicated at the base of each bar.

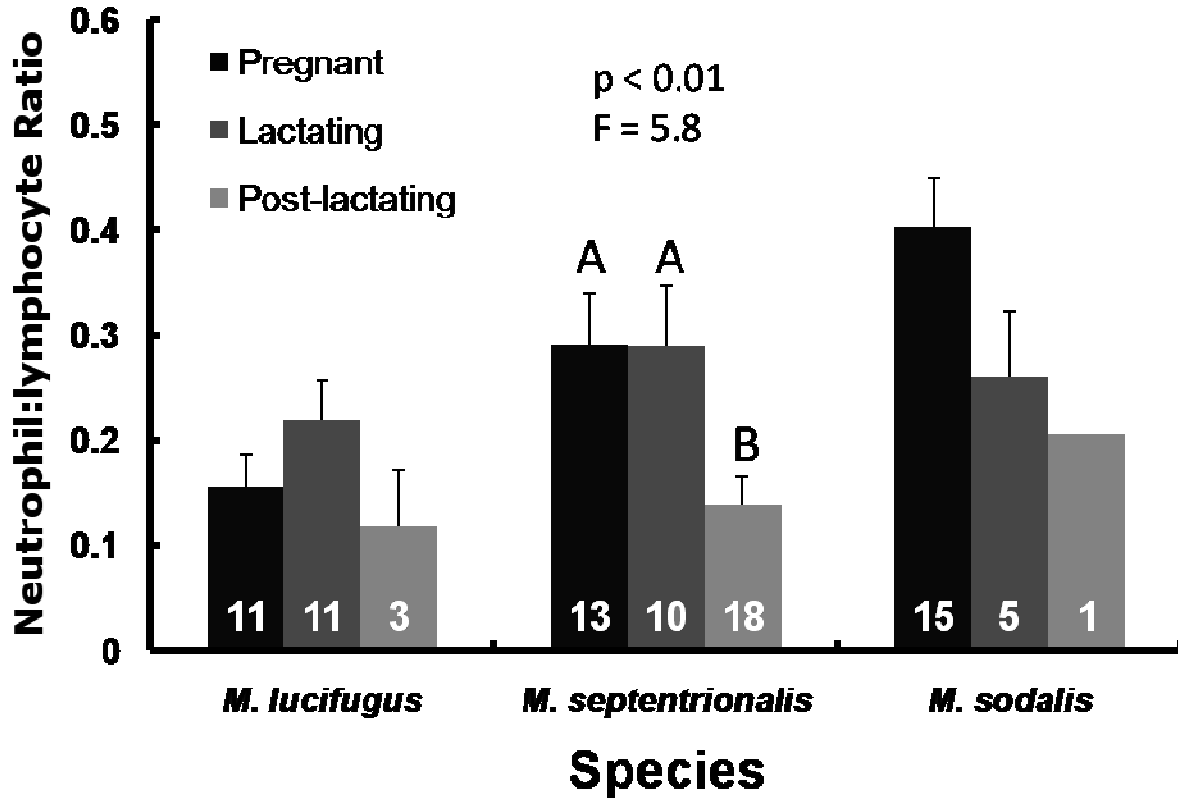


FIG. 6.—Neutrophil:lymphocyte ratios of females by species and reproductive status for three species in the genus *Myotis*. Letters (A and B) represent significant differences. Lines represent standard error. Sample sizes are indicated at the base of each bar.

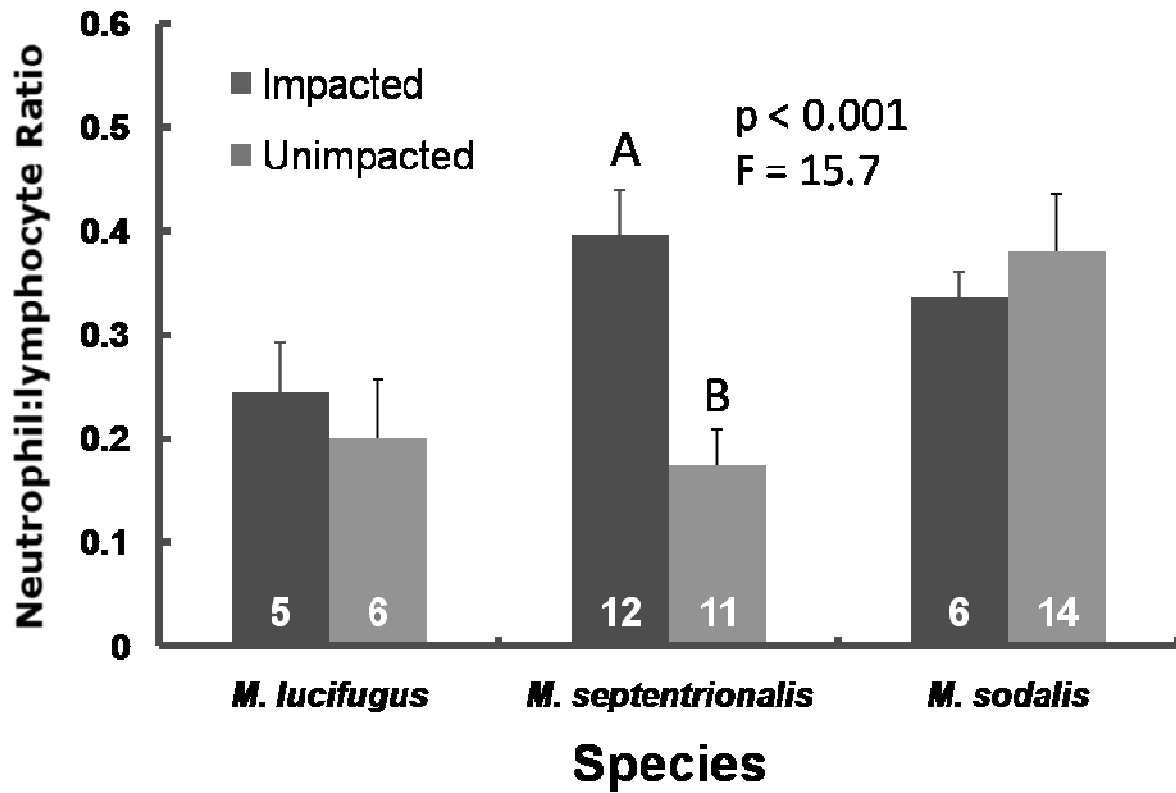


FIG. 7.—Neutrophil:lymphocyte ratios for reproductively active female *Myotis septentrionalis* were significantly higher at impacted sites. Letters (A and B) represent significant differences. Lines represent standard error. Sample sizes are indicated at the base of each bar.

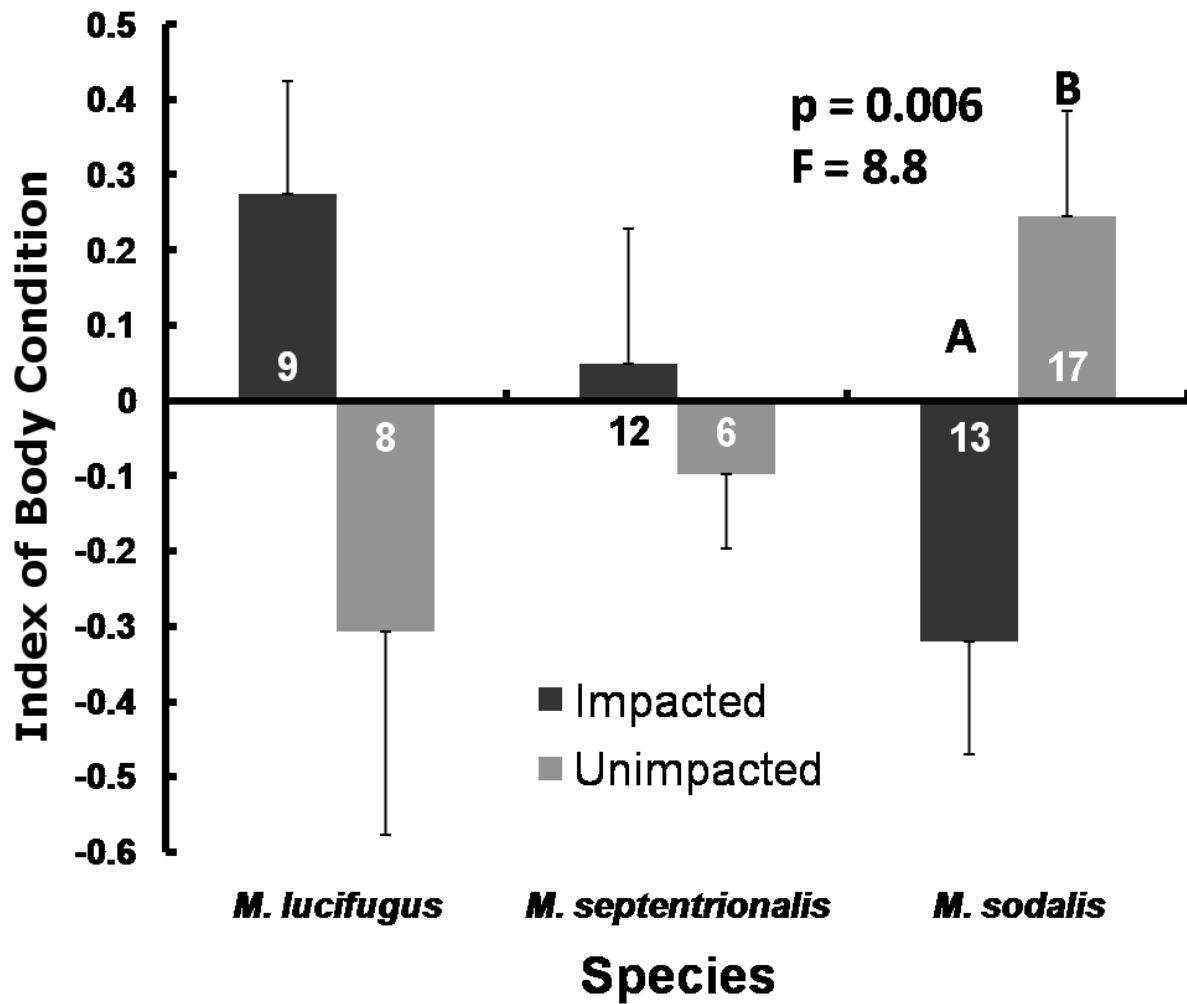


FIG. 8.—Indexes of body condition for female *Myotis sodalis* were lower at impacted sites. Letters (A and B) represent significant differences. Lines represent standard error. Sample sizes are indicated at the base of each bar.

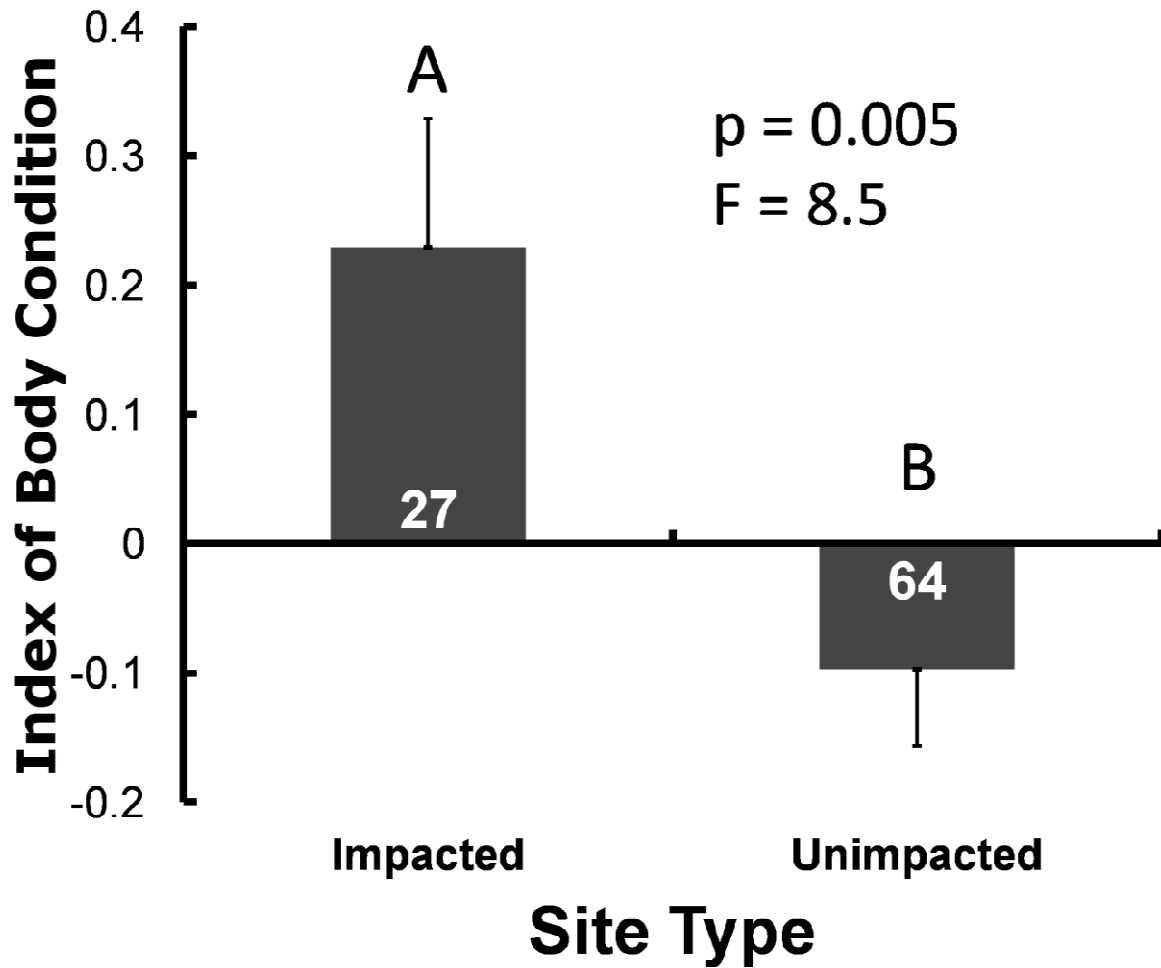


FIG. 9.—Indexes of body condition for male *Myotis septentrionalis* were higher at impacted sites. Letters (A and B) represent significant differences. Lines represent standard error. Sample sizes are indicated at the base of each bar.

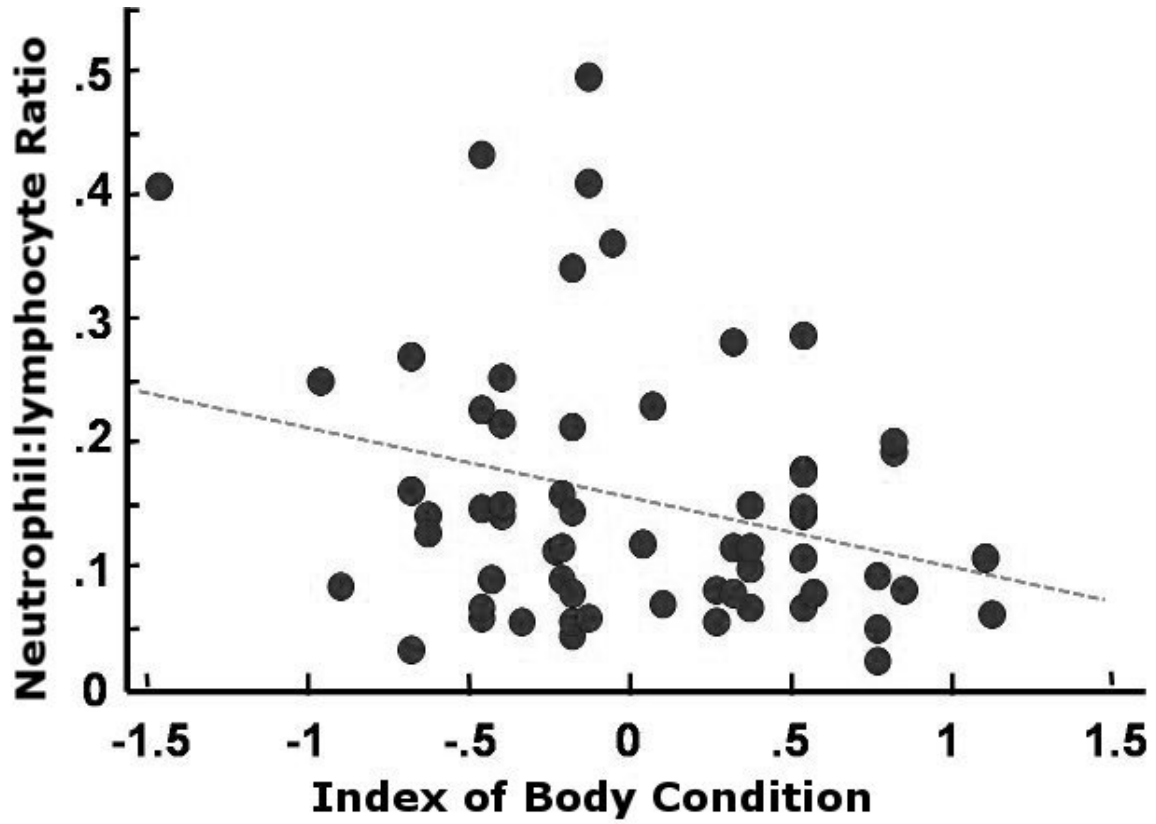


FIG. 10.—Neutrophil:lymphocyte ratio and index of body condition are negatively correlated in male *Myotis septentrionalis* ($P = 0.024$, $F = 5.416$, $R^2 = 0.085$).

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