Short Communication

Vocal behaviour, parasitic infection, chronic stress and body condition in rufous-naped wrens (*Campylorhynchus rufinucha*)

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We explored the relationship between haemosporidian infection (infected vs. uninfected), parasite load, heterophil:lymphocyte ratio, body condition and vocal behaviour of the Rufous-naped Wren *Campylorhynchus rufinucha*. Our results, albeit based on a small sample size, showed no evidence of an association between vocal behaviour and infection status, heterophil:lymphocyte ratio or body condition. Infections did not vary between localities. Our results showed, however, that infection status was associated with a higher song rate, and that in males and females belonging to the same reproductive pair there was a strong positive correlation in terms of infection status and parasite load.

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Parasite-mediated sexual selection theory suggests that females prefer males giving more energetic displays that may reflect relatively higher resistance to parasites (Hamilton & Zuk 1982). Four hypotheses attempt to explain the association between the expression of secondary sexual characteristics and parasitic infection (reviewed in Garamszegi 2005). The Hamilton and Zuk (resistance gene) hypothesis suggests that female preference is mediated by resistance genes, where hosts coevolve with parasites generating heritable resistance (Hamilton & Zuk 1982). Consequently, females could benefit by producing offspring with partial resistance to parasites and choosing a healthy male that could provide better resources (Borgia & Collis 1989). The contagion indicator hypothesis states that females that prefer males with elaborate sexual characteristics will avoid damage caused by parasites that could be transmissible between individuals (Able 1996). The efficient parenting hypothesis suggests that females choosing males with elaborate ornaments could benefit, as the males might provide greater resources for parental care compared with parasitized males that cannot maintain such ornaments in good shape (Hamilton 1990). Finally, the parasite avoidance hypothesis suggests that females can reduce the probability of contracting parasites either directly or indirectly (e.g. via vectors) if they choose parasite-free males (Borgia 1986, Borgia & Collis 1989) or territories with a lower probability of infection in the case of vector-borne parasites.

Multiple studies have shown that infection status and/or parasite load can influence avian acoustic signalling (e.g. Garamszegi *et al.* 2004, Bischoff *et al.* 2009) and birds' stress responses (e.g. heterophil:lymphocyte (H:L) ratio; Townsend *et al.* 2018, Becker *et al.* 2019). However, such effects could be also related to sex and body condition (Bustillo-de la Rosa *et al.* 2022), suggesting that bird songs may have evolved as secondary sexual traits to transmit information about males' health status (Garamszegi 2005).

Here, we investigate the associations of infection status and parasite load (i.e. parasitaemia) with vocal behaviour, H:L ratio and body condition of Rufous-naped Wrens *Campylorhynchus rufinucha*. This species inhabits Neotropical dry forests from south-western Mexico to north-western Costa Rica along the Pacific slope (with an isolated population in central Veracruz), for which previous detailed studies on genetics, ecology, song and behaviour have been developed (e.g. Bradley & Mennill 2009, Vázquez-Miranda *et al.* 2009, Sosa-López *et al.* 2013, Ku-Peralta *et al.* 2020, Quiroz-Oliva & Sosa-López 2022), providing a solid framework to test the influence of parasites on life history. We determined whether (1) infection status and parasite load are

associated with body condition and the H:L ratio, (2) infection status, parasite load, H:L ratio and body condition are associated with acoustic traits, and (3) infection status and parasite load are correlated between members of a reproductive pair.

METHODS

Study site and body condition

From 18 April to 5 July 2017, we sampled six sites in lowland tropical deciduous forest around La Joya lagoon, Tonalá, coastal Chiapas, Mexico (Appendix S1). We captured 50 individuals from 32 territories using mist-nets, and banded them with a unique colour combination and a metallic ring with a unique alphanumeric combination. We captured both females and males in 17 territories. In one territory, we captured four individuals (the pair and two juveniles; juveniles were not included in the analyses). In the remaining territories, we captured only females in 11 and only males in three territories. We took eight morphological measurements following Pyle *et al.* (1997; see Appendix S2). We sexed 32 individuals in the field by manually inspecting the brood patch on females and the cloacal protuberance in males (Ralph *et al.* 1996), and sexed the remainder using a DNA test (Griffiths *et al.* 1998). We were unable to determine the sex of one individual. We used the scaled mass index $\left(\hat{M}_i\right)$ to assess body condition following Peig and Green (2009, 2010, Appendix S2).

Haemoparasite identification and H:L ratio index calculation

We collected blood from the brachial vein and prepared four blood smears (fixed in absolute methanol for 4 min in the field, and stained with Giemsa in the lab) for each bird for quantification of haemoparasites following Valkiūnas (2005) and Santiago-Alarcon and Carbó-Ramírez (2015). We used an optical microscope (Nikon Eclipse Ni) to determine infection status at 600× and, if infected, we determined the parasite load and performed a leucocyte differential count (i.e. lymphocytes, monocytes, eosinophils, heterophils and basophils) using the best two smears at 1000× amplification following Campbell and Ellis (2007). We used the H:L ratio to index chronic stress (Davis & Maerz 2008; Appendix S3).

We estimated prevalence and parasite load in a total count of 10 000 erythrocytes. Additionally, we performed three PCR protocols for the amplification of a fragment of the mtDNA cytochrome b gene (*c.* 479–1100 bp) of avian haemosporidians (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*) following first Pacheco *et al.* (2018), then Beadell and Fleischer (2005), and finally Hellgren *et al.* (2004). Although limited funding prevented us from sequencing the positive samples, the use of both microscopy and three PCR protocol, gives us high confidence in the estimated prevalence of avian haemosporidians.

Acoustic analysis

Previous studies have suggested that the dawn chorus in Rufous-naped Wrens peaks between 06:45 and 11:00 h (CST) (Quiroz-Oliva & Sosa-López 2022); thus, we recorded banded birds in their territories during this period. On average, we gathered 5.43 h (± 0.14) of focal recordings in a period of 2.84 days (± 1.34) per territory (Appendix S2).

We expressed repertoire size as vocal complexity, defined as the number of song versions that one bird performs (Catchpole & Slater 2008). We used all recordings collected for each bird, categorizing *a priori* each song by type (following Sosa-López & Mennill 2014a, 2014b). We classified song types into those with distinct phrases (following Quiroz-Oliva & Sosa-López 2022), thus classifying two songs as the same type if phrases were the same, regardless of the number of times a phrase was repeated within a bout (Fig. 1). We built a catalogue of song types for each bird and computed asymptotic curves, using the number of songs recorded against the number of new song types, to determine whether we had achieved sampling close to an individual's complete repertoire (Fig. 2a). Our analysis suggested that *c.* 40 song recordings (regardless of type) would be needed to have a good representation of a bird's repertoire (Fig. 2a). We reached this number of recordings for 10 individuals (Fig. 2b).

For structural analysis, we selected high-quality recordings with a high signal-to-noise ratio. We selected the most common song type in the population (i.e. shared by all individuals; Fig. 1). We gathered 105 songs from 21 birds (an average of 5.2 ± 2.7 songs per bird). Then, we obtained an individual mean value for eight acoustic variables: song length, phrase length, minimum and maximum frequencies, bandwidth, peak frequency, rhythm and the average number of phrases within the song (Appendix S2).

To measure vocal performance, we identified all songs emitted between 06:45 and 11:00 h (CST), annotating the time between the first and last song, and calculated the singing rate as the number of times that a bird sings a song per unit of time (Møller 1991). This variable was calculated per day, and we calculated the average rate per bird (over the 2 or 3 days of recordings) for 19 individuals.

Statistical analyses

A principal component analysis (PCA) yielded two components explaining 78.6% of the variance in the acoustic
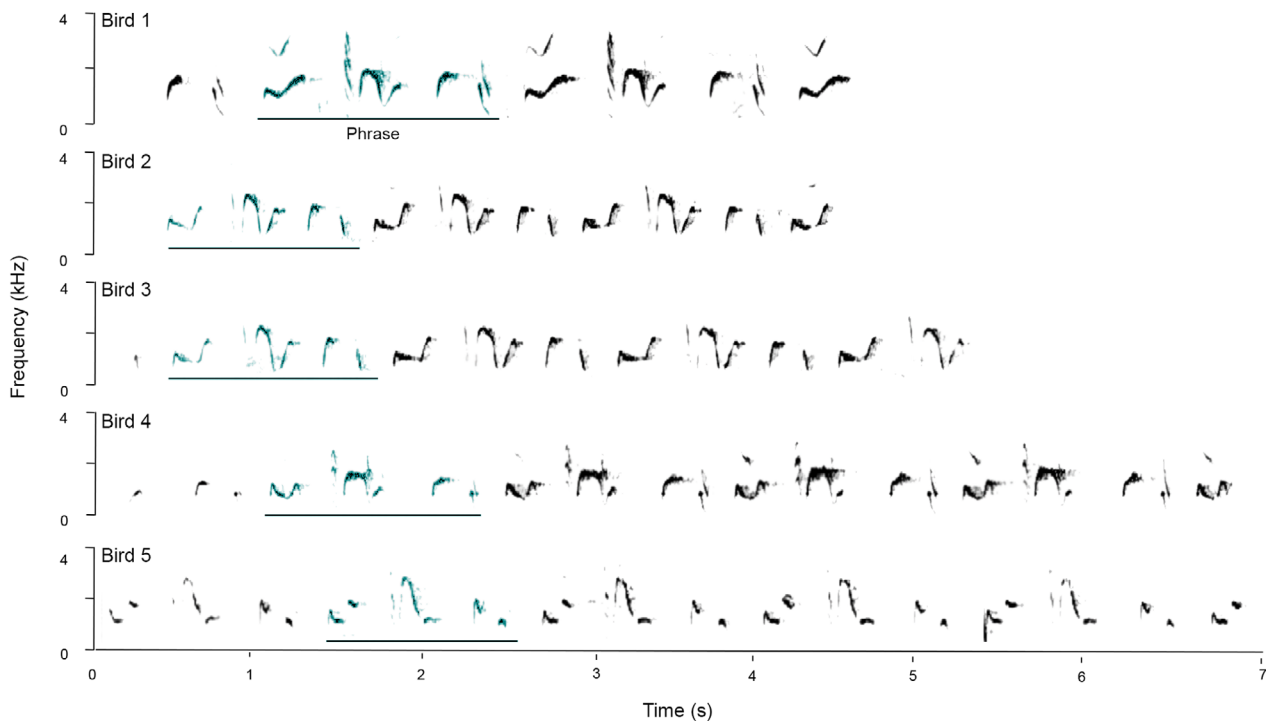
Figure 1. Spectrograms of Rufous-naped Wren songs. The image shows examples of the song type 'J' belonging to five birds. The phrases are underlined within each song (phrases are shown in green in the online version).

measurements. The first component (PC1) explained 46.5% of the variance and was correlated with variables related to frequency, and the second component (PC2) explained 32.1% of the variance and was correlated with variables related to time (additional methodological information is given in Appendix S3).

We fitted a set of generalized linear models (GLMs) including as dependent variables: infection status (0 = non-infected, 1 = infected), parasite load, song complexity, song characteristics (PCA scores) and vocal performance. We included the following as explanatory variables: body condition, H:L ratio, sex and infection status (Table 1). We used a Spearman rank correlation test to assess the relationship between infection status and parasite load between members of territorial pairs. We tested whether the probability of a bird being infected varied between sites, with infection status as the dependent variable and territory ID as a fixed factor. We applied Akaike's information criterion (AIC) model selection approach to compare univariate models with a null model. Univariate models with a delta (Δ) AIC value ≤ 2 were considered to explain the data better than the other models (Akaike 1987). The PCA and correlations were performed in SPSS v.21 (IBM Corp., Armonk, NY, USA), and GLMs were performed using the MASS package in R v3.3.3 (R

Foundation for Statistical Computing, Vienna; Venables and Ripley 2002).

RESULTS

We identified parasites from the genera *Plasmodium*, *Haemoproteus*, *Leucocytozoon* and *Trypanosoma* and nematode larvae (microfilariae) in 14 of 50 individuals (six females and eight males). *Plasmodium* was detected in 12 individuals (24%) and nematode larvae in two. The prevalence was 20% for females and 40% for males. Parasite load varied substantially, with counts ranging from 0 to 488 parasites per 10 000 erythrocytes (Appendix S3). There was no significant association of infection status or parasite load with body condition or H:L ratio, and neither infection status nor parasite load varied significantly with sex (Table 1).

Females and males did not differ in repertoire size (7.3 ± 0.51, $n = 6$; and 8.0 ± 1.41, $n = 4$, respectively). Infection status, body condition, H:L ratio and sex were not related to song complexity, PC1 or PC2 (Table 1). Vocal performance was not associated with sex but it was significantly affected by infection status, with infected individuals having a higher song rate (Table 1, Fig. 3). Infection status ($r = 0.78$, $P < 0.05$; $n = 16$) and parasite load ($r = 0.83$, $P < 0.05$; $n = 16$) were
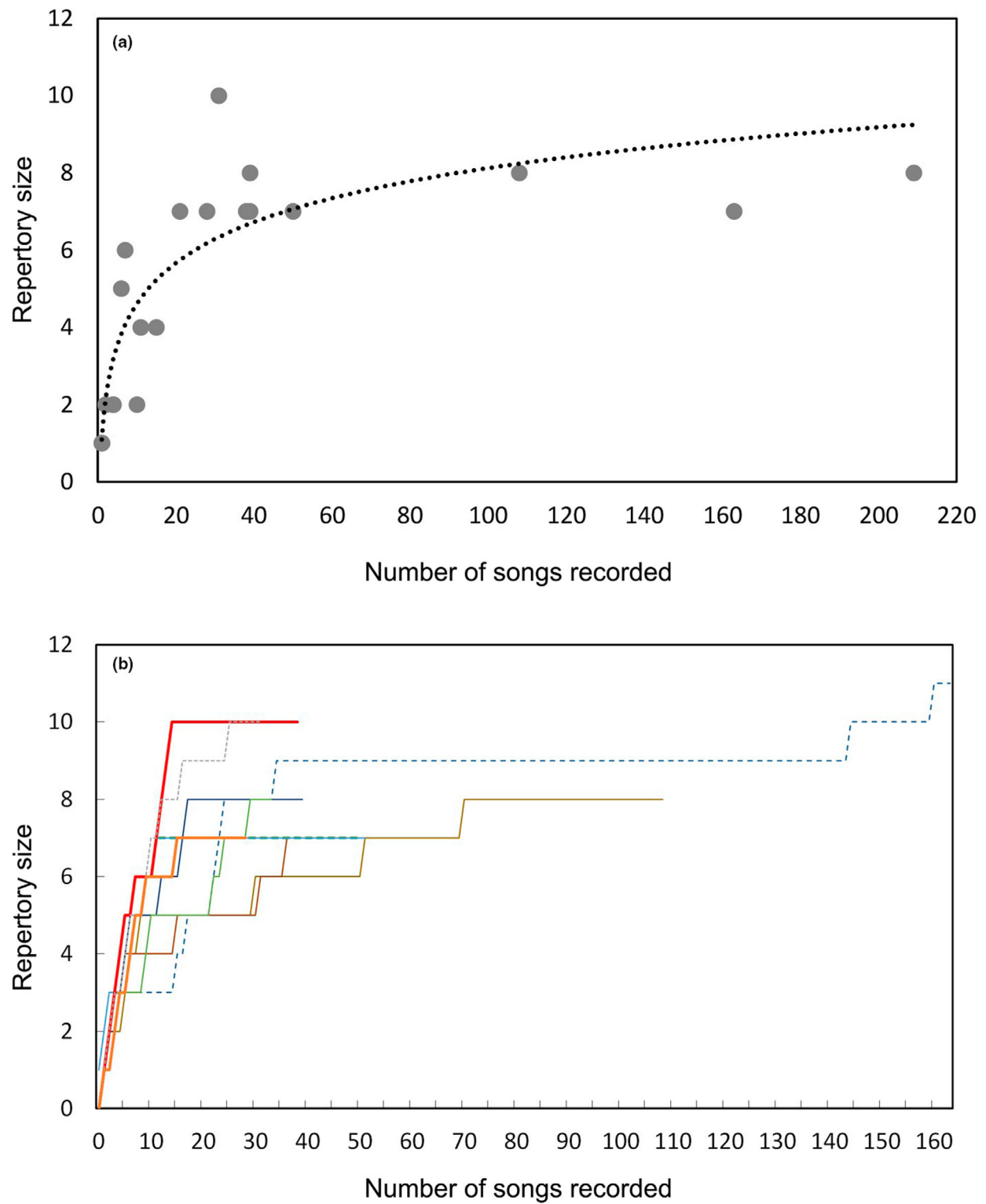
Figure 2. Graphs depicting the size of the repertoire as a function of the recorded songs in Rufous-naped Wrens. (a) Number of total songs recorded against the repertoire size of 19 birds, for which we gathered recordings. (b) Graph depicting asymptotic curves for 10 birds for which we recorded more than 20 songs.

Table 1. Results of GLMs to identify factors associated with infection status, parasite load, song complexity, fine structural characteristics of song and vocal performance. Univariate models with a delta (Δ) AIC value ≥ 2 below the null model are in bold type.

Dependent variables	*n*	AIC	Explanatory variables	Estimate	se	*t/z* Value
Infection status[a]	49	60.63	Null	−0.92	0.32	−2.89
	49	62.35	Body condition[d]	0.17	0.31	0.53
	49	61.59	H:L ratio	2.32	2.29	1.01
	49	60.49	Sex	−0.94	0.65	−1.45
Parasite load[b]	12	121.07	Null	4.54	0.51	8.89
	12	122.49	Body condition[d]	0.65	0.53	1.24
	12	122.34	H:L ratio	−6.21	5.32	−1.16
	12	122.82	Sex	0.53	1.08	0.49
Song complexity[b] (Repertoire size)	10	30.63	Null	7.6	0.31	24.88
	10	32.27	Infection status	−0.38	0.69	−0.55
	10	45.69	Body condition[d]	2.03	0.11	17.65
	10	45.86	H:L ratio	−0.15	2.31	−0.06
	10	31.78	Sex	0.57	0.68	0.84
Fine structural characteristics of song[c] (PC1)	20	60.69	Null	0.01	0.23	−0.05
	20	61.83	Infection status	0.52	0.58	0.89
	20	61.73	H:L ratio	−4.63	4.89	−0.95
	20	60.64	Body condition[d]	−0.13	0.09	−1.39
	20	61.63	Sex	0.47	0.47	0.99
Fine structural characteristics of song[c] (PC2)	20	58.86	Null	−0.07	0.22	−0.3
	20	60.33	Infection status	−0.39	0.55	−0.69
	20	60.31	H:L ratio	3.33	4.72	0.71
	20	57.23	Body condition[d]	0.16	0.09	1.89
	20	59.94	Sex	−0.41	0.45	−0.92
Vocal performance[c,e] (Singing rate)	20	28.85	Null	1.08	0.10	10.48
	20	30.31	Body condition[d]	−0.08	0.11	−0.699
	20	**26.38**	**Infection status**	**0.50**	**0.24**	**2.119**
	20	28.10	Sex	−0.32	0.20	−1.627

Distribution used in GLM: [a]binomial; [b]negative binomial; [c]Gaussian. Data transformation: [d]z-score; [e]Log$_{10}$.
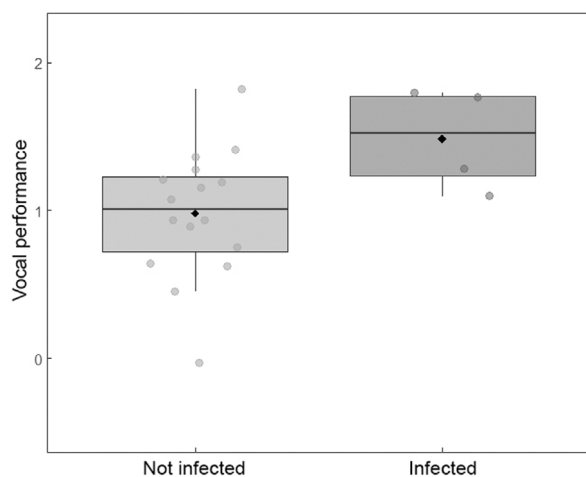
Figure 3. Box plot showing how vocal performance (as measured by number of song bouts per minute) differs according to infection status. Box plots show the median, first (lower) and third (upper) quartile, and whiskers denote values that are not outliers. Dots show the raw data and the black diamond symbols indicate the mean.

positively correlated between members of pairs. Finally, there were no significant differences in prevalence among localities (Appendix S4).

DISCUSSION

We found no evidence of an association between infection and body condition in Rufous-naped Wrens. Previous studies in birds have shown contrasting results, with some suggesting an association between infection and body condition (e.g. Buchanan *et al.* 1999, Garamszegi *et al.* 2004, Fleskes *et al.* 2017), but others not (e.g. Spencer *et al.* 2005, Santiago-Alarcon *et al.* 2013, 2019, 2020). Multiple factors could explain these contradictory results, such as varying sample sizes or differences in study site, bird populations and types of parasite (Santiago-Alarcon *et al.* 2019). Also, different genetic variants or lineages within the same genus or species of parasite can have different effects on the same host species (e.g. Palinauskas *et al.* 2008, 2011). Further, body condition could vary with multiple factors (e.g. variation in bird weight with the time of the day, breeding status, moult; see review in Lovette & Fitzpatrick 2016), and

the infections that we detected might have occurred just a few days or several weeks previously, hindering our ability to find an association of infection with body condition. Another plausible explanation is that the infected birds that we followed were those that survived the infection because of their high quality. It is also thought that coinfections have higher virulence and consume a greater amount of host resources than infections by a single species, but the parasite species (even the genetic lineage) might be as important as the number of parasites (McKenzie et al. 2006, Choisy & De Roode 2010, Palinauskas et al. 2011, 2020).

We found no evidence of an association between parasitism and H:L ratio. A possible explanation is that the effect of blood parasites is small or that the effect is only evident in certain scenarios. For instance, Becker et al. (2019) found that blood parasites only impact the H:L ratio when birds undergo a physical challenge such as migration. While some studies have found high H:L ratios to indicate ability to control and repel parasite infections (e.g. Minias 2019), others have suggested that high ratios are the result of active infections (e.g. Davis et al. 2008) or are associated with body condition (Bustillo-de la Rosa et al. 2022). Indeed, there are multiple studies with contrasting results between parasites and haematological variables, including the H:L ratio (e.g. Ots et al. 1998, Cornelius et al. 2014, Santiago-Alarcon et al. 2020). Additionally, it has been suggested that haemosporidian pathogenicity in wild birds produces mortality in the severe acute stage of infection (Permin & Juhl 2002, Atkinson & Samuel 2010, Palinauskas et al. 2011). Surviving individuals may show immunity to parasites, with presence of chronic infection but with lower costs to their fitness than during the acute or relapse stages of infection (Valkiūnas 2005, Bensch et al. 2007, Valkiūnas & Atkinson 2020).

We found no evidence of an association between repertoire size and haemosporidian infection. This contradicts studies suggesting that infection in wild birds may affect the development of the high vocal centre, the brain nucleus necessary for learning and producing vocalizations that mediates the expression of bird songs (Jarvis et al. 2005, Nottebohm 2005). In addition, and contrary to our expectation, we found that Plasmodium-infected Wrens produced higher song rates in comparison with healthy individuals (e.g. Møller 1991, Garamszegi et al. 2004, Gilman et al. 2007). Although the number of infected birds in our study was rather low, there are some plausible biological explanations for these results. Variation in vocal behaviour may be the result of biological traits such as age, body condition, testosterone levels and immune response (Gil & Gahr 2002). It is also possible that some host species may be more sensitive to infections than others, and Wrens may have coevolved with local parasites (e.g. between malaria and Common Starlings Sturnus vulgaris, Palinauskas

et al. 2008; recently between P. relictum and Hawaiian endemic honeycreepers, Woodworth et al. 2005) or that infected birds might have survived the acute infection that is now maintained as a chronic infection; or perhaps parasitized birds may show better vocal performance due to temporal variation during the sampling period (e.g. Buchanan et al. 1999).

Our finding that infection status and parasitaemia are correlated between members of a pair supports both the contagion indicator and the parasite avoidance hypotheses. This outcome is biologically feasible because Rufous-naped Wrens remain in pairs for at least 4 months, creating an opportunity for parasite transmission between paired birds (Valkiūnas & Atkinson 2020). Yet, our results showed a positive relationship between infection status and vocal performance, making it challenging to propose a mechanism that explains how females could avoid the contagion using vocal behaviours as a cue in this bird species.

Few studies have analysed associations between vocal behaviour and parasitic infections, and they have shown inconsistent results. Therefore, it remains unclear how parasitic infection affects acoustic signalling in birds. We found no evidence of an association between vocal traits and birds' health. Our results also suggest that Plasmodium spp. have a minor impact on vocal behaviours, as infected birds have a similar repertoire size and structural characteristics of their song compared with uninfected birds. Indeed, our results suggest that females did not avoid contagion, indicating that vocal behaviours may not reflect birds' health in this species. Of course, our findings do not imply that parasitic infections do not entail a cost. A larger sample size, or other variables not measured in this study (e.g. haematocrit, testosterone, age, quality of territory, parental care, survival rate), could shed light on these costs; thus, our results should be interpreted with caution. Future studies could include a greater range of vocal and life history traits to improve our understanding of the relationship between parasitic infections and the vocal behaviour of birds, which could be complemented with experimental infections in semi-captive conditions.

AUTHOR CONTRIBUTIONS

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

ETHICAL NOTE

None.

FUNDING

Data Availability Statement

The data have been archived at: Meza-Montes, Estefanía; Fernández-Gómez, Ronald A.; Llanes-Quevedo, Alexander; Navarro-Sigüenza, Adolfo; Santiago-Alarcon, Diego; J Roberto, Sosa Lopez (2022) Vocal behaviour, parasitic infection, chronic stress, and body condition in Rufous-naped Wrens (*Campylorhynchus rufinucha*). Mendeley Data, V1, doi: 10.17632/v57k7w5jpk.1.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1. Supplementary figure.
Appendix S2. Extended methods.
Appendix S3. Supplementary tables.
Appendix S4. Additional statistical results.