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Physiological and Ecological Correlates of the Cellular and Humoral Innate Immune Responses in an Insular Desert Bat: The Fish-Eating Myotis (*Myotis vivesi*)

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Citation: Otálora-Ardila, A.; Flores-Martínez, J.J.; Rosales, C.; Salame-Méndez, A.; Montalvo., L.G.H. Physiological and Ecological Correlates of the Cellular and Humoral Innate Immune Responses in an Insular Desert Bat: The Fish-Eating Myotis (*Myotis vivesi*). *Diversity* **2022**, *14*, 781. https://doi.org/10.3390/d14100781

Academic Editors: Carmi Korine, Fulgencio Lisón and Michael Wink

Received: 8 July 2022
Accepted: 14 September 2022
Published: 21 September 2022

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Abstract: The immune response is affected by aridity, but it has been rarely examined in desert-dwelling bats. For two consecutive years, we examined the seasonal variation in the innate immune response of an insular desert bat, the fish-eating myotis (*Myotis vivesi*), in relation to its reproductive activity and ectoparasite load. We evaluated the reproductive activity based on external morphological traits and testosterone levels in the plasma for males and progesterone and estradiol for females. We injected phytohemagglutinin (PHA) into the footpads of the bats to estimate the innate cellular response, and we measured the bacterial killing ability (BKA) of the blood plasma to determine the innate humoral response. Both the external morphological traits and hormone levels indicate that the females were pregnant in spring and lactating in summer, and that the males were reproductively active in autumn, when mating probably occurred. The swelling response of the female and male bats was lower in spring. The BKA in the males did not vary seasonally; the BKA in the females varied seasonally but only in the first year of the study, with lower values in spring and summer. The BKA in spring was lower in the first year of the study, when the females appeared to be in early pregnancy, compared to the second year, when the females were in advanced pregnancy. The swelling increased as the body mass and body conditions of the males increased, but the BKA was not correlated with body mass or body condition in either sex. Ectoparasite abundance and prevalence did not vary among seasons. Ectoparasite abundance was not correlated with the PHA response in both sexes; it was not correlated with the BKA in females, but it was inversely correlated in males. Of the three hormones measured, only estradiol was correlated with the immune response: females with higher estradiol levels had a higher PHA response and BKA. Our findings indicate that the cellular and humoral innate immune responses of the fish-eating myotis varied throughout the year, following the seasonal reproductive pattern of the species. Our evaluation of the proximal factors affecting the expression of the immune response points to the potential immunoregulatory role of sex hormones and body mass.

Keywords: bats; dry habitats; gulf of California; immune response; insular ecosystems; Sonoran Desert

1. Introduction

Parasites are present during the lifetime of wild animals, often having a deleterious impact on their survival [1]. Parasites might reduce an animal's fitness, decrease reproductive success, diminish adult conditions and survival, and serve as vectors of other pathogens [2]. Animals, in turn, respond to parasites with an immune system composed of physical and chemical barriers [3]. The immune response is vital for animal survival, and its expression might vary over time [4], regulated by physiological tradeoffs between the reproductive and the immune systems [5]. Reproduction is one of the costliest activities in which wild animals engage during their lifetime and affects their immune response and their ability to cope with parasites. Rising concentrations of steroid hormones during reproduction regulate the immune response [6]. In general, testosterone functions as an immunosuppressor, and estrogens as immunoenhancers [7], although the effect might vary depending on the immune response analyzed [8]. Parasites, in turn, reproduce more intensely when the hosts are reproductively active [9–11], probably due to a depressed immune response and/or behavioral and ecological changes favoring infestation [12,13].

The nature of the interrelations among immunity, reproductive activity, and parasitism might depend on the sex examined. For instance, reproductive females of some bird species [14,15] and mammals [16,17] have a lower immune response and a higher parasite load than non-reproductive females. In turn, reptile males have a higher parasite load and a higher immune response at the end of their mating season [18], and testosterone levels are inversely related to immune resistance to parasites in mammals and reptiles [19,20].

Bats are the second most diverse mammalian order and are widely distributed worldwide [21]. Bats are natural hosts of a significant number of disease vectors, including bacteria, protozoa, and viruses [22,23], and the study of their immune system has gained increasing attention recently [24–26]. A handful of bat studies have examined the relationship of variations in immune function with reproductive activity and parasite burden [27–31]. A common feature of these studies is that they have examined only arthropod-eating species despite the remarkable dietary diversification found in bats and the link between dietary habits and the expression of the immune response [32]. More remarkably, these studies have not included species that live in habitats characterized by aridity and geographical isolation, two factors expected to shape immune expression in animals [33].

We examined the seasonal variation of the innate immune response of a desert carnivorous bat, the fish-eating myotis (*Myotis vivesi*), in relation to reproductive activity and parasite load. Carnivorous bats may face a higher risk of exposure to infectious diseases than bats with other feeding habits [32] and consequently be particularly sensitive to seasonal variations of immunocompetence. The fish-eating myotis feeds regularly on marine fish and crustacea throughout the year [34,35], and it is endemic to desert islands in the Gulf of California, Mexico [36]. Parasite pressure and immune response might differ between animals living in arid and more mesic environments [37,38], and between animals with insular and continental geographic ranges [39]. The loss of polymorphism in the major histocompatibility complex (MHC) of the fish-eating myotis is compelling evidence that a geographic distribution restricted to desert islands has played a role in the organization of its immune system [40]. Studies on desert bats have indicated that their immune system and parasite pressure might be particularly sensitive to the quality of their scarce water sources [41,42]. In this study, we measured the inflammatory response after a phytohemagglutinin (PHA) challenge and the bacterial killing ability (BKA) of plasma for two consecutive years in male and female adults of the fish-eating myotis. PHA is a plant protein that induces an inflammatory process in the place of an injection, a component of the cellular innate immune response [43,44]. The BKA is used to estimate the ability of complement proteins contained in the blood plasma to kill pathogens [45]. We evaluated the reproductive activity based on external morphological traits and the plasma levels of testosterone for males, and progesterone and estradiol for females. The interrelation of the PHA response and the BKA with bat reproductive activity and parasite burden has not been tested simultaneously in the same species, but a few studies have reported inconsistent

patterns. Pregnant individuals of the greater mouse-eared bat (*M. myotis*) had a higher ectoparasite load and a lower PHA response than lactating individuals [27], and the BKA was higher in the greater sac-winged bat (*Saccopteryx bilineata*) infected with endoparasites [29], but it did not vary with the reproductive activity of male and female Daubenton´s bats (*M. daubentonii*; 31). The relationship between steroid sex hormones and the immune response has been largely ignored in bats, although one study described a decrease in the plasma testosterone in males of Seba's short-tailed bat (*Carollia perspicillata*) after an immune challenge [46]. We tested the hypothesis that the PHA response and the BKA activity of the fish-eating myotis would be significantly associated with the reproductive activity and ectoparasite load. We predicted that the expression of both immunological factors would be lower and that the parasite burden would be higher in gestating and lactating females and in reproductive males than in non-reproductive individuals. Accordingly, we expected a negative relationship between the immune response and the testosterone, progesterone, and estradiol levels, and a positive relationship with the parasite load.

2. Materials and Methods

2.1. Study Area

We conducted the study on Partida Norte Island (28°52′30″ N, 113°02′17″ W), a 1.4-km^2 island located in the midriff region of the Gulf of California, Mexico. This island holds the largest known colony of fish-eating myotis (~8000 adults; 47). The population structure in Partida Norte fluctuates throughout the year: in autumn and winter, it is equally composed of males and females, whereas in spring and summer the island population is mainly composed of pregnant and lactating females, respectively [36,47].

We visited the island in winter (December 2012 and February 2014), spring (April 2013 and 2014), summer (July 2013 and 2014), and autumn (October 2013 and November 2014) and collected only adult individuals of male and female fish-eating myotis. We captured between 13 and 34 individuals in their diurnal roost in each season, and we registered their sex, body mass, forearm length, and reproductive status based on external signs (females: non-reproductive, gestating, and lactating; males: non-scrotal and scrotal). We measured each individual´s body mass (XSXScale, China; ±0.01 g) and forearm length (Mitutoyo CD-6, Mexico; ±0.01 mm) to estimate the scaled mass index as an estimator of the body condition index (BCI) [48]. Females with external signs of pregnancy were excluded because their body mass included the fetus´ body mass. We then processed the bats to determine the parasite load, immune response, and steroid hormone contents. We took a blood sample (200 µL) from the bat´s antebrachial vein within 2 h after capture before the immune challenge. The blood was centrifuged directly in the field, and plasma aliquots were stored frozen in liquid nitrogen for 30 to 95 days. We collected samples from a total of 194 individuals (116 females and 78 males), but due to logistic restrictions, it was not possible to obtain all these parameters from the same individuals in all cases. For example, the plasma volumes were not always sufficient to measure both the immune and hormone parameters from the same individual, or constraints in processing time did not always allow for counting the parasites from all individuals. All the procedures were minimally invasive, and the bats were released in the place of capture within an hour after the last measurement or sample collection after being rehydrated with fresh water and fed with a small piece of fresh fish.

2.2. Parasite Load

The bats were checked for five minutes, and ectoparasites (flies and mites) were collected from the dorsal and ventral body parts (neck, head, eyes, mouth, foot and claws, forearm, genital area, wing membrane, and uropatagium). Ectoparasites were stored in ethanol at 70% and posteriorly counted in the lab. We considered the ectoparasite load as the total number of ectoparasites per bat. We estimated the ectoparasite prevalence, mean intensity, and mean abundance for each season and the bat sex with the program

Quantitative Parasitology–QPweb 1.0.15 [49] with a 95% confidence interval. We collected ectoparasites in almost all seasons throughout two sampled years, except autumn 2014.

2.3. Immune Challenge

We challenged bats (116 females, 71 males) in the field with phytohemagglutinin (PHA). We injected 50 µL of PHA (PHA-P, No. L8754, Sigma-Aldrich, Toluca, Mexico) from a solution of 3 mg of PHA diluted in 1 mL of PBS in the right footpad of each individual. As a control, 50 µL of PBS was injected into the left footpad. The average thickness (from three measures) of both footpads was measured with a digital caliper (Mitutoyo CD-6, Mexico; ±0.01 mm) before and 3, 6, and 12 h after the PHA or PBS injection. The PHA response was measured as the swelling index (*S*) 3, 6, and 12 h after the injection as

$$S = (THtPHA - TH0PHA) - (THtPBS - TH0PBS),$$

where *THt* is the footpad thickness 6 or 12 h after the injection, and *TH*0 is the footpad thickness before the injection of PHA or PBS [50]. A swelling index of 0 occurred when the swelling in the PHA-injected foot was equal to that in the PBS-injected foot, indicating no immune response.

2.4. Bacterial Killing Ability

We measured the BKA of the plasma collected from the bats (110 females and 68 males). We carried out BKA experiments at the Instituto de Investigaciones Biomédicas of the Universidad Nacional Autonoma de México. We performed in vitro experiments of the BKA of the bat plasma against *Escherichia coli* following and adapting a method previously used for bats [32]. *E. coli* (ATCC#8739, Microbiologics, San Diego, CA, USA) was diluted so that 200–300 colony-forming units (CFU) were present in 50 µL of solution, and 3 µL of plasma was diluted to 1:100 in 297 µL of RPMI-1640 medium (11875093, ThermoFisher Scientific, Waltham, MA, USA) supplemented with FBS 5% (Gibco, Grand-Island, NY, USA). The bacterial dilution (20 µL) and plasma dilutions (280 µL) were mixed and incubated for 60 min at 37 °C (mammalian body temperature). After incubation, two plates were prepared using a 50-µL aliquot of the plasma/bacteria mixture spread on LB Broth medium (L30221, Sigma Aldrich, St Louis, MO, USA). A control mixture was made with 60 µL of the bacterial dilution and 840 µL of media, and three control plates were prepared immediately using a 50-µL aliquot per plate. Both the plasma and control plates were incubated for ~14 h. The number of CFUs was visually counted, and the mean of the plasma and the control plates were estimated to quantify the bactericidal ability of the plasma for each bat individual as

$$BKA = 1 - (mean\ CFU\ in\ plasma\ plate / mean\ CFU\ in\ control\ plate)\ .$$

2.5. Steroid Hormone Analyses

The plasma concentrations of progesterone (P_4) and estradiol (E_2) for the females and testosterone (T) for the males were determined in duplicate, using an enzyme-linked immuno-sorbent assay (ELISA) kit (Diagnostic Systems, Webster, Tx, USA; Accu-Bind, Monobind Inc., Lake Forest, CA, USA). A standard curve was obtained for each steroid in duplicate; the manufacturer supplied the control steroid. The specificity of the antiserum for the P_4, T, and E_2, as well as the linearity described by the manufacturer of each kit, were validated by making assays in both the solutions of the hormones provided in the kit and the previously purified P_4, T, and E_2 (Sigma Chemical, St. Louis, MO, USA). The percentage of recovery (linearity) for P_4 was 97 ± 2.2, with 94.5 ± 0.88 for T and 89.4 ± 2.4 for E_2. The assay detectable sensitivity (minimum concentration) for P_4 was 130 pg, with 40 pg for T and 7 pg for E_2. According to the manufacturer's indications, the ELISA plates with the samples and reagents were incubated with steroid-specific antibodies. After excess antibody removal in PBS-Tween, a secondary HRPO-conjugated antibody was added. The sandwich interaction was detected by substrate addition and quantified at 450 and 620 o

630 nm in an ELISA plate reader (Microplate Reader, MR 600, Dynatech Product® Dynatech, Chantilly, VA, USA). A standard concentration curve was determined for each hormone.

2.6. Data Analysis

Because we could not obtain all measurements from the same individuals in several cases, we were unable to test models that simultaneously included the effects of all variables. We compared the body mass and BCI among seasons with separate analyses of variance (ANOVA) for each sex. We excluded females with external signs of pregnancy for the reason mentioned above. The content values for the sex hormones were not normally distributed, and they were log-transformed for analyses. We compared the progesterone, estradiol, and testosterone values among seasons using ANOVA. We further compared the progesterone and estradiol values among reproductive categories because we found more than one category in spring, but we did not include non-lactating females in summer because of a small sample size (n = 3). We did not include males from spring in the comparison because of a small sample size (n = 2). We did not include the year as a factor in these analyses because virtually all samples in each season were collected in the same year. We compared the swelling indices at 3, 6, and 12 h after the PHA injection using repeated measure ANOVA separately for each bat sex. We then compared the swelling indices among seasons using the time period with the highest value (S_{maxper}) with factorial ANOVA with the year and season of the sample collection as factors. We defined years as "year 1" (from December 2012 to October 2013) and "year 2" (from February 2014 to October 2014) for this and subsequent analyses. We also compared the maximum swelling value registered for each individual within the 3–12-h period after the PHA injection ($S_{maxindiv}$) with factorial ANOVA because we noticed individual variation at the time when they reached the largest swelling value. The BKA indices were compared separately for each sex with generalized linear models (GLM) with a Gamma distribution. We included the year and season as factors and excluded outliers from the analysis. We used Tukey´s HSD for unequal sample sizes for post-hoc comparisons following the ANOVAs, and Kruskal –Wallis tests after the GLM when applicable. A previous work showed that PHA-induced swelling is significantly correlated with the body mass of lactating female bats [27], but the body condition was not significantly related to the swelling and BKA in female bats [51]. Since the body mass and body condition were highly correlated in females (r = 0.85, $p < 0.00001$, n = 114) and males (r = 0.96, $p < 0.00001$, n = 77), we only present the tests of the relationship of the body mass with the swelling indices (Pearson correlation analyses) and with the BKA (Spearman Rank correlation analyses) for each sex regardless of reproductive status (excluding females with external signs of pregnancy) and separately for lactating female´s swelling indices. The results of the tests of the correlation of the BCI with the immune indices follow the same patterns as with the body mass (not reported). We compared the ectoparasite load among seasons separately for each bat sex with Fisher´s exact tests for prevalence and with bootstrap one-way analyses of variance with 1000 replications for intensity and abundance using the program Quantitative Parasitology–QPweb 1.0.15 [49]. We tested the relationship between the swelling indices and the log-transformed values of the sex hormone concentration using Pearson correlation analyses for males and females. We tested the relationship between the BKA and sex hormone concentrations using Spearman Rank correlation analyses. We tested the relationship between the log-transformed values of the ectoparasite abundance (log x + 1 for females) and the swelling indices using Pearson correlation analyses for males and females. We tested the relationship between the BKA and the ectoparasite abundance using Spearman Rank correlation analyses for males and females. The analyses were conducted in STATISTICA [52].

3. Results

3.1. Body Mass, Reproductive Condition, and Sexual Hormones

There were no significant seasonal differences in body mass among females ($F_{3, 110}$ = 1.23, p = 0.30; Table 1), but the BCI did vary seasonally ($F_{3, 110}$ = 2.85, 0 = 0.04; Table 1): females had a lower BCI in spring than in autumn (p = 0.04). Females captured in December 2012 (n = 14), October 2013 (n = 17), February 2014 (n = 17), and November 2014 (n = 9) did not show any external evidence of reproductive activity. In April, we captured pregnant females in 2013 (n = 5) and 2014 (n = 11) and females with no evidence of pregnancy in both years (2013: n = 18; 2014: n = 4). In July, most females were lactating (2013: n = 18; 2014: n = 11), with a few individuals with no sign of reproductive activity (2013: n = 4; 2014: n = 3). There were no significant differences in the progesterone content among seasons ($F_{3, 58}$ = 2.23, p = 0.09; Figure 1A), but differences among the reproductive categories were significant ($F_{3, 58}$ = 3.55, p < 0.0001; Figure 1B): females captured in spring with no external signs of pregnancy had higher progesterone content than females with external signs of pregnancy (p = 0.0001), lactating females (0.0001), and females with no external evidence of reproductive activity captured in winter and autumn (0.001). There were no significant differences in the estradiol content among seasons ($F_{3, 58}$ = 2.16, p = 0.10; Figure 1C) or reproductive categories ($F_{3, 55}$ = 2.16, p = 0.10; Figure 1D).

Table 1. Body mass (BM) and body condition index (BCI) of female and male fish-eating myotis (*Myotis vivesi*) during four seasons in Partida Norte Island, Mexico. The BCI was estimated using the scaled max index (Peig and Green 2009). Females with external signs of pregnancy were not included because their body mass was confounded by the body mass of the fetus. Values are mean ± SE.

Bat sex	Season	BM (g)	BCI	n
Female	Winter	28.53 ± 0.50	204.55 ± 3.05	31
	Spring	28.25 ± 0.59	197.57 ± 3.62	2
	Summer	28.21 ± 0.46	200.69 ± 2.83	36
	Autumn	29.52 ± 0.56	210.92 ±3.40	25
Male	Winter	25.83 ± 0.49	187.15 ± 3.44	18
	Spring	24.80 ± 0.55	178.48 ± 3.90	14
	Summer	27.35 ± 0.41	196.80 ± 2.86	26
	Autumn	27.56 ± 0.48	199.20 ± 3.34	19

There were significant seasonal differences in the body mass among males ($F_{3, 73}$ = 6.70, p = 0.0004; Table 1): males in spring had a lower body mass than in autumn (p = 0.002) and summer (p = 0.002). Similarly, the BCI differed seasonally ($F_{3, 73}$ = 7.14, p = 0.0002; Table 1): males in spring had a lower BCI than in autumn (p = 0.002) and summer (p = 0.007). Most males captured in autumn had scrotal testicles (October 2013: n = 14; November 2013: n = 3), with a few individuals with abdominal testicles (October 2013: n = 2). In winter (December 2012: n = 5; February 2014: n = 13), spring (April 2013: n = 6; April 2014: n = 6), and summer (July 2013: n = 11; July 2014: n = 14), all males had abdominal testicles. There were significant differences in the testosterone content among seasons ($F_{2, 41}$ = 71.36, p < 0.0001; Figure 1E): males in autumn had higher values than in summer (p = 0.0001) and winter (p = 0.0001).

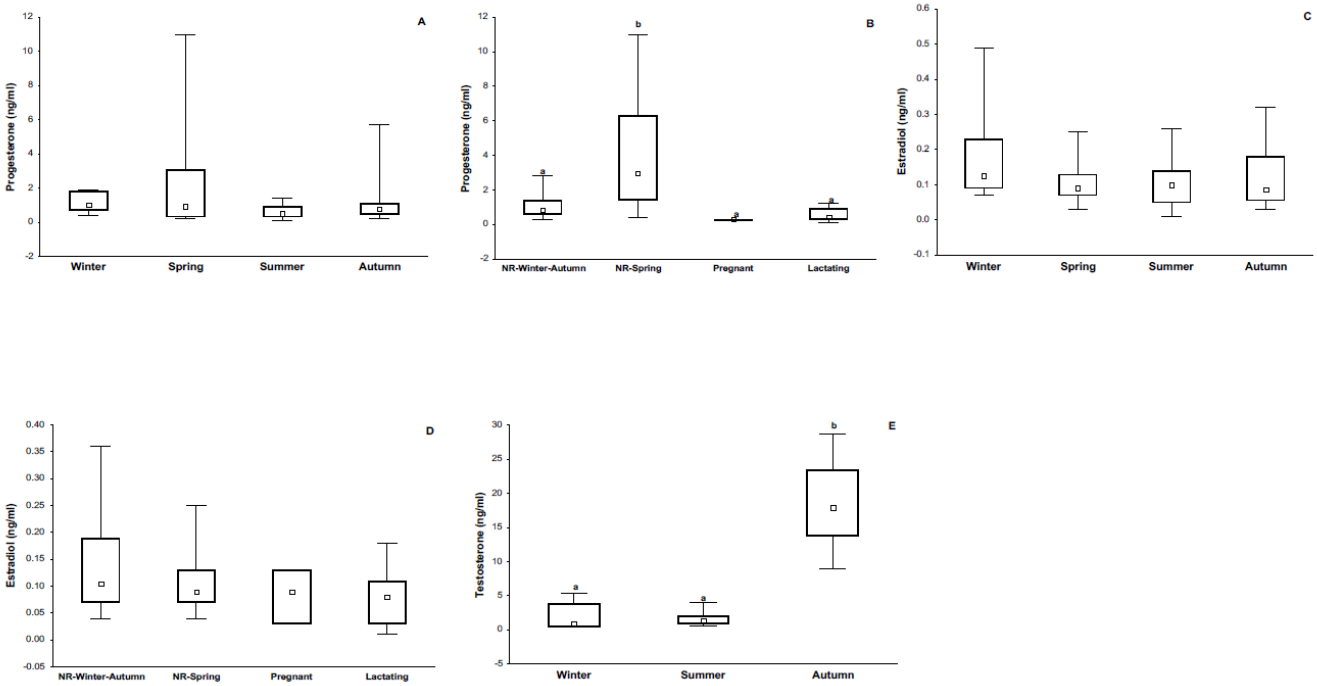

Figure 1. Concentration of steroid sex hormones in the plasma of female and male fish-eating myotis (*Myotis vivesi*) in Partida Norte Island, Mexico. The values were compared among seasons for both sexes (**A**,**C**,**E**) and among the reproductive categories for females (**B**,**D**). The values are the median (square), interquartile range (box), and 5th and 95th percentiles (whiskers). Only significant differences among the $\log_{10}$-transformed hormone concentration values are pointed out using different letters after factorial analysis of variance. NR = females with no external signs of reproductive activity.

3.2. Phytohemagglutinin (PHA) Measurements

The swelling indices for females varied among the measurement periods ($F_{2, 236} = 8.12$, $p = 0.0003$), with the S_{maxper} occurring 6 h after the PHA injection (3 h: 0.81 ± 0.05 mm; 6 h: 1.04 ± 0.05 mm; 12 h: 0.91 ± 0.04 mm). The S_{maxper} varied significantly among seasons ($F_{3, 111} = 2.98$, $p = 0.03$; Figure 2A), with no effect of the year ($F_{1, 111} = 1.53$, $p = 0.22$) or the year–season interaction ($F_{3, 111} = 0.37$, $p = 0.77$): the mean value was lower in spring than in autumn ($p = 0.05$;). The $S_{maxindiv}$ varied significantly among seasons ($F_{3, 111} = 5.17$, $p = 0.002$; Figure 2B), with no effect of year ($F_{1, 111} = 3.62$, $p = 0.06$) or the year–season interaction ($F_{3, 111} = 0.70$, $p = 0.55$): the mean value was lower in spring than in autumn ($p = 0.01$), winter ($p = 0.01$), and summer ($p = 0.01$). Neither the swelling indices were significantly correlated with the body mass for all females (S_{maxper} r = 0.18, $p = 0.06$; $S_{maxindiv}$: r = 0.05, $p = 0.58$, $n = 107$) nor for lactating females (S_{maxper} r = 0.13, $p = 0.45$; $S_{maxindiv}$: r = 0.09, $p = 0.58$, $n = 36$).

The swelling indices for males varied among the measurement periods ($F_{2, 142} = 9.76$, $p = 0.0001$), with the S_{maxper} 6 h (1.07 ± 0.06 mm; 3 h: 0.78 ± 0.06 mm; 12 h: 1.03 ± 0.06 mm) after the PHA injection. The S_{maxper} varied significantly among seasons ($F_{3, 66} = 3.63$, $p = 0.01$; Figure 2C), with no effect of the year ($F_{1, 66} = 0.60$, $p = 0.44$) or the year–season interaction ($F_{3, 66} = 1.83$, $p = 0.15$): the mean value in spring was lower than in summer ($p = 0.04$). In contrast, the $S_{maxindiv}$ was not affected by the year ($F_{1, 66} = 0.40$, $p = 0.52$; Figure 2D) or the year–season interaction ($F_{3, 66} = 0.81$, $p = 0.49$), although the effect of the season was nearly significant ($F_{3, 66} = 12.51$, $p = 0.06$), with a trend to lower values in spring than in summer. The S_{maxper} was significantly correlated with the body mass (r = 0.34, $p = 0.002$, $n = 75$) but the $S_{maxindiv}$ was not (body mass: r = 0.12, $p = 0.28$).

In females ($n = 57$), the progesterone content was not correlated with any inflammation index (S_{maxper}: r = 0.12, p =0.37; $S_{maxindiv}$: r = 0.20, $p = 0.14$; Figure 3A,B). In contrast, the estradiol content was significantly correlated with the $S_{maxindiv}$ (r = 0.32, $p = 0.02$; Figure 3C) and it had a nearly significant correlation with the S_{maxper} (r = 0.25, $p = 0.07$; Figure 3D).

Among males (n = 45), the testosterone content was not significantly correlated with any inflammation index (S_{maxper}: r = 0.18, p = 0.21; $S_{maxindiv}$: r = 0.05, p = 0.70; Figure 3E,F).

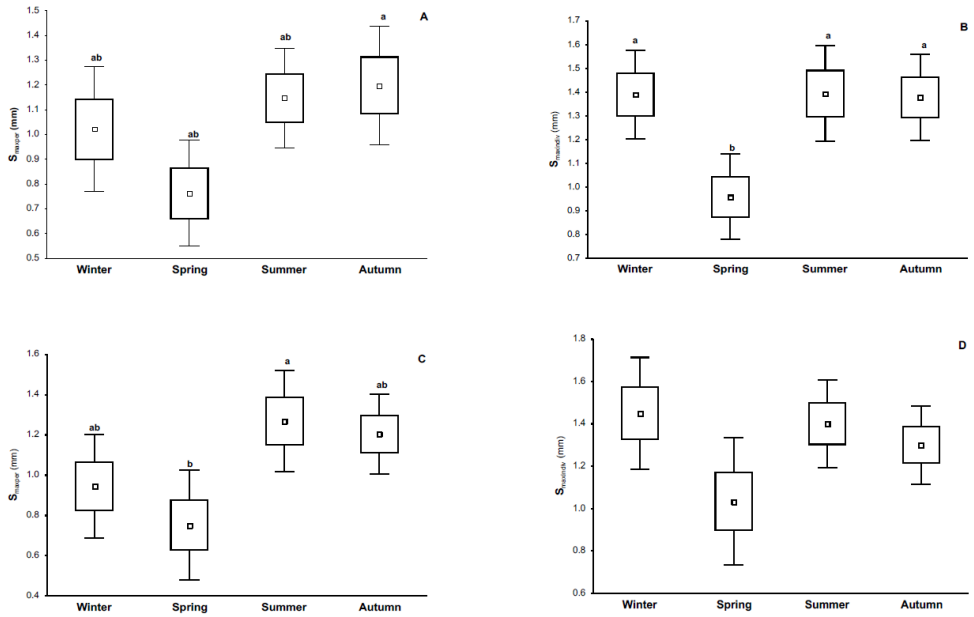

Figure 2. Swelling index 6 h after injection of phytohemagglutinin (S_{maxper}) in female (**A**) and male (**C**) fish-eating myotis in Partida Norte Island, Mexico, and maximum swelling value registered for each individual within the 3–12-h period after injection ($S_{maxindiv}$) in females (**B**) and males (**D**). Values are mean (square), standard error (box), and minimum and maximum values (whiskers). Only significant differences after factorial analysis of variance are pointed out using different letters.

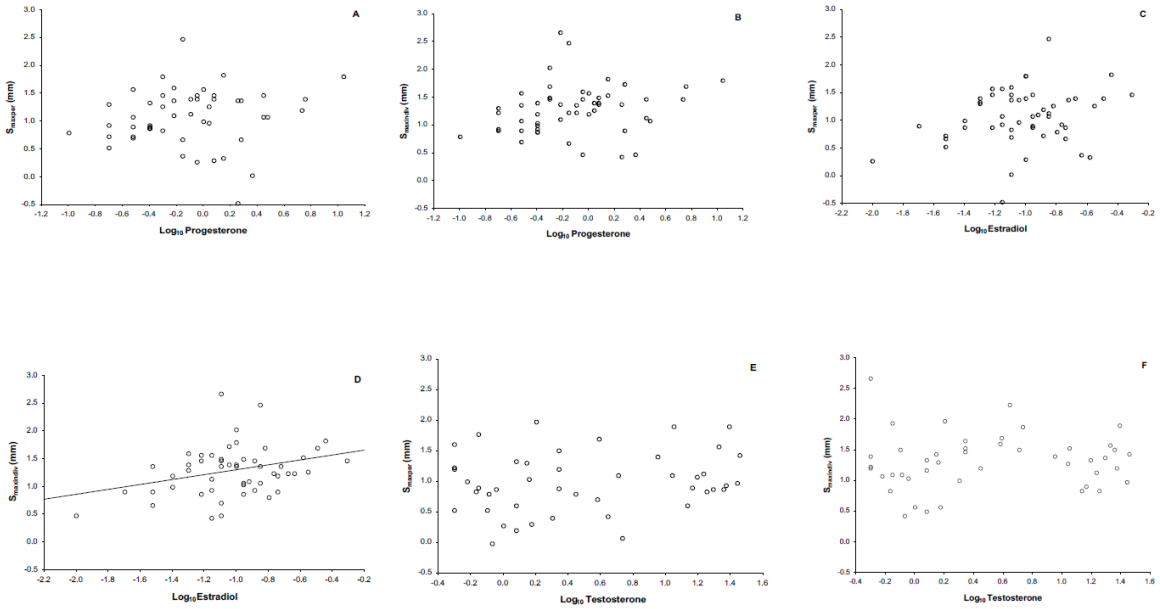

Figure 3. Pearson correlation analyses between swelling indices (S_{maxper} and $S_{maxindiv}$) and steroid sex hormone concentration in female (**A**–**D**) and male (**E**,**F**) fish-eating myotis in Partida Norte Island, Mexico. The regression line is shown only when the correlation was significant.

3.3. Bacteria Killing Ability

The BKA in females was not affected by year (χ^2_1 = 3.04, p = 0.08), but the effects of the season (χ^2_3 = 10.65, p = 0.01) and the year–season interaction (χ^2_3 = 16.89, p = 0.0007) were significant (Figure 4A): the BKA values were lower in spring (p = 0.001) and summer

($p < 0.0001$) than in winter but only in year 1 of the measurements and with no differences among seasons in year 2 ($p > 0.67$). Interestingly, females in spring and summer in year 2 had a higher BKA than in spring ($p = 0.002$) and summer ($p = 0.01$) in year 1, respectively. The BKA in males was affected by year ($\chi^2_1 = 4.72$, $p = 0.02$) but it was not affected by season ($\chi^2_3 = 2.52$, $p = 0.47$) or the year–season interaction ($\chi^2_3 = 6.62$, $p = 0.09$; Figure 4B). The BKA was not significantly correlated with body mass in females (all females: R = 0.06, $p = 0.51$, $n = 102$; lactating females: R = −0.08, $p = 0.62$, $n = 33$) or males (R = 0.12, $p = 0.31$, $n = 70$). The BKA in females ($n = 55$) was not correlated with the progesterone content (R = 0.13, $p = 0.32$; Figure 5A), but it was significantly correlated with the estradiol content (R = 0.34, $p = 0.01$; Figure 5B). The BKA in males ($n = 44$) was not significantly correlated with the testosterone content (R = −0.09, $p = 0.52$; Figure 5C).

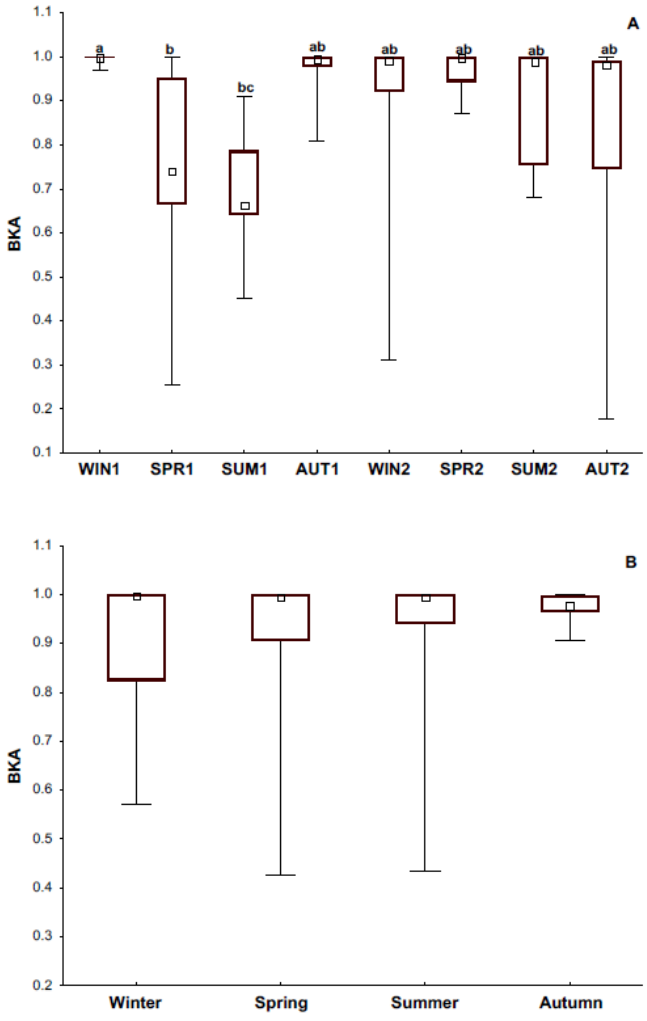

Figure 4. Bacterial killing ability (BKA) in plasma of fish-eating myotis collected for two years in Partida Norte Island, Mexico. BKA values of females (**A**) were significantly affected by the year–season interaction, but the values for males (**B**) were not. Values are median (square), interquartile range (box), and 5th and 95th percentiles (whiskers). Only significant differences after generalized linear models are pointed out using different letters.

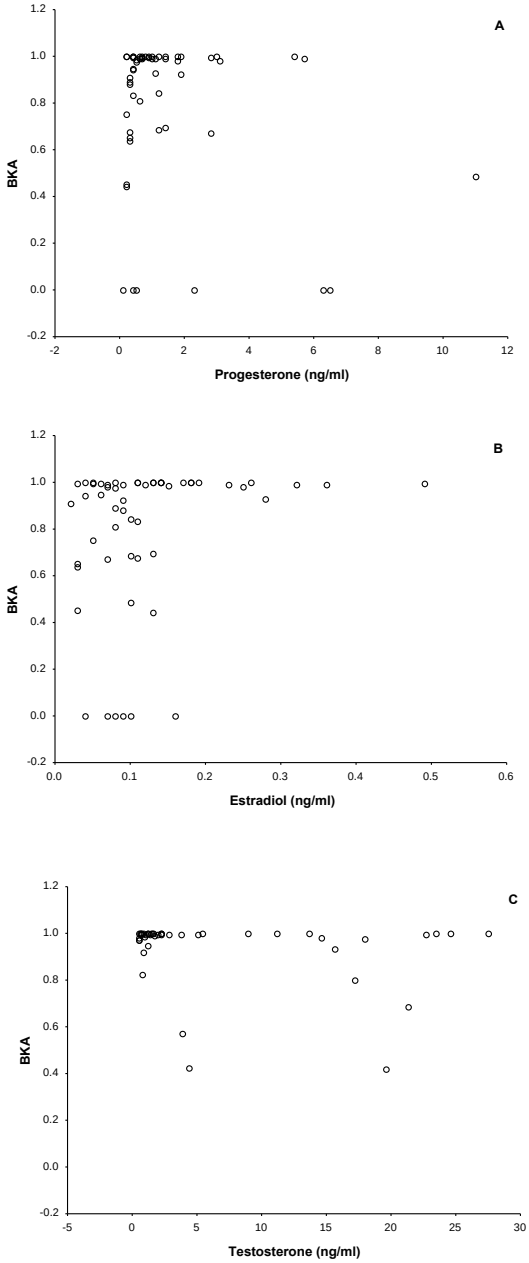

Figure 5. Spearman Rank correlation analyses between bacterial killing ability (BKA) and steroid sex hormone concentration in female (**A**,**B**) and male (**C**) fish-eating myotis in Partida Norte Island, Mexico. Only BKA and estradiol concentration were significantly correlated.

3.4. Ectoparasite Load

In total, we collected 1151 and 493 ectoparasites from 121 female and 72 male bats examined, respectively. Parasite prevalence did not vary seasonally in the female ($p = 0.39$; Table 1) or male bats ($p = 1.0$; Table 2). The mean intensity ($p = 0.07$) and abundance ($p = 0.07$) in the female bats did not vary among seasons, although both parameters showed a trend towards lower values in spring and autumn (Table 2). Neither intensity nor abundance varied significantly among seasons in males ($p = 0.25$; Table 2). Parasite abundance was not significantly correlated with the inflammation indices in females (S_{maxper}: r = 0.04, $p = 0.66$; $S_{maxindiv}$: r = 0.03, $p = 0.68$; $n = 110$; Figure 6A,B) and males (S_{maxper}: r = 0.007, $p = 0.95$; $S_{maxindiv}$: r = 0.04, $p = 0.69$; $n = 71$; Figure 6C,D). Parasite abundance was not significantly correlated with the BKA in females (R = −0.12, $p = 0.23$, $n = 108$; Figure 7A) but the correlation was significant for males (r = −0.24, $p = 0.04$, $n = 65$; Figure 7B).

Table 2. Prevalence, intensity, and abundance of ectoparasites in female and male fish-eating myotis (*Myotis vivesi*) during four seasons in Partida Norte Island, Mexico. Values are mean (95% confidence interval) and were obtained with the program Quantitative Parasitology–QPweb 1.0.13 (Reiczigel et al. 2019). We used the Clopper–Pearson method for the prevalence confidence interval and the bias-corrected and accelerated bootstrap method, with 2000 replications for intensity and abundance confidence intervals.

Bat sex	Season	Prevalence (%)	Intensity	Abundance	n
Female	Winter	96.8 (83.3–99.9)	11.9 (8.3–20.0)	11.5 (8.2–19.6)	31
	Spring	100.0 (90.5–100.0)	5.9 (4.7–7.6)	5.9 (4.7–7.6)	37
	Summer	100.0 (90.3–100.0)	11.1 (9.5–13.0)	11.1 (9.5–12.9)	36
	Autumn	100.0 (80.5–100.0)	7.7. (5.7–10.6)	7.7. (5.7–10.8)	17
Male	Winter	100.0 (85.1–100.0)	5.72 (4.0–9.4)	5.7 (4.0–9.4)	18
	Spring	100.0 (76.8–100.0)	7.57 (5.1–10.1)	7.57 (5.1–10.2)	14
	Summer	100.0 (86.3–100.0)	5.0 (3.9–6.3)	5.0 (3.8–6.2)	25
	Autumn	100.0 (78.2–100.0)	7.6 (5.5–10.3)	7.6 (5.4–10.2)	15

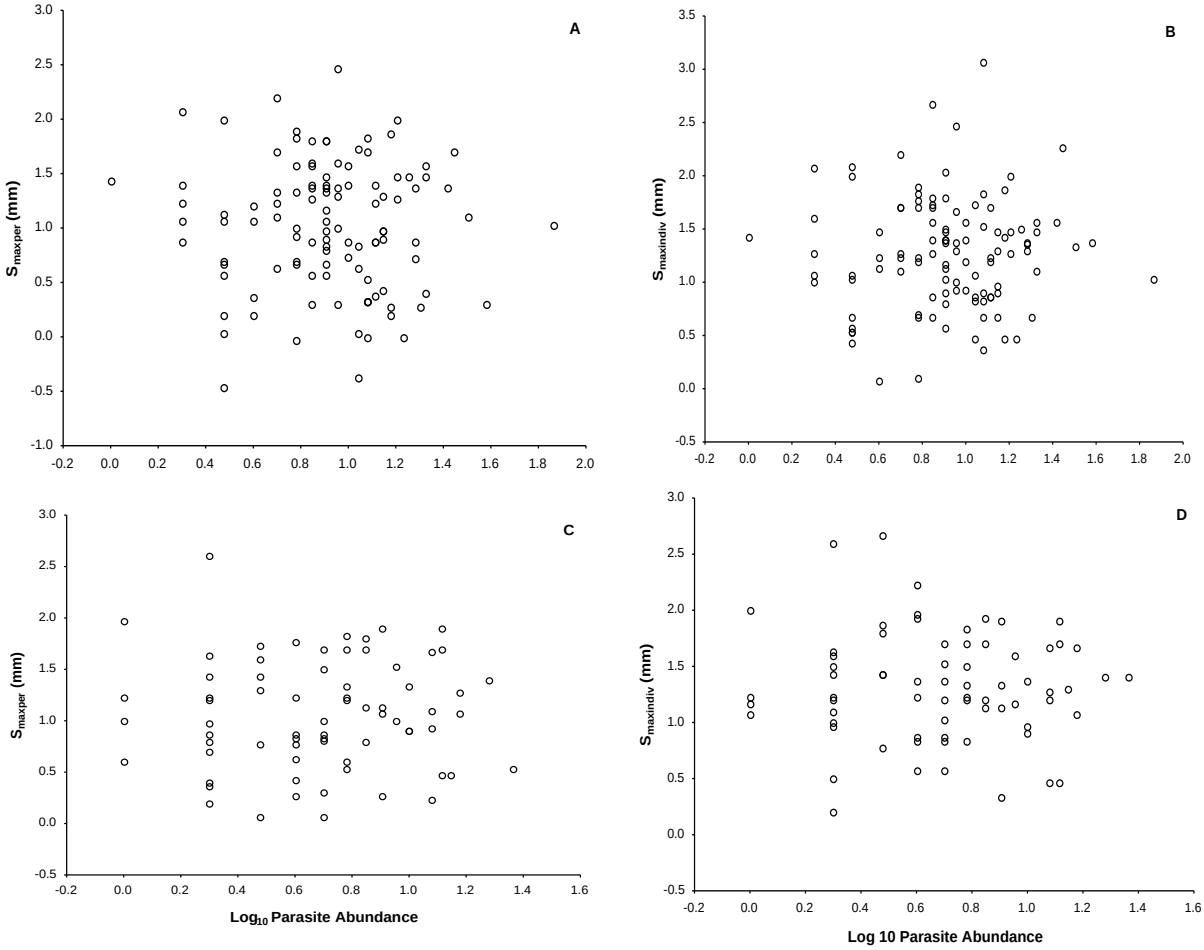

Figure 6. Pearson correlation analyses between swelling indices (S_{maxper} and $S_{maxindiv}$) and ectoparasite abundance in female (**A**,**B**) and male (**C**,**D**) fish-eating myotis bats in Partida Norte island. No correlation was significant.

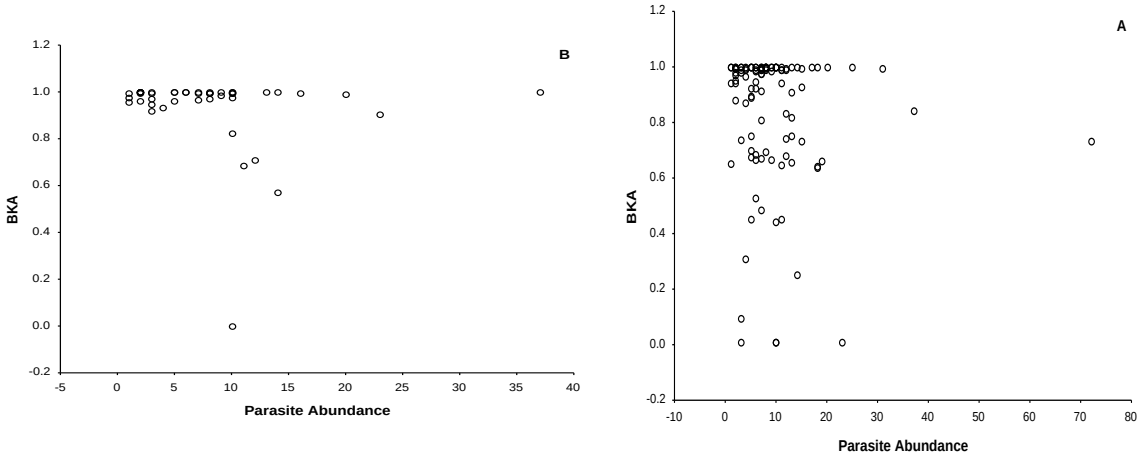

Figure 7. Spearman Rank correlation analyses between bacterial killing ability (BKA) and ectoparasite abundance in female (**A**) and male (**B**) bats in Partida Norte Island, Mexico. Parasite abundance was significantly correlated only with BKA in males.

4. Discussion

4.1. Immune Response and Its Relationship with Season and Reproductive Condition

In general, the swelling response of female and male fish-eating myotis was lower in spring. Seasonal changes in the swelling response of bats to PHA have been examined only in two species: mouse-eared bats and the fish-eating myotis. Pregnant mouse-eared bats had less swelling than non-reproductive and lactating females [27] 6 h after PHA injection, whereas in a study that did not include the pregnancy season, non-reproductive females of fish-eating myotis had a lower swelling response in early winter than lactating females and the swelling did not differ among males at the same post-injection period [53]. In spring, some females of fish-eating myotis had external evidence of pregnancy, and some did not. However, the progesterone levels of females with no external sign of pregnancy in spring were the highest recorded in our study, suggesting that the females in this period were in early pregnancy. Progesterone values are an adequate proxy for bat reproductive status; for instance, progesterone values are very low in non-reproductive females of black myotis (*M. nigricans*), peaking in early pregnancy with a slight decrease in advanced pregnancy [54]. Higher testosterone levels were measured in autumn, but the two males in which we measured the testosterone in spring had similarly high values (19.6 and 14.6 ng/mL). During spring, most males leave the colony, and the roosting groups are formed of a single male and several females [36,47]. The biological reason behind this pattern is unknown, but it is probable that the remaining males mated with several females. Unfortunately, we did not have access to more plasma samples to test if high testosterone is a general pattern for males in spring, but it might indicate territorial behavior. For instance, testosterone is higher in males of the greater sac-winged bat engaged in defending harems during mating [55]. All things considered, it appears that the cellular innate response is downregulated in spring when females of fish-eating myotis are pregnant and when males roosting with several females are probably involved in maintaining their territory.

The BKA varied seasonally only in female fish-eating myotis depending on the year examined. The BKA in year 1 was lower in spring and summer than in winter and that in spring and summer in year 2. Females collected in spring in year 1 were probably in early pregnancy, whereas females in year 2 were in a more advanced pregnancy stage. Therefore, it appears that the pregnancy stage might influence the BKA in fish-eating myotis similar to the effect on other immune responses in other bats [27]. The females in summer in year 1 were all lactating, and in year 2, they were either lactating or not. However, the lactating females in year 1 had a lower BKA than the lactating (median = 0.91) and non-lactating females (median = 0.98) in year 2, which indicates that the interannual difference was not related to differences in reproductive activity. Only one study previously examined the

relationship between the BKA and reproduction in bats, reporting no differences among the reproductive categories in male (non-reproductive and reproductive) and female (non-reproductive, gestating, lactating, and post-lactating) Daubenton's bats [31].

4.2. Immune Response and Its Relationship with Parasite Load and Body Mass

We found no significant correlation between the ectoparasite load and the swelling indices in fishing bats, but body mass appeared to play a role in the extent of inflammation. Previous work with pregnant mouse-eared bats showed that the swelling response of females was associated with the ectoparasite load and body condition. Females in early pregnancy had a lower swelling index and higher ectoparasite abundance than females in advanced pregnancy and lactation, and the swelling index of lactating females increased as the body mass increased [27]. In contrast, PHA-induced inflammation was not related to the body condition of females of Brazilian free-tailed bats (*Tadarida brasiliensis*) at four maternity roosts [51]. The lack of a relationship between the swelling and the parasite load in fish-eating myotis is not surprising given that the ectoparasite abundance was relatively stable throughout the different seasons in both females and males. In contrast to other colonial bats, fish-eating myotis roost under rockslides in groups of few individuals throughout the year (3–5 individuals per roost; 36), which probably helps to maintain ectoparasite transmission among individual bats at the same rate among seasons. The swelling increased as the body mass (and body condition) of male fish-eating myotis increased, indicating that bats in a better nutritional condition responded more strongly to the PHA challenge. Additional evidence supporting this scenario is given by the fact that the lowest inflammation response was found in spring, when males had the lowest body mass. Interestingly, the energy cost of inflammation measured for the fish-eating myotis is null: the resting metabolic rate and body mass of the bats injected with PHA were not significantly different from those of the bats injected with a control substance [56]. No detectable energetic cost of PHA-induced inflammation has been found in other mammals, suggesting that the cost might be expressed in other terms [57] that are probably related to body mass and/or body condition.

The BKA of female fish-eating myotis was not significantly related to the ectoparasite load or body mass. In contrast, although it was not related to body mass, the BKA was inversely related to the ectoparasite load: bats with lower parasite abundance had a higher BKA. Findings in other bats show no relationship of BKA with body mass (Daubenton's bat; 28), body condition (Greater sac-winged bat; 29), or ectoparasite load (Daubenton's bat; 28). However, parasites might play a role in the magnitude of the BKA, although not in the same direction found with male fish-eating myotis. For instance, the BKA is higher in greater sac-winged bats with trypanosome infections than in uninfected individuals and tends to be higher in bats infected with nematodes [29].

4.3. Immune Response and Its Relationship with Steroid Sex Hormones

A significant correlation between female sex hormones and the immune response was found only with estradiol: females with higher estradiol levels had a higher $S_{maxindiv}$ and BKA. Neither progesterone in females nor testosterone in males had a significant association with any index of the immune response. In general, the correlation of testosterone with male immune performance is negative, indicating an immunosuppressive effect of this hormone [8]. However, an increasing number of publications indicate that the immune response rather depresses testosterone [30,46,58]. Previous studies with other taxa have found contrasting relationships between testosterone levels and PHA-induced swelling and BKA. The swelling response to PHA increased with the baseline values of testosterone in free-ranging tree sparrows (*Passer montanus*; [59]), but an experimental treatment with testosterone decreased the swelling response in common wall lizards (*Podarcis muralis*; [60]), dark-eyed juncos (*Junco hyemalis*; [61]), and European starlings (*Sturnus vulgaris*; [62]), but not in house sparrows (*P. domesticus*; [63]). Testosterone had a positive correlation with the BKA in free-living Grant´s gazelle (*Nanger granti*; [64]), but this relationship was negative

in red-winged blackbirds (*Agelaius phoeniceus*; [65]) and in American alligators (*Alligator mississippiensis*; [66]). Among bats, the relationship between testosterone levels and the immune response has been examined in a handful of studies, but with different immune components from those included in our study. For instance, there was no significant relationship between testosterone levels and the dinitrofluorobenzene-induced swelling response in Seba's short-tailed bat [46], nor with lytic and agglutination immune responses in big brown bats (*Eptesicus fuscus*; [30]). Furthermore, greater sac-winged bats showed no correlation between testosterone levels and parasite infestation [55].

In contrast with testosterone, most studies on the relationships between immune response and progesterone and estradiol levels have been conducted with humans and laboratory animal models. Both positive and negative effects on immune function have been reported for estradiol depending on the immune function examined [8]. For instance, estradiol administration enhanced the number of IgG- and IgM- producing cells [67] and the level of IgG [68] in mice. It increased IgG plasma levels in zebra fish (*Dario rerio*; [69]), and it augmented lymphocyte proliferation following PHA administration in Siberian hamsters (*Phodopus sungorus*; [70]). In contrast, estradiol administration depressed the swelling response [68,71] and the production of interleukin-6 in mice [71]. Recent evidence in humans also indicates a protective effect of estradiol against coronavirus disease 2019 [72]. Progesterone can have both stimulatory and suppressive effects on the immune system but is typically regarded as immunosuppressive and anti-inflammatory [12], especially when compared to estradiol. For instance, rather than progesterone, estradiol enhanced immunity during malaria infection in mice [73]. In rats, estradiol augmented IgG and IgA production, whereas progesterone reduced IgA production [74], and estradiol elevated circulating levels of key inflammatory mediators after an in vivo endotoxin challenge in mice [75]. Our findings of a positive correlation of inflammation and BKA with estradiol but not with progesterone are in line with the preponderant role of estradiol as an enhancer of the immune response found in model animals.

5. Concluding Remarks

Similar to other bats in more mesic environments, the cellular and humoral innate immune responses of fish-eating myotis varied throughout the year, following the seasonal reproductive pattern of the species. Our evaluation of the proximal factors affecting the expression of the immune response is not conclusive since we could not measure all variables simultaneously from all individuals, but it points to the potential immunoregulatory role of sex hormones and body mass. With a few exceptions [41,42], eco-immunological studies have largely ignored bats in arid zones. Arid environments are limited in food and water availability, and the health of bats that live in these zones is highly vulnerable to anthropogenic impacts [41,42]. For instance, its unique marine-based feeding habits expose fish-eating myotis to the presence of heavy metals in the food chain [35], which might, in turn, affect their immune response [76]. Further research on the immune ecology of insular desert bats is warranted, as they are particularly vulnerable to anthropogenic environmental changes [77].

Author Contributions: Conceptualization, A.O.-A. and L.G.H.M.; Methodology, A.O.-A., J.J.F.-M., C.R., A.S.-M. and L.G.H.M.; Formal Analysis, A.O.-A. and L.G.H.M.; Investigation, A.O.-A., J.J.F.-M., C.R., A.S.-M. and L.G.H.M.; Resources, J.J.F.-M. and L.G.H.M.; Data Curation, A.O.-A. and L.G.H.M.; Writing—Original Draft Preparation, A.O.-A. and L.G.H.M.; Writing—Review and Editing, J.J.F.-M., C.R., and A.S.-M.; Supervision, L.G.H.M.; Project Administration, L.G.H.M.; Funding Acquisition, A.S.-M. and L.G.H.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Dirección General de Asuntos del Personal Académico [PAPIIT # IN202113] to LGHM, and by the Division of Biological and Health Sciences in UAM-I (DCBS-144.03.07) to ASM. AOA acknowledges the scholarship provided by the National Council of Science and Technology, and the support of the Posgrado en Ciencias Biológicas UNAM.

Institutional Review Board Statement: This study was carried out in strict accordance with the recommendations and permits approved by the Secretaría de Gobernación (#013/13 and #052/13) and from the Dirección General de Vida Silvestre (2082/12 and 1947/13), Mexico.

Data Availability Statement: The data presented in this study are available on request to the corresponding author.

Acknowledgments: Transport to Partida Norte Island was generously provided by the Secretaría de Marina-Armada de México. The Prescott College Kino Bay Center and the Área de Protección de Flora y Fauna Islas del Golfo de California-Baja California provided invaluable logistic support.

Conflicts of Interest: The authors declare no conflict of interest.

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