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RESEARCH ARTICLE

## Constitutive innate immunity of tropical House Wrens varies with season and reproductive activity

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### ABSTRACT

In lowland Neotropical regions, where air temperature and day length remain relatively constant year round, seasonality is determined primarily by changes in rainfall. The wet season triggers the start of breeding for many Neotropical birds but also alters the antigenic environment, likely increasing the risk of disease transmission. We explored 2 hypotheses about temporal variation in constitutive innate immunity of a Neotropical bird, the House Wren (*Troglodytes aedon*). The antigen response hypothesis proposes that Neotropical wrens upregulate their immune function in the wet season either in anticipation of or in response to vectors that become more prevalent. The resource constraint hypothesis proposes that during periods of putative high resource demand, such as when parents are feeding young, immune function should be compromised and downregulated. Controlling for reproductive stage, we found that microbicidal capacity of blood against *Escherichia coli* was higher in the wet than the dry season, consistent with the antigen response hypothesis. Phagocytosis of *E. coli* and *Staphylococcus aureus* did not differ between wet and dry seasons. Microbicidal capacity and H/L ratio of tropical House Wrens did not vary among reproductive stages, and our data offered no support for the idea that immune function is compromised during the period when parents are feeding young.

**Keywords:** birds, microbicidal capacity of blood, phagocytosis, seasonality, tropics

### La inmunidad innata constitutiva de *Troglodytes aedon* varía con la estación y la actividad reproductiva

### RESUMEN

En las regiones neotropicales bajas, donde la temperatura del aire y la duración del día permanecen relativamente constantes a lo largo de todo el año, la estacionalidad está determinada principalmente por cambios en la precipitación. La estación húmeda desencadena el inicio de la época de cría para muchas aves neotropicales, pero también altera el ambiente antigénico, probablemente aumentando el riesgo de transmisión de enfermedades. Evaluamos dos hipótesis sobre la variación temporal en la inmunidad innata constitutiva de un ave neotropical, *Troglodytes aedon*. La hipótesis de respuesta antigénica propone que *T. aedon* regula hacia arriba su función inmune en la estación húmeda ya sea en anticipación o en respuesta a los vectores que se vuelven más prevalentes. La hipótesis de restricción de recursos propone que durante los períodos de supuesta alta demanda de recursos, como cuando los progenitores están alimentando a los juveniles, la función inmune debería verse comprometida y regulada hacia abajo. Una vez que controlamos por el estadio reproductivo, encontramos que la capacidad microbicida de la sangre en contra de *Escherichia coli* fue más alta en la estación húmeda que en la estación seca, apoyando la hipótesis de respuesta antigénica. La fagocitosis de *E. coli* y *Staphylococcus aureus* no difirió entre las estaciones húmeda y seca. La capacidad microbicida y la relación H/L de *T. aedon* no varió entre los estadios reproductivos, y nuestros datos no apoyaron la idea de que la función inmune esté comprometida durante el periodo cuando los progenitores están alimentando a los juveniles.

**Palabras clave:** ave, capacidad microbicida de la sangre, estacionalidad, fagocitosis, trópicos

### INTRODUCTION

The field of ecological immunity has burgeoned in the last decades providing us with information on the operation of the vertebrate immune system in natural environments. Some studies have indicated that the immune functioning

of birds and mammals is suppressed during putative periods of low food availability or high resource demand, such as cold winters (Dowell 2001, Nelson et al. 2002, Gasparini et al. 2006), or when parents are caring for young (e.g., Richner et al. 1995, Sheldon and Verhulst 1996; for reviews, see Knowles et al. 2009, Tieleman 2018) or molting

(Moreno-Rueda 2010, Merrill et al. 2015). An alternative hypothesis is that the pathogenic pressure of the environment shapes immune systems (Moyer et al. 2002, Horrocks et al. 2011, 2012a; Tieleman 2018). For example, humid regions may pose a higher risk of infection by endoparasites and ectoparasites than arid or semi-arid environments (Moyer et al. 2002, Valera et al. 2003, Froeschke et al. 2010). Likewise, seasonal changes in environmental conditions can have associated changes in risk of infection (Horrocks et al. 2011, 2012b). For example, in tropical environments, prevalence of avian malaria (Hernández-Lara et al. 2017) and bacterial infections (Pascual et al. 2002, Desvars et al. 2013) are higher in the rainy season. Moreover, vectors transmitting diseases, such as mosquitos, increase in the rainy season, likely leading to more infections (Altizer et al. 2006). Because pathogen populations likely vary through the annual cycle, it is conceivable that host populations could evolve a programmed ramping of their immune system prior to periods when pathogens are most common (Nelson et al. 2002, Horrocks et al. 2011). For tropical birds that experience wet and dry seasons, one might hypothesize that they are exposed to more pathogens during the wet season than during the dry season (Patz et al. 2000). This may suggest that tropical birds ought to elevate their immune function during the wet season in anticipation of encountering more disease vectors during this season.

Workload differs between nonbreeding and breeding animals (Bryant and Westerterp 1980, Anava et al. 2002, Hambly et al. 2007). Also within the breeding season, specific stages of reproduction require elevated energy expenditure, such as when parents are feeding young. This may create a situation of negative energy or nutrient balance (Nelson et al. 2002, Tulp et al. 2002, Bourgeon et al. 2010). Reputedly, the resulting resource trade-off between self-maintenance and reproduction can compromise immune function, if maintenance of the immune system and its upregulation have energetic or nutritional costs (Sheldon and Verhulst 1996, Martin et al. 2003, Schmid-Hempel 2003, Klasing 2004). In some studies, lactating mammals and parent birds that have high feeding frequencies show compromised immune function and increased parasite burdens, putatively as a result of resource reallocation into current reproductive effort and away from the immune system, while other studies do not find support for such an immunity cost of reproduction (Nordling et al. 1998, Knowles et al. 2009, Evans et al. 2015, Ibañez et al. 2018, Tieleman 2018).

Studies comparing variation in immunity with season or workload in species native to the tropics are relatively rare (but see Tieleman et al. 2008, Herrera et al. 2016, Ndithia et al. 2017). In many tropical areas, ambient temperature and food resources vary less throughout the year than in temperate areas, making seasonal food scarcity an unlikely

factor in patterns of immune function in birds that live there. In some tropical regions, rainfall varies considerably within the year. In the Panamanian Neotropics there is a clear distinction between the rainy season and the dry season and, in a number of species, breeding occurs in both seasons (Freed 1987, Tieleman et al. 2008). This provides the opportunity to uncouple rainfall and reproductive stage in order to determine the independent influences of workload and environment on the immune system.

Because the innate arm of the immune system is complex, any one measure of its capacity is likely to fall short of adequately describing it. Different immune measures often show opposing patterns or no patterns at all. In this study we have adopted multiple assays that focus on different components of the constitutive innate immune system. The microbial-killing (Tieleman et al. 2005, Millet et al. 2007) and phagocytosis assays (Millet et al. 2007) that we use here provide a relatively integrative and quantitative view of innate immune function in birds. These assays have been useful in predicting susceptibility of humans to a variety of bacterial infections and thus are related to the ability to combat disease (Keusch et al. 1975).

*Escherichia coli* is a gram-negative bacterium, mainly killed by humoral components of the immune system (Matson et al. 2006b, Millet et al. 2007). *Staphylococcus aureus* is a gram-positive bacterium, and *Candida albicans* is a yeast-like fungus, both mainly killed by the cellular parts of the immune system (Davies et al. 1999, Matson et al. 2006b). Moreover, these are all common microorganisms that birds are likely to encounter in nature (Millet et al. 2007). By integrating immune components present in the cellular and plasma fractions of whole blood, our microorganism-killing and phagocytosis assays take a functional approach, providing a measure of blood's ability to act against pathogens (Tieleman et al. 2005, Matson et al. 2006a, 2006b; Millet et al. 2007). Additionally, we measured the ratio between heterophils and lymphocytes (H/L), a measure commonly used as an immunological indicator of stress (Davis et al. 2008).

House Wrens (*Troglodytes aedon*) provide a model system for examining connections among life history, physiology, and environment. Populations of nonmigratory Neotropical House Wrens have small clutch sizes of 3–4 eggs and long incubation periods of 15–18 days. In this paper, we explored 2 hypotheses. By comparing immune function of wrens in the dry and wet seasons, we tested the hypothesis that House Wrens have an enhanced immune function during the wet season in anticipation of a period when bacterial vectors are more prevalent. In addition, because periods of high resource demand are thought to compromise immune function, we predicted that parents during the nestling phase would have lower microbial-killing ability compared with birds during nonbreeding

or incubation phases (Moreno et al. 2001). H/L ratio is predicted to be higher in stressful periods. On the one hand, we could argue that in the dry season recourses are limited, which could lead to energetic stress. On the other hand we predict that pathogens are more abundant in the rainy season, which could lead to immunological stress. Thus, the antigen response and resource constraint hypotheses make contrasting predictions regarding seasonal patterns in H/L ratios.

## MATERIAL AND METHODS

We conducted our study during March–July 2004 and May–June 2005, in Gamboa and Summit Botanical Gardens, Republic of Panama (9°N, 79°W; Walker 2007). Both sites are surrounded by humid lowland tropical forests with average annual temperature ( $T_a$ ) of 25°C and a rainy season from late April until December. Although day length is relatively constant in the tropics, this region experiences a wet and dry season. In 2004, the wet season began on April 29, and in 2005 on May 6 (Smithsonian Tropical Research Institute, Environmental Science Program).

We recorded whether pairs of House Wrens were breeding or nonbreeding. For breeding pairs, we distinguished 2 stages: incubation or feeding young. Wrens ( $n = 55$ ) were captured using mist nets placed close to nests or within their territory. At capture we recorded mass ( $\pm 0.1$ g) using a Pesola scale (calibrated against a Mettler balance). We collected a 100- $\mu$ L blood sample in sterile heparinized hematocrit tubes by puncturing the brachial vein with a sterile needle. Prior to collecting blood, we wiped the under-wing with alcohol and allowed the skin to dry. Blood samples were collected within 5 min of capture to minimize the effect of elevated corticosterone levels upon the immune system (Matson et al. 2006b, Millet et al. 2007). We sealed hematocrit tubes with clay and transported them back to the lab. We began our assays within 60 min of extracting blood.

### Microbicidal Assay

We assessed the capacity of fresh whole blood of wrens to kill *E. coli* (ATCC #8739; MicroBioLogics, St. Cloud, Minnesota, USA) during the 2004 and 2005 field seasons. In 2005, we also incorporated assays using *S. aureus* (ATCC #6538; MicroBioLogics) and *C. albicans* (ATCC #10231; MicroBioLogics). For detailed descriptions of the assays and procedures see Tieleman et al. (2005) and Millet et al. (2007). In short, we mixed 20  $\mu$ L blood and 180  $\mu$ L CO<sub>2</sub>-independent medium, and mixed this with the microbial suspensions. We modified concentrations of all microbial suspensions to yield approximately 150–200 colonies per 75  $\mu$ L of diluted blood sample. We incubated the blood–*E. coli* suspension for 30 min, the blood–*S. aureus*

suspension for 180 min, and the blood–*C. albicans* suspension for 60 min at 41°C. We plated the blood–microorganism suspension on agar plates. For each session we made 1–5 controls by plating microorganism–media suspensions without House Wren blood on agar plates. We took the average number of CFU on control plates if  $N > 1$ . All agar plates were incubated until CFU were visible. Antimicrobial activity was calculated as the percentage of microorganisms killed. Data for killing of *S. aureus* and *C. albicans* were only taken during the wet season, thus in our comparisons for wet and dry season we only used *E. coli*.

### Phagocytosis Assay

We conducted phagocytosis assays in a sterile laminar flow hood. We diluted blood 1:20 in sterile CO<sub>2</sub>-free medium with 5% fetal calf serum and 1% penicillin–streptomycin solution. We added 66  $\mu$ L of blood solution to each well on a chamber slide (Millet et al. 2007). Fluorescently labeled dead bacteria (at 20 mg mL<sup>-1</sup>; Molecular Probes, Eugene, Oregon, USA) were reconstituted in PBS with 2 mM sodium azide, the latter to inhibit any bacterial growth. In each of 4 wells we added 250  $\mu$ L of fluorescently labeled *E. coli* (E-2864) solution and in 4 other wells *S. aureus* (S-2854). We then placed the slide in an incubator for 15 min at 41°C. Immediately after incubation, we removed the slide and placed it on ice, ending phagocytosis. The slide wells were washed with medium and adhering cells (primarily monocytes) were fixed on the slide. We used a fluorescent microscope to count a minimum of 100 white blood cells per slide. For each white blood cell, we recorded the presence or absence of fluorescent bacteria inside. Additionally, we did counts of different macrophage white blood cell types (cell counts are provided in Appendix Table 3).

### Heterophil-to-Lymphocyte Ratio

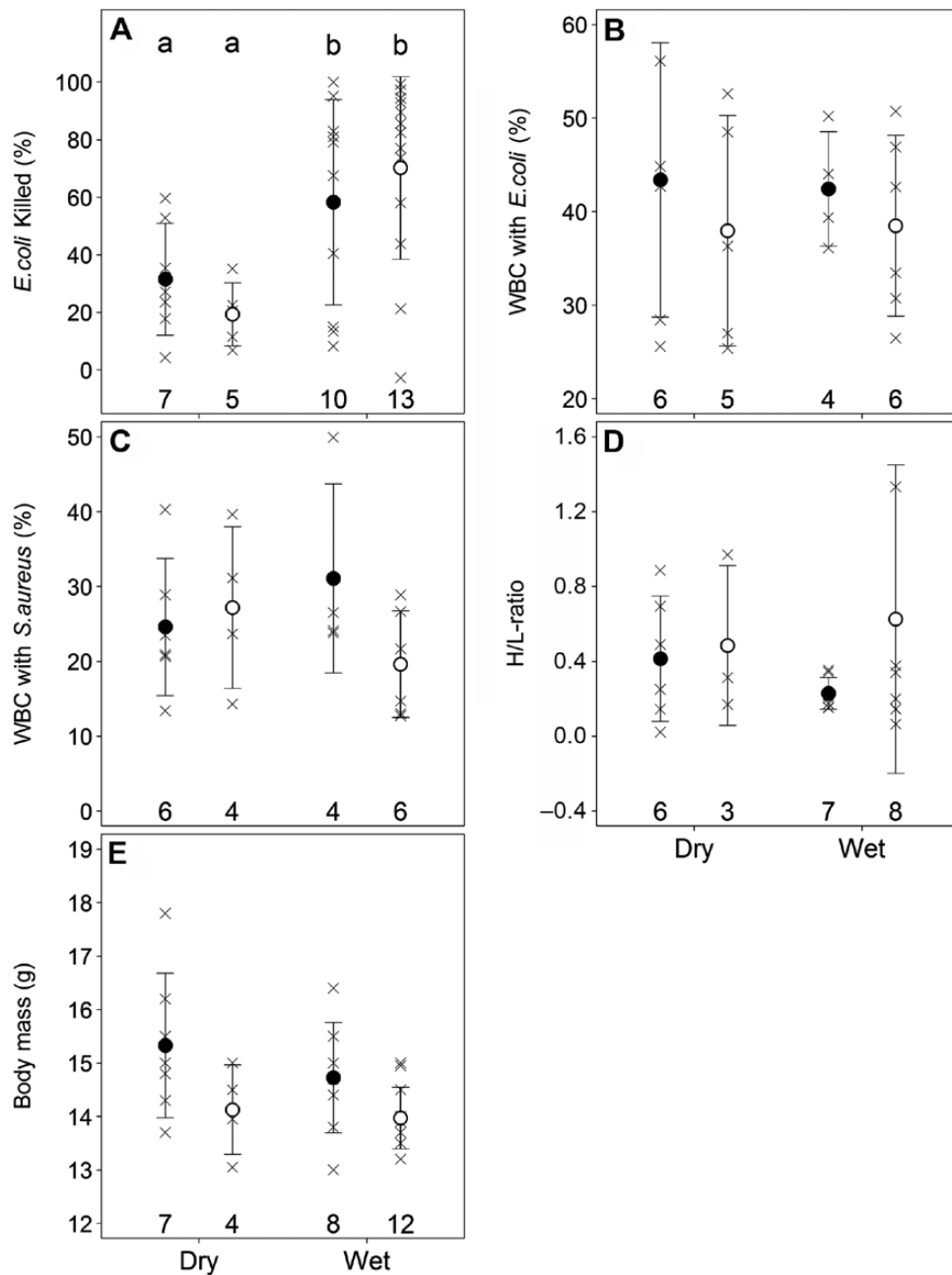
To count heterophils and lymphocytes, we prepared blood smears, air-dried them, and fixed them in methanol. We stained the slides using Wright-Giemsa stain (Bennett 1970). On each slide, we counted 100 white blood cells, recording the number of heterophils, and lymphocytes. We calculated H/L ratio as (number of heterophils)/(number of lymphocytes). Raw cell counts are provided (Appendix Table 3).

### STATISTICAL ANALYSIS

Body mass was normally distributed (Shapiro test:  $W = 0.98$ ,  $P = 0.91$ ) and was analyzed using linear regression. Microbicidal capacity, phagocytosis, and H/L ratio were analyzed with a beta regression for proportions. Additionally we performed nonparametric Kruskal-Wallis tests on variables that were not normally distributed, with

main effects stage and season. The results of these analyses did not qualitatively differ from beta regression, and we therefore do not report them. Tukey post hoc tests for the linear models, and pairwise contrasts with Tukey adjustment for the beta regression analyses, were used to determine significance for specific group means. Significant contrasts ( $P < 0.05$ ) found with post hoc tests are shown with letters in the figures. We used Program R (R Development Core Team 2010) for all statistical analyses.

Because we collected data on incubating House Wrens only during the wet season we analyzed the data in 2 separate sets. For the first set of analyses we selected nonbreeding and nestling-feeding House Wrens in the wet and dry season (thus excluding incubating birds). This dataset was used to investigate the effects of season, stage (nonbreeding or nestling-feeding), and their interaction on microbicidal ability against *E. coli*, phagocytosis of *E. coli* and *S. aureus*, H/L ratio, and body mass. For the second set



**FIGURE 1.** (A) Microbicidal capacity against *E. coli*, (B) phagocytosis against *E. coli* and (C) *S. aureus*, (D) H/L ratio, and (E) body mass of (filled circles) nonbreeding birds and (open circles) nestling-feeding House Wrens during dry and wet seasons in Panama. Values are presented as means  $\pm$  SD, individual data points are gray crosses. Numbers refer to sample sizes. Letters indicate significant differences.

**TABLE 1.** Results of the linear model and the beta regression models analyzing immune indices and body mass of House Wrens in Panama. Models included season (wet, dry), reproductive stage (nestling-feeding, nonbreeding), and the covariates sex and year. *P* values are given after Bonferroni correction for multiple comparisons.

df	Microbicidal capacity		Phagocytosis		<i>S. aureus</i> <sup>a</sup>		H/L ratio		Body mass	
	<i>E. coli</i>		<i>E. coli</i> <sup>a</sup>		<i>S. aureus</i> <sup>a</sup>		H/L ratio		Body mass	
	$\chi^2$	<i>P</i>	$\chi^2$	<i>P</i>	$\chi^2$	<i>P</i>	$\chi^2$	<i>P</i>	<i>F</i>	<i>P</i>
Season*stage	1	1.00	0.02	1.00	2.76	0.40	0.46	1.00	0.52	1.00
Season	1	0.003	0.00	1.00	0.01	1.00	0.20	1.00	1.24	1.00
Stage	1	0.55	1.05	1.00	1.12	1.00	1.72	0.76	6.93	0.054
Sex	1	2.45	0.00	1.00	0.35	1.00	1.71	0.76	1.51	0.92
Year	1	1.19	—	—	—	—	1.52	0.44	0.18	1.00

<sup>a</sup> Only measured in a single year.

of analyses we selected only House Wrens in the wet season, and investigated the effect of the 3 stages (nonbreeding, incubation, and nestling-feeding) on microbicidal ability against *E. coli* and *S. aureus*, *C. albicans*, H/L ratio, and body mass. In both datasets we included year and sex.

We deleted terms with backward elimination, always leaving stage and (if appropriate) season in the model. The null hypothesis was rejected at  $P < 0.05$ , with a Bonferroni correction for multiple comparisons. Because of the recent discussions about the best methods to analyze data (i.e. information theory vs. hypothesis-testing approach; Guthery et al. 2005, Grueber et al. 2011), we additionally analyzed the data using an information criterion approach, but this yielded qualitatively similar results, and we do not provide details of these analyses.

## RESULTS

### Effects of Season and Reproductive Stage

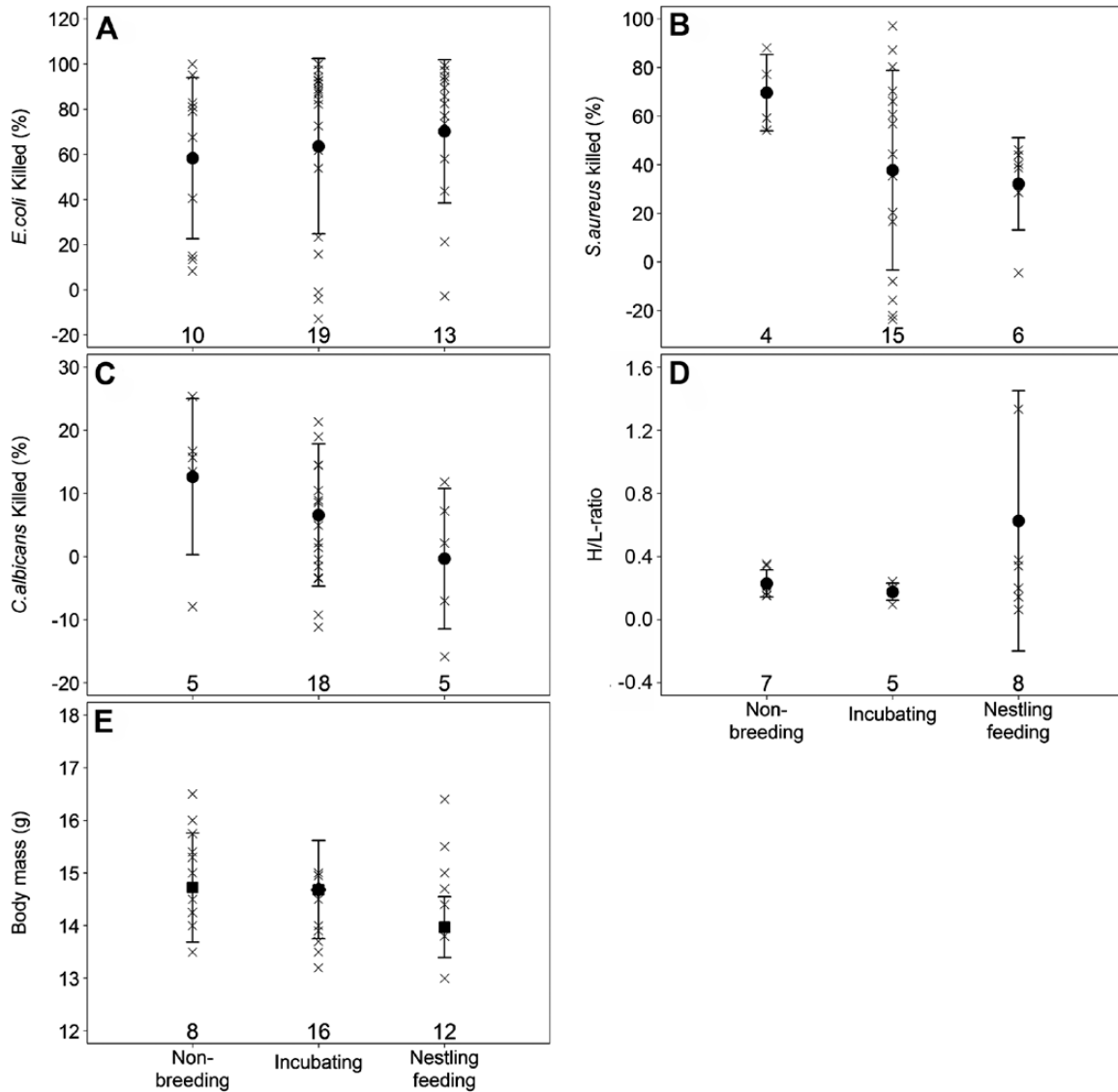
Controlling for reproductive stage, House Wrens in the wet season had a significantly higher microbicidal ability against *E. coli* than those in the dry season (Figure 1A, Table 1). House Wrens did not differ in any of the other variables between dry and wet season (Figure 1B–E, Table 1).

### Effect of Breeding Stage within the Wet Season

In the dataset of the wet season, which included incubation, we found no significant differences between stages (Figure 2, Table 2).

## DISCUSSION

Quantifying various measures of the constitutive innate immune system of Neotropical House Wrens, we found some but limited variation in immunity with season and none with reproductive stage. Our results partly supported the hypothesis that birds in the wet season have an elevated immune system. Blood taken from wrens during the wet season killed significantly more *E. coli* cells than blood taken during the dry season, but phagocytosis did not differ between seasons. Killing of *E. coli* can for a large part be attributed to proteins in the plasma (e.g., complement) and not to cellular parts of the innate immune system (Matson et al. 2006b, Millet et al. 2007). Similar variation in plasma vs. cellular parts of immunity has been found in birds after exercise (Nebel et al. 2012). Although maintenance of the constitutive innate immune system in general is likely to be not very costly, differences in costs between the cellular and humoral immunity could underlie the variation in effects found between killing of *E. coli* and phagocytosis (Klasing 2004, Lee 2006). Our second hypothesis was that, when parents were feeding young, they would display a lower immune function as a result of reallocation of resources



**FIGURE 2.** (A) Microbicidal capacity against *E. coli*, (B) *S. aureus*, and (C) *C. albicans*, (D) H/L ratio, and (E) body mass of House Wrens during nonbreeding, incubation, and chick-feeding in the wet season in Panama. Values are presented as means  $\pm$  SD, individual data points are gray crosses. Numbers refer to sample sizes.

**TABLE 2.** Results of the linear model and the beta regression models analyzing immune indices and body mass of House Wrens in Panama during the wet season. Models included stage (nonbreeding, incubation, nestling-feeding), and the covariates sex and year. Microbicidal abilities against *S. aureus* and *C. albicans* were only measured in one year. *P* values are given after Bonferroni correction for multiple comparisons.

	df	Microbicidal capacity									
		<i>E. coli</i>		<i>S. aureus</i> <sup>a</sup>		<i>C. albicans</i> <sup>a</sup>		H/L ratio		Body mass	
		$\chi^2$	<i>P</i>	$\chi^2$	<i>P</i>	$\chi^2$	<i>P</i>	$\chi^2$	<i>P</i>	<i>F</i>	<i>P</i>
Stage	2	0.54	1.00	1.20	1.00	2.89	0.95	2.53	1.00	3.5305	0.16
Sex	1	0.98	1.00	0.19	1.00	0.05	1.00	1.58	0.83	5.4663	0.10
Year	1	0.23	1.00	–	–	–	–	1.83	0.35	0.5452	0.93

<sup>a</sup> Only measured in a single year.

to parental care. This hypothesis was not supported. The immune indices did not differ between breeding stages. In addition, House Wrens did not differ in H/L ratio and body mass between the different stages, suggesting there is no variation in energetic constraints within the wet season.

Seasonal changes in immune function can be driven by predictable stressors, which in temperate regions may be anticipated by monitoring photoperiod (Nelson and Demas 1996). In tropical regions, changes in photoperiod are slight, so tropical birds may use other environmental cues that vary with season, such as rainfall, to time preparatory adjustments in immune function, as they are known to do with reproduction (Dawson et al. 2001, Scheuerlein and Gwinner 2002, Hau et al. 2008, de Araujo et al. 2017). We propose that the immune system of tropical House Wrens has been shaped by natural selection to upregulate components of the constitutive innate arm during the wet season, when pathogen abundance is likely to be higher. In support of this idea, incidences of vector- and waterborne tropical diseases, such as malaria, dengue fever, and cholera, typically exhibit peak rates during the early rainy season (Pascual et al. 2002, Altizer et al. 2006). Moreover, prevalence of blood parasites was highest during the wet season, in birds from Jamaica and Costa Rica (Bennett et al. 1980, Young et al. 1993, Sebaio et al. 2012). Experiments are needed to provide insight into whether the patterns we observed in upregulation of bactericidal competence reflect anticipation of, or response to, the transition between seasons. Maintenance of constitutive innate immunity components during periods of increased energetic demand suggests that food resources are not limiting energy intake during our study or that constitutive innate defensive systems are not especially costly. Our study also stresses that multiple assays should be used in studies of the immune system to gain a more integrated understanding of mechanisms shaping variation in immune function.

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**Author contributions:** B.I.T., J.B.W., and K.K. conceived the idea, design, and experiment (supervised research, formulated question or hypothesis). B.I.T. and J.B.W. performed the experiments (collected data, conducted the research). B.I.T. and M.A.V. wrote the paper. B.I.T. and K.K. developed or designed the methods. M.A.V. analyzed the data. K.K. and J.B.W. contributed substantial materials, resources, or funding.

**Data availability:** Analyses reported in this article can be reproduced using the data provided by Tieleman et al. (2019).

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APPENDIX TABLE 3. Sample size, average number, and standard deviations of white blood cell counts of House Wrens in the wet and dry season, excluding incubating birds, and in the 3 different stages in the wet season.

	<i>n</i>	Heterophils		Lymphocytes		Eosinophils		Monocytes		Basophils	
		Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
Season											
	9	21.60	12.50	60.82	17.46	7.23	6.32	9.79	4.52	0.56	1.01
Dry											
	15	18.86	12.27	62.39	16.70	9.83	7.54	8.65	5.66	0.27	0.80
Wet											
Stage											
	5	13.00	3.08	75.18	6.42	6.62	2.60	5.20	1.79	0.00	0.00
Incubating											
	8	21.13	16.37	53.56	18.65	13.85	8.48	10.95	6.65	0.50	1.07
Nestling-feeding											
	7	16.27	4.93	72.47	4.72	5.23	1.65	6.01	2.84	0.00	0.00
Nonbreeding											