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Experimental Evidence That Blood Parasite Infection Affects Incubation Patterns in a Cavity-Nesting Songbird

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ABSTRACT

Avian chronic hemoparasite infections occur commonly in wild birds, causing adverse effects on host fitness and breeding success. However, the potential impact of such infections on the incubation behavior has been scarcely experimentally studied. We reduced the infection of hemoparasites in wild-breeding female pied flycatchers (*Ficedula hypoleuca*) through medication with primaquine to test the possible effects on incubation patterns compared with non-medicated control females. As predicted, medicated females significantly reduced their parasite infection compared to control females. This had a direct significant effect on the female behavior, as medicated females were able to have longer incubation sessions, while control females reduced the time devoted to each incubation session. In addition, females from both treatment groups spent less time incubating as incubation progressed, with control females showing a greater reduction. In contrast, the average length of recess sessions did not vary across treatment groups. Moreover, incubation sessions were more frequently interrupted when clutches were smaller. However, these changes had no apparent effects on immediate fitness. To our knowledge, this is the first study showing changes in individual incubation patterns in response to parasites in a wild-bird population, adding to previous studies showing that blood parasites have detrimental effects on bird reproductive success.

1 | Introduction

Reproduction is a critical stage in the life cycle of animals, and individuals usually allocate a significant amount of energy to this activity to enhance their reproductive fitness (Bell 1980). In birds, for instance, breeding consists of several costly activities such as nest building, egg incubation, and offspring provisioning. Among these activities, incubation is a crucial period as most species are required to engage in contact incubation for an extended period of time to ensure an optimal environment for embryonic growth and development (Deeming 2002). In many cases, incubation is performed solely by the female. As the sole caregiver, females face a trade-off between time spent foraging and self-maintenance, and the incubation requirements of the developing embryo (Stearns 1998; Reid et al. 2002; Boulton et al. 2010). Additionally, if food resources are scarce at the beginning of the breeding season (Perrins 1970), this trade-off between self-maintenance and reproduction can entail higher costs for females (Koski et al. 2020). This can be compensated, in part, with the assistance of males, since male incubation feeding is more frequent when the conditions are worse, for example, the temperature is lower (Amininasab et al. 2017).

Parasites play an important ecological role in all ecosystems, exerting extreme selection pressures on the different organisms involved in parasite–host interactions (Price 1980). Hemoparasites represent a very diverse group and are usually transmitted by blood-sucking insects that act as vectors. Several hemoparasites

infect birds, including protozoa such as *Trypanosoma* or those belonging to the order Haemosporida (Apicomplexa), such as *Haemoproteus*, *Leucocytozoon*, and *Plasmodium*, but also some pluricellular organisms such as filarial nematodes. Once in the vertebrate hosts, most of these parasites reproduce asexually in internal organs before reaching the peripheral blood. This prepatent period varies among species, but it spans several days (Valkiūnas 2005). In general, these parasites cause chronic infections in wild birds with relapses (defined as the appearance of secondary parasitemia after a latent stage of infection or a significant increase of parasitemia during the period of chronic infection) occurring during stressful situations for the hosts (Møller 1997; Merino et al. 2000; Scheuerlein and Ricklefs 2004; Valkiūnas 2005). The consequences of parasitic infections in birds extend beyond immediate health concerns and can influence individual reproductive success by changing the expression of sexually selected ornaments or modifying reproductive behavior (Hamilton and Zuk 1982; Allander 1997; Møller 1997; Ots and Horak 1998; Wiehn et al. 1999; Brawner et al. 2000; Fitze and Richner 2002; Hõrak et al. 2004; Lozano 1994; Mougeot et al. 2009). For instance, the detrimental impact of parasites on behavioral traits, such as the song in male birds considered an acoustic sign of health (Garamszegi 2005), may differ from their effects on olfaction or plumage-based ornaments, with a positive relationship between immune defense or parasite infection and the complexity of bird song (Garamzegi et al. 2003; Spencer et al. 2005). Moreover, behavioral traits are likely to be more dynamic signals compared to ornaments, and alterations in signaling content, such as infestation status, become immediately apparent. For example, domestic rabbits *Oryctolagus cuniculus*, experimentally infected with *Psoroptes cuniculi* decrease rearing behavior as early as 2 days post-infestation (Hallal-Calleros et al. 2013) and house sparrow (*Passer domesticus*) females experimentally infested with coccidian parasites reject highly infested males in mate choice tests (Cantarero et al. 2023). Modifications in behavioral parameters can be identified at an earlier stage compared to other clinical symptoms. However, the ability of hosts to maintain infections below the threshold above which their effects become evident makes it difficult to demonstrate their pathogenicity (Atkinson and Van Riper 1991; Weatherhead and Bennett 1991). Therefore, to rigorously test the behavioral effects of parasitic infection in wild birds, an experimental approach in which natural infection levels are reduced in the wild is necessary.

Several previous studies have used experimental manipulation of hemoparasite infections in wild bird populations using medication (Merino et al. 2000; Sanz et al. 2001; Marzal et al. 2005; Tomás et al. 2005, 2007; Knowles et al. 2010; Martínez-de la Puente et al. 2010; Schoepf et al. 2022), showing that reduction in parasitemia can lead to increases in reproductive success (e.g., hatching success, provisioning rates, and fledging success). For instance, Marzal et al. (2005) reported that treated birds had larger clutches and broods, with increases of 18% at laying, 39% at hatching, and 42% at fledging. The observed increase in hatching success suggests that treatment influenced egg quality and/or incubation behavior.

Given that parasite infection consumes birds' energy resources through direct or indirect effects, such as the need to mount an immune response, parental effort capacity may also be negatively affected (Sheldon and Verhulst 1996). However, only one of these studies has investigated the effects of hemoparasite infections on incubation patterns (Schoepf et al. 2022). In that study, medicated females laid heavier clutches, invested more in incubation and provisioning behavior, and produced more fledglings than control females, and their nestlings had higher hematocrit, higher blood glucose, and lower reactive oxygen metabolites than nestlings of control females. However, they did not compare changes in behavior before and after treatment to check for the effect of medication on individual incubation behavior.

In this study, we conducted a medication experiment in a wild European pied flycatcher (*Ficedula hypoleuca*) population to test the effects of hemoparasite infection on female incubation behavior. Because incubation is an energetically demanding reproductive phase, carried out by females alone for a prolonged period, hemoparasite infections may play a central role in determining reproductive success. Therefore, we expect that a decrease in hemoparasite infection among medicated females, result in a beneficial impact on incubation patterns in terms of investing more time on incubation and less on self-maintenance, and on reproductive success in terms of improved hatching success and survival and quality of the nestlings, due to the fact that females could devote more energy to feeding the nestlings.

2 | Materials and Methods

2.1 | Study Area and Species

The study was carried out in the spring of 2023 within a population of pied flycatchers that breed in nest boxes in a montane forest of Pyrenean oak (*Quercus pyrenaica*) in Valsaín, central Spain (40°54'N, 04°01'W). Among the 465 nest-boxes available, we selected 100 of the 112 occupied by pied flycatchers. Nest-boxes in our study area are made of pinewood and measure 17.5 cm high, 11.7 cm wide, and 12.5 cm deep (about 2500 cm^3 inside; see Lambrechts et al. 2010 for more information).

Egg laying within the studied pied flycatcher population typically commences in mid-May (Cantarero et al. 2013b), with a typical clutch size of six eggs (Lundberg and Alatalo 1992) and low hatching asynchrony, with hatching of eggs in the clutch occurring within a short period of time (Lundberg and Alatalo 1992; Fuertes-Recuero et al. 2024). They are typically single-brooded with biparental care. The female takes on the responsibility of incubation and brooding independently, receiving part of her food from her mate, and both parents usually contribute to feeding the nestlings (Cantarero et al. 2014). No instances of brooding are observed once nestlings reach 7 days of age (Sanz and Moreno 1995).

On the fifth day of incubation, females were sequentially and randomly assigned to a treatment, since a previous study tested the prevalence of blood parasites in this population and found that 35% of females were infected with *Haemoproteus* and 16% with *Trypanosoma* (Sanz et al. 2001). Then, females were captured in the nest-box during the daytime without traps, as they are not easily frightened away from the nest at this stage. A blood sample was extracted from the brachial vein, immediately smeared,

air-dried, and fixed with absolute ethanol to assess the presence of parasite infections once the experiment was over (see below). Then, females were subcutaneously injected into the back, with either 0.01 mg of primaquine (Sigma, St Louis, MO, USA) diluted in 0.1 mL of saline solution ($N = 50$) or the same quantity of pure saline solution for controls ($N = 50$), following the procedure reported by Merino et al. (2000). Primaquine, known for its antimalarial activity when administered at 10 mg kg^{-1} (Graczyk et al. 1994; Mayorga et al. 1997a), exhibits some undesirable side effects, including gastrointestinal disturbances and the potential development of methemoglobinemia and hemolytic anemia, which are dose-dependent (Mayorga et al. 1997b). To minimize these effects, we opted for a low-concentration single dose. It is crucial to note that the toxicity of primaquine precludes the occurrence of any beneficial side-effects, aside from the reduction in blood parasitemia. Additionally, females were weighed to the nearest 0.01 g with a digital balance. Females were individually identified based on their existing rings, or if needed, they were newly ringed using numbered metal rings. The exact age of the females older than 1 year was established if they were ringed as nestlings in the study area. The age of unringed females was determined based on the color and wear of the inner great wing coverts according to Jenni and Winkler (1994) and Svensson (1984). Although it was not possible to determine the exact age of females ringed in previous years as adults, we can calculate a minimum age. Thus, birds ringed as adults for the first time were considered with a minimum age of 2 years and when captured in following years were aged as minimum of 3, 4, etc., years.

Three days after the observed hatching date, when all eggs should have hatched (considering hatching day as day 1), we recorded the number of nestlings and computed hatching success as the proportion of eggs that successfully hatched. When nestlings were 7 days old, all adult birds were captured by using a passive trap placed inside their nest-box. The trapping mechanism was deployed for a maximum of 1 h and withdrawn once both adults were captured. Identification of birds was accomplished through their existing rings or were ringed with uniquely numbered rings if needed. The following measurements were taken on all birds: tarsus length with a digital caliper to the nearest 0.01 mm, and weight as indicated above. The body condition of the parents was calculated by dividing body mass by $(\text{tarsus length})^3$, following Griggio and Hoi (2010). For females, we also calculated body condition at incubation by using the length of the tarsus taken at day 7 of nestling age, since it is not expected that the length of the tarsus would vary in so few days. Additionally, a second blood smear from females was collected at this capture to assess the effect of our manipulation on hemoparasites. The same blood smear fixing process described above was also used with these samples. Nestlings were ringed at 13 days old, and measures of tarsus length and mass were taken as described above. Nestling's body condition was also calculated in the same way as for adults. Fledging success was computed as the proportion of hatched nestlings that successfully fledged.

2.2 | Video Recordings

On days 3 and 11 of incubation, we recorded nest activity for about 90 min (84.35 ± SE 10.04, $N = 197$) with digital video cameras (Sony Dcr-sr190, with an extra battery Sony np-fh100) placed 20 m away from the nest-box tree and recording an area of approximately 1 m around the nest-box. Three females (two from the primaquine group and one control) deserted during incubation; therefore, the total number of recordings was $N = 197$. Thus, we obtained two incubation recordings for each nest, pre- and post-female treatment. All films were recorded between 8 a.m. and 1 p.m., and we found no significant effects of the time of day on behavioral variables, as indicated by correlation tests (effect of hour $p > 0.30$ in all cases).

2.3 | Behavioral Data Analysis

Recordings were scored in the free VLC Media Player software (VideoLan 2006). We estimated the average duration in minutes of incubation sessions and recess sessions (time between incubation sessions when females were out of the nest-box) as well as the proportion of time spent by the female inside the nest-box or "egg attendance," which encompassed the time devoted to incubating and turning the eggs (Cantarero et al. 2013a).

2.4 | Parasite Assessment

Once the experiment was over, blood smears were stained with Diff-Quik (PanReac AppliChem) in the laboratory, previous to searching for parasites with the aid of an Olympus BX-41 microscope as follows. Half a smear was scanned at 200× magnification in search of large parasites such as *Trypanosoma* and microfilariae, whereas intra-erythrocytic parasites, such as *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* were searched under oil immersion at 1000 magnification. We quantified the number of extraerythrocytic parasites in one-half of the smear, scanning its longitudinal and vertical axes, counting the number of fields scanned, and transforming parasite numbers to parasites per 100 fields (Merino and Potti 1995). Intraerythrocytic parasites were quantified as the number of parasites per 2000 erythrocytes (Godfray et al. 1987). When the infection was very slight (i.e., less than 1 parasite per 10000 erythrocytes), we assigned an infection value of 0.1 parasites per 2000 erythrocytes, that is, just half of 1 parasite per 10000 erythrocytes. We assigned this value after having checked more than 10 000 erythrocytes (Fargallo and Merino 1999). We were unable to obtain blood samples from some females in the second capture, so we had two samples from 47 medicated females and 47 controls. We calculated infection intensity for the most abundant parasites, *Haemoproteus* and *Leucocytozoon*. Due to the limited occurrence of the rest of the hemoparasites, quantifying infection intensities proves challenging. Consequently, we opted for a presence-absence of parasites when conducting analyses on these parasites. It is crucial to consider that due to the prepatent times of these infections, which vary from 5 to 13 days, samples from infected individuals may appear as uninfected due to the parasites being still in internal organs and not present in peripheral blood (Merino and Potti 1995, Valkiūnas 2005). Although molecular methods are more sensitive for detecting low-level infections, microscopy was selected as the sole diagnostic approach since it is a robust method for assessing reductions in infection following treatment (Merino et al. 2000), consistent with the primary aim of the present study.

2.5 | Data Analysis

First, we conducted non-parametric analyses (Wilcoxon signed-rank test) on the infection intensities of the most abundant parasites (*Haemoproteus* and *Leucocytozoon*) to test whether there were differences in infection intensity in the pre-treatment phase, and also to assess whether the medication affected infection intensity by comparing the pre- and post-treatment phases in each treatment group separately. Then, we checked the effect of medication on infection by all hemoparasites studied in females, by comparing the number of infected and uninfected females before (day 5 of incubation) and after treatment (day 7 of nestling age) using a Chi-square contingency table test for each treatment group (control and medicated females separately). In addition, we used the McNemar test to analyze the infection differences between pre- and post-treatment samples in each group. We used linear models (LMs) to test whether females assigned to both treatment groups differed in weight, tarsus, wing length, and laying date before the beginning of the experiment. We also test for initial differences in female age and clutch size by using generalized linear models (GLMs) with a Poisson distribution.

We tested whether treatment had an overall effect on female body condition by fitting a linear mixed model (LMM), with "female body condition" as the dependent variable, "treatment (control vs. medicated)" and "stage (pre- vs. post-treatment)" and their interaction as fixed factors, and "Female ID" as a random effect. In addition, we investigate potential behavioral changes in incubation patterns due to the reduction of blood parasite infection caused by the medication by using LMMs. Four different models were conducted using different dependent variables: (1) "Average length of incubation sessions" that was calculated as the total minutes a female spent inside the nest-box (avoiding those sessions in which the female was already inside the nest when the recording began or ended) divided by the number of times the female entered; (2) "Hourly number of incubation sessions"; (3) "Average length of recess sessions" that was calculated as the total time a female spent outside the nest box (avoiding those sessions in which the female was already outside the nest when the recording began or ended) divided by the number of times the female came out of the nest box; and (4) "Hourly number of recess sessions." "Treatment (control vs. medicated)," "phase (pre- vs. post-treatment),"' and their interaction were introduced as fixed factors while controlling for clutch size, laying date, female weight at incubation, and female age as covariates. "Female ID" was included as a random structure to account for repeated measures. The dependent variables in these models were log-transformed to improve the normality of model residuals. To further investigate whether the reduction in blood parasite infection caused by the medication affected incubation behavior we fitted an additional generalized linear mixed model (GLMM, family = binomial) on the "Proportion of total time spent incubating" (time spent incubating/total time recorded) that was modeled using the same fixed and random structure of the previous models.

Analyses were conducted on the total sample of females included in the experiment because even those females found uninfected by searching for parasites in blood smears can in fact be infected. This is due to the characteristics of many of these diseases that can maintain parasites in internal organs and out of peripheral blood for relatively long periods (Valkiūnas 2005). Some birds are free of blood parasites on both samples, while most appear infected at least on one of the occasions. These are possibly infected from the beginning because prepatent periods for blood parasites of birds are several days, but we cannot rule out completely the possibility that infections occurred after treatment. However, we also repeated analyses using only females that were found infected in at least one of the samplings (N = 53; control = 30, medicated = 23), thus considering as uninfected those that were found uninfected in both samplings (N = 41; control = 17, medicated = 24). By doing so, we avoid including uninfected females in the analyses that may suffer from a negative side effect of medication, thus obscuring the effects of the reduction of blood parasites on incubation patterns.

A different set of analyses was further conducted for the incubation pattern variables by grouping individuals into four categories: medicated infected and uninfected females, and control infected and uninfected females. These models were used to clarify whether the effects of primaquine medication depended on the observed infection status of individuals at the beginning of the treatment. In these models, we used the same set of covariates and random structure as the previous analyses.

Lastly, we explored whether the reduction in blood parasite infection following medication affected hatching success, fledging success, and nestling body condition at fledging. For fledging and hatching success, we ran two GLMs (family "quasibinomial" to account for overdispersion) with "treatment," "clutch size," and "laying date" as independent variables. For "Nestling body condition" at fledging, we instead run a separate LM with "treatment," "clutch size," and "laying date" as independent variables.

We performed all LMMs and GLMMs with the "lmer" and "glmer" functions, respectively, in the "lme4" package (Bates et al. 2015), while for the zero-inflated models, we used the "glmmTMB" package (Brooks et al. 2017) in the R environment (R Core Team 2017). To test the significance of the main effects and interaction terms in the LMMs, we estimated degrees of freedom and p values of the F tests with the Kenward–Roger approximation implemented in the "pbkrtest" package (Halekoh and Højsgaard 2014). To assess differences between treatment groups and stages or phases, we carried out post hoc tests using the "emmeans" function in the "emmeans" package (Lenth 2020). The significance of the fixed effects in the GLMM model was estimated with the function "Anova," implemented by the "car" package. Overdispersion in the GLM models was assessed using the "performance" package (Lüdecke et al. 2021). Unless otherwise stated, we used a backward removal procedure, consisting of the reduction of the models to the significant variables by sequential elimination of variables (the interaction terms were tested first) with the highest p value. Significance was taken at α = 0.05. Model assumptions were assessed via the "performance" package (Lüdecke et al. 2021).

3 | Results

We did not find significant differences in weight, tarsus, wing length, age, clutch size, and laying date between females assigned

TABLE 1 | Results of Chi-square tests comparing the number of European pied flycatcher females infected and uninfected by any parasite analyzed, depending on the treatment and its stage (pre- or post-treatment).

Treatment	Pre-treatment		Post-treatment		Statistics
	Infected	Not infected	Infected	Not infected	
Medicated	22	25	9	38	$\chi^2 = 11.80$; $\mathbf{p < 0.001}$
Control	26	21	20	27	$\chi^2 = 0.04$; $p = 0.840$

p values < 0.05 are indicated in bold.

TABLE 2 | Mean infection intensity of *Haemoproteus* and *Leucocytozoon* in both treatment groups (control and medicated), before and after treatment. Non-parametric Wilcoxon tests between stages are shown for each treatment.

Control				
	Pre-treatment	Post-treatment	*W*	*p* value
Haemoproteus	3.97 ± 7.752	0.98 ± 2.723	55.5	**0.007**
Leucocytozoon	0.19 ± 0.711	0.2 ± 0.768	34.5	0.893
Medicated				
	Pre-treatment	**Post-treatment**	***W***	***p* value**
Haemoproteus	10.29 ± 25.856	0.62 ± 2.130	10.0	**0.003**
Leucocytozoon	0.4 ± 1.14	0.0 ± 0.0	0.0	**0.011**

p values < 0.05 are indicated in bold.

to different treatments before the beginning of the experiment (all *p* values > 0.05).

3.1 | Hemoparasite Infection and Primaquine Treatment

The most common hemoparasite infecting pied flycatcher females before treatment was *Haemoproteus* (38%), followed by *Leucocytozoon* (14%), *Trypanosoma* (7%), microfilariae (5%), and *Plasmodium* (2%), with a percentage of mixed infections of 9.6%. The number of females infected or uninfected by any parasite analyzed or their intensity of infection by *Haemoproteus* and *Leucocytozoon* did not differ between treatments before the beginning of the experiment (Chi-square test and Wilcoxon test $p > 0.05$, respectively)

After treatment, the percentage of females infected decreased, especially in the case of *Haemoproteus* (18%) and *Leucocytozoon* (7%), but also in the case of *Trypanosoma* (6%) and microfilariae (3%), with a percentage of mixed infections of 6.4%. However, there was a slight increase in the case of *Plasmodium* (3%) (see Tables S1 and S2 for more information about the number of infected individuals and the intensity of parasite infections). As expected, in the medicated group, the number of infected and uninfected females varied significantly between stages (Table 1). Specifically, the number of infected females was significantly reduced after treatment (McNemar, $\chi^2 = 4.75$, $p = 0.0311$). However, in the control group, there were no significant differences in the number of infected and uninfected females before and after treatment (Table 1). In addition, we found that *Haemoproteus* infection intensity significantly decreased after treatment in both groups, but was more pronounced in the primaquine group (Table 2). However, in the case of *Leucocytozoon*, a significant reduction was observed after treatment only in the primaquine group (Table 2).

3.2 | Incubation Patterns

We found a significant interaction between treatment and phase of the average length of incubation sessions ($F_{3,130} = 3.06$, $p = 0.030$; Table 3a). Specifically, post hoc tests showed that the average length of incubation sessions differed between the pre- and the post-treatment phase in the control group ($t = 3.02$, $p = 0.017$, Table S4), with the incubation sessions being shorter in the post-treatment phase (Figure 1a), but not in the medicated group ($t = -0.01$, $p = 0.998$; Figure 1a). Also, we found that the laying date was negatively related to the average length of incubation sessions ($F_{1,94} = 12.01$, $p = 0.008$). The hourly number of incubation sessions was significantly affected by phase ($F_{1,98} = 6.28$, $p = 0.013$), increasing in the post-treatment phase compared to the pre-treatment phase (Figure 1b and Table 3b) but not in interaction between treatment type ($F_{3,129} = 2.57$, $p = 0.056$). In contrast, the average length of recess sessions did not vary across treatment groups and phases (respectively $F_{3,92} = 1.15$, $p = 0.332$; $F_{1,97} = 0.18$, $p = 0.671$; Figure 1c and Table 3c), but females significantly increased the hourly number of recess sessions between phases in both groups ($F_{1,98} = 10.04$, $p = 0.002$; Figure 1d and Table 3d). Also, the hourly number of recess sessions was negatively related to clutch size ($F_{1,97} = 6.14$, $p = 0.015$); that is, there was a greater number of recess sessions when the clutch size was smaller. In addition, we found that females significantly decreased the proportion of time spent incubating between the two phases depending on treatment. Specifically, females in the

TABLE 3 | Model estimates of the effects of treatment groups and phases on incubation variables.

Variable	Estimate	SE	*F*	df	*p* value
(a) LMM for average length of incubation sessions					
Intercept	3.37	0.18			
Phase (Post-)	−0.25	0.08			
Treatment (Medicated)	−0.11	0.08			
Clutch size			1.14	1,95	0.287
Laying date	−0.01	0.00	12.01	1,94	**0.008**
Female weight at incubation			1.45	1,95	0.231
Female age			0.13	1,91	0.720
Phase (Post-) × Treatment (Medicated)	0.26	0.11	3.06	3,130	**0.030**
(b) LMM for hourly number of incubation sessions					
Intercept	0.95	0.03			
Phase (Post-)	0.12	0.05	6.28	1,98	**0.014**
Treatment (Medicated)			0.02	1,94	0.867
Clutch size			2.82	1,97	0.096
Laying date			1.51	1,95	0.221
Female weight at incubation			0.47	1,95	0.491
Female age			0.55	1,93	0.460
Phase (Post-) × Treatment (Medicated)			2.57	3,129	0.056
(c) LMM for average length of recess sessions					
Intercept	1.56	0.03			
Phase (Post-)			0.17	1,97	0.680
Treatment (Medicated)			1.69	1,97	0.196
Clutch size			1.75	1,96	0.189
Laying date			1.07	1,94	0.302
Female weight at incubation			0.04	1,95	0.826
Female age			1.93	1,95	0.168
Phase (Post-) × Treatment (Medicated)			0.79	3,129	0.501
(d) LMM for hourly number of recess sessions					
Intercept	1.63	0.28			
Phase (Post-)	0.18	0.05	10.04	1,96	**0.002**
Treatment (Medicated)			0.76	1,94	0.384
Clutch size	−0.11	0.05	6.14	1,96	**0.015**
Laying date			0.75	1,96	0.387
Female weight at incubation			0.16	1,94	0.690
Female age			0.98	1,94	0.325
Phase (Post-) × Treatment (Medicated)			0.00	1,96	0.995

The values of *F*, df, and *p* are given for the variables before being removed from the full model. Estimates and SEs are given for a model containing only significant terms, including those belonging to significant interactions (shown in bold). The control group and pre-treatment phase are, respectively, the treatment group and the phase of reference.

control group reduced incubation time more markedly than medicated females ($\chi^2 = 13.07$, df = 1, $p = < 0.001$; Figure 1e and Table 4). Regarding these results, we found these same effects when we restricted the analyses only to females that had been found infected in any of the phases, regardless of the treatment (more details in Tables S5 and S6).

When we repeated these analyses using the infection status (infected/uninfected) for each treatment groups (control/primaquine), we found the same patterns of the previous analyses except in the case of females from the control groups that significantly decreased the length of the incubation session irrespective of their infection status (Figure 2 and Table 5a; more details in

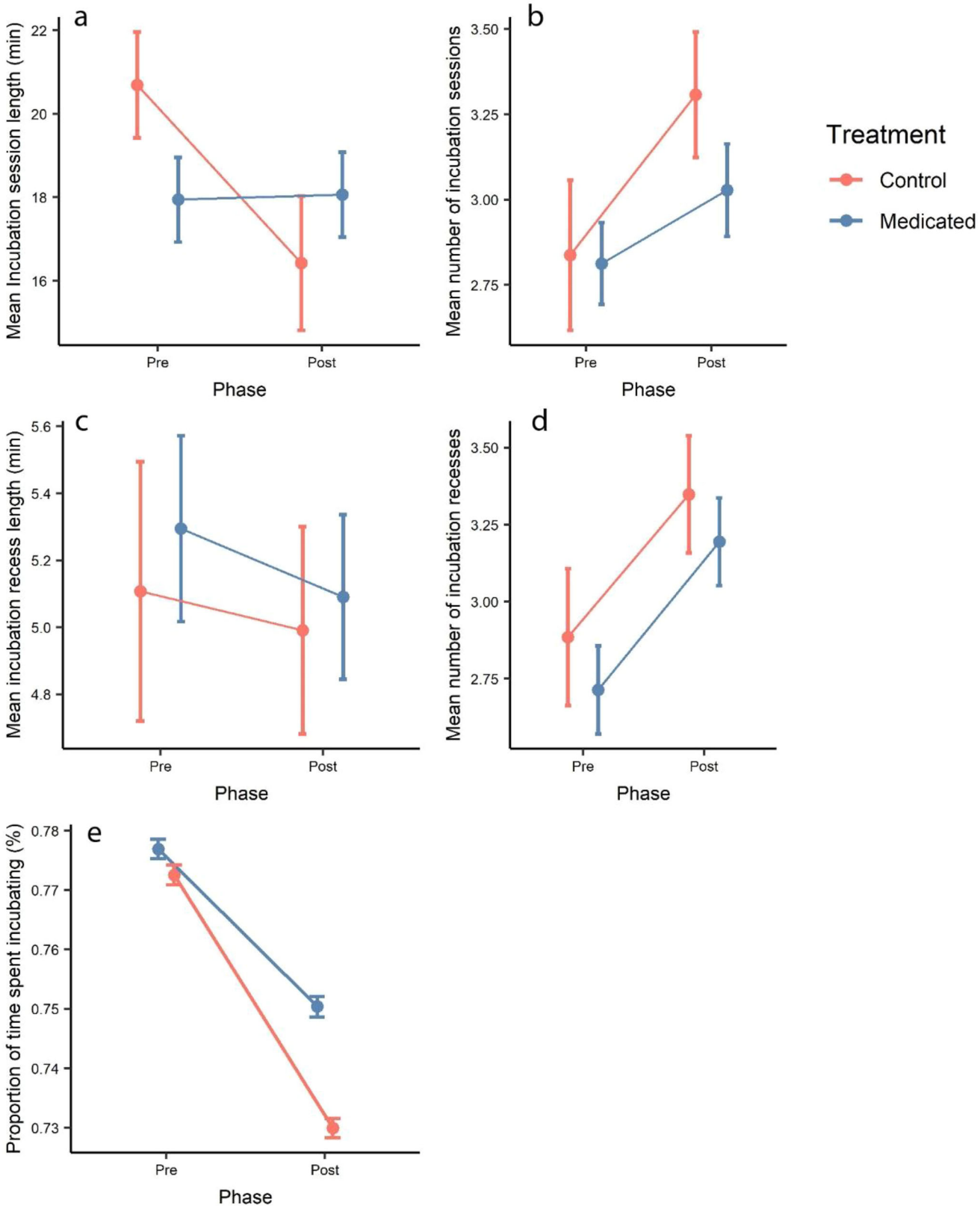

FIGURE 1 | Effects of treatment groups (medicated with primaquine vs. control) and phases (pre and post treatment manipulation) on incubation variables (a–e). Bars for panels (a)–(d) show means ± SE of the mean. Bars for panel (e) show the predicted means ± SE from the glmer model. Predicted means are plotted using the function cat_plot implemented in the "interactions" package.

TABLE 4 | Model estimates of the effects of treatment groups and the proportion of incubation time.

GLMM for proportion of time spent incubating	Estimate	SE	χ^2	df	*p* value
Intercept	1.77	0.18			
Phase (post-)	−0.15	0.01			
Treatment (medicated)	−0.02	0.06			
Clutch size			0.15	1	0.698
Laying date	−0.01	0.00	7.49	1	**0.006**
Female weight at incubation			1.30	1	0.253
Female age			0.01	1	0.888
Phase (post-) × Treatment (medicated)	−0.03	0.01	13.07	1	**< 0.001**

The values of *F*, df, and *p* are given for the variables before being removed from the full model. Estimates and SEs are given for a model containing only significant terms, including those belonging to significant interactions (shown in bold). The control group and pre-treatment phase are, respectively, the treatment group and the phase of reference.

Table S7 for post hoc tests). The hourly number of incubation sessions significantly differed between phases (Figure 2 and Table 5b) but not in interaction with the four infection status by treatment groups ($F_{7,147} = 1.60$, $p = 0.138$; Table 6). Length of incubation recesses did not vary across phases or infection status by treatment groups (Figure 2 and Table 5c), whereas females significantly increased the hourly number of recess sessions between phases (Figure 2 and Table 5d). Lastly, females from all groups significantly reduced the total time spent incubating (%) between phases in interaction with group (Figure 2 and Table 6). More specifically, infected females from the control group exhibited a more pronounced decrease in time spent incubating as compared to infected females from the primaquine group (Table 7).

3.3 | Body Condition and Reproductive Success

Female body condition was not significantly related to the reduction of infection, although it decreased significantly between pre- and post-treatment stages ($t = -12.47$, $p = < 0.001$; Table S3). On the other hand, hatching success, fledgling success, and nestling body condition at fledging were unaffected by treatment (Table 8 and Table S8).

4 | Discussion

Previous experimental studies in birds, which have managed to reduce blood infection through medication, reported detrimental effects of hemoparasites on breeding performance, negatively affecting breeding success and clutch size (Merino et al. 2000; Marzal et al. 2005; Knowles et al. 2010; Schoepf et al. 2022). Similarly, negative effects of other types of parasites, such as helminths, have been found on maternal investment (Reed et al. 2008). Consistent with these studies, our results show that hemoparasite infection in female European pied flycatchers mediated incubation patterns. Primaquine medication reduced parasitic infection in the blood of female birds. The number of infected individuals in the group of medicated females after treatment was significantly reduced, while in the control group, the number of infected individuals did not change significantly. Additionally, we found that (1) *Haemoproteus* infection intensity decreased after treatment in both groups, but decrease was more pronounced in the primaquine group, and (2) the intensity of *Leucocytozoon* infection was reduced only in the medicated group. There was a clear effect of reducing hemoparasite infection on the time females spent in each incubation session. Medicated females had longer incubation sessions in the post-treatment phase, while control females, probably due to the negative effect of hemoparasite infection, reduced the time they devoted to each incubation session. Notably, this pattern was observed regardless of the females' detected infection status, possibly because the medication may have also reduced undetected infections, such as tissue-phase stages not visible in blood smears. However, the reduction of infection did not affect the time females spent in each recess session, meaning they invested the same time on self-maintenance and feeding activities in each session outside the nest-box. There was also no effect on the number of incubation and recess sessions, both increasing in the post-treatment phase. Similar results were shown by Schoepf et al. (2022) in a study medicating red-winged blackbird females (*Agelaius phoeniceus*) with a combination of chloroquine and primaquine in single or multiple doses administered before egg-laying. Medicated red-winged blackbirds invested more in incubation than control females as occurs with pied flycatcher females, but we here additionally show differential changes in individual incubation patterns comparing incubation behavior before and after the treatment between control and medicated females. It is also possible that different parasites or mixed infections by several parasites produce differential effects on females and their behavior (see, e.g., Marzal et al. 2008), but infections by all parasites and mixed infections were reduced after treatment, avoiding separate analyses by each parasite or mixed infections. Therefore, the effects reported here are probably due to the sum of effects by different parasites.

The fact that medicated females had longer incubation sessions is most likely due to the existence of a trade-off between reproductive effort and immunity (Sheldon and Verhulst 1996). That is, medicated females might improve resource allocation to parental care by alleviating the burden imposed by parasites and/or by diminishing investment in immune responses (Sheldon and Verhulst 1996) as seen in other studies analyzing tolerance

TABLE 5 | Model estimates of the effects of infection status (infected/uninfected) by treatment (control/primaquine) groups on incubation variables.

Variable	Estimate	SE	*F*	df	*p* value
(a) LMM for average length of incubation sessions					
Intercept	3.50	0.18			
Phase (post-)	−0.30	0.10			
Group (uninfected-control)	−0.01	0.11			
Group (infected-medicated)	−0.00	0.11			
Group (uninfected-medicated)	−0.23	0.11			
Clutch size			1.31	1,92	0.255
Laying date	−0.01	0.00	12.10	1,94	**<0.001**
Female weight at incubation			0.61	1,93	0.434
Female age			0.50	1,89	0.478
Phase (post-) × Group (uninfected-control)	0.03	0.15	2.87	7,148	**0.007**
Phase (post-) × Group (infected-medicated)	0.28	0.14			
Phase (post-) × Group (uninfected-medicated)	0.35	0.15			
(b) LMM for hourly number of incubation sessions					
Intercept	0.95	0.04			
Phase (post-)	0.13	0.05	6.28	1,97	**0.014**
Group (uninfected-control)			1.15	3,93	0.333
Clutch size			2.82	1,97	0.096
Laying date			1.51	1,95	0.221
Female weight at incubation			0.29	1,92	0.587
Female age			0.99	1,90	0.320
Phase (post-) × Group (uninfected-control)			1.60	7,147	0.138
(c) LMM for average length of recess sessions					
Intercept	1.20	0.27			
Phase (post-)			0.18	1,97	0.67
Group (uninfected-control)			1.15	3,93	0.332
Clutch size			1.75	1,96	0.189
Laying date			0.78	1,92	0.377
Female weight at incubation			0.00	1,92	0.932
Female age			1.56	1,95	0.214
Phase (post-) × Group (uninfected-control)			0.86	7,146	0.533
(d) LMM for hourly number of recess sessions					
Intercept	1.63	0.28			
Phase (post-)	0.18	0.05	10.04	1,98	**0.002**
Group (uninfected-control)			0.63	3,92	0.594
Clutch size	−0.11	0.05	6.14	1,96	**0.015**
Laying date			0.75	1,96	0.387
Female weight at incubation			0.11	1,92	0.741
Female age			0.98	1,94	0.325
Phase (post-) × Group (uninfected-control)			0.10	3,94	0.995

The values of *F*, df, and *p* are given for the variables before being removed from the full model. Estimates and SEs are given for a model containing only significant terms, including those belonging to significant interactions (shown in bold). Control group, pre-treatment phase, and infected group are, respectively, the treatment group, phase, and infection status of reference.

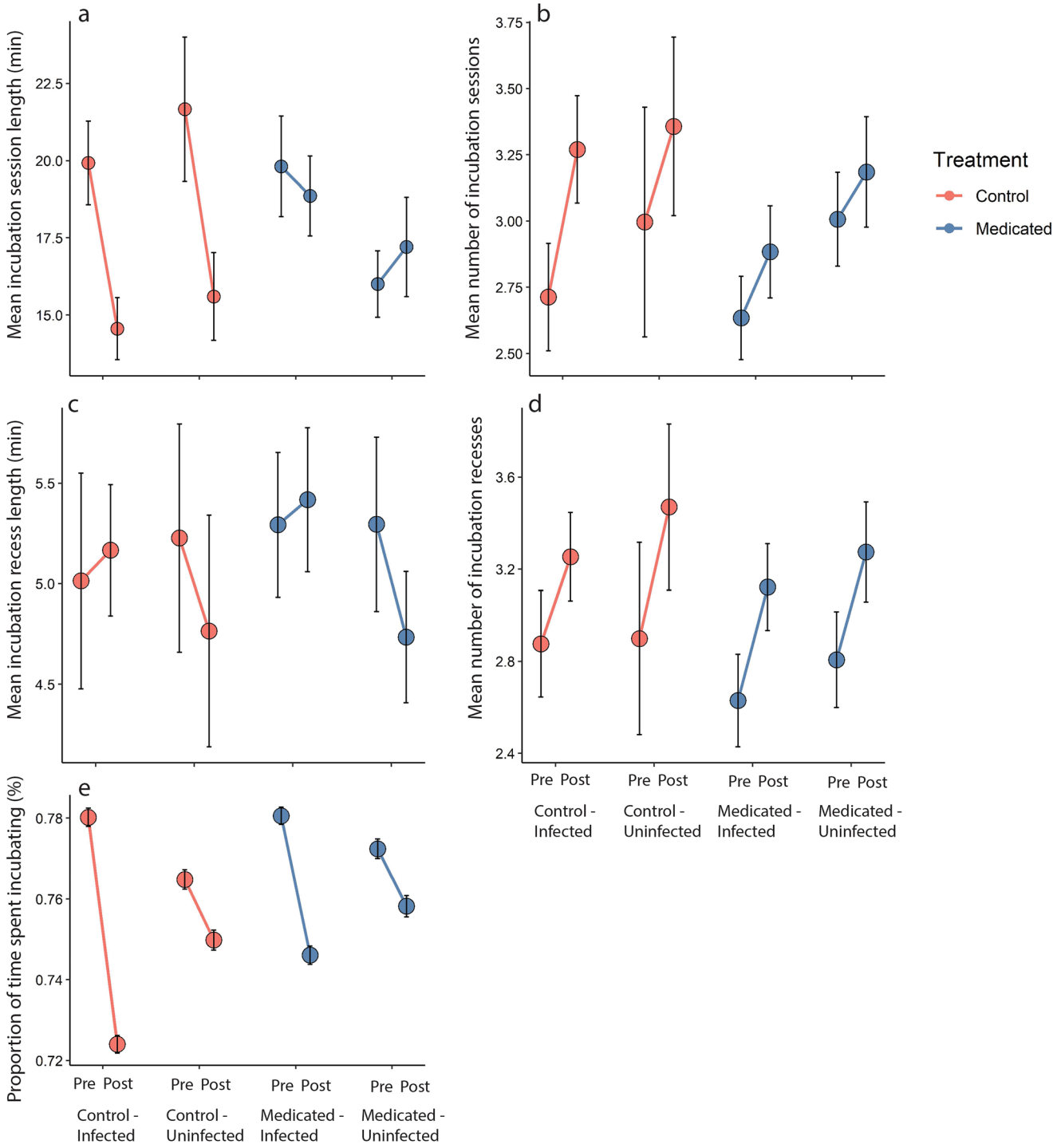

FIGURE 2 | Effects of infection status (infected/uninfected) by treatment (control/primaquine) groups and phases (pre and post medication manipulation) on incubation variables (a–e). Bars for panels (a)–(d) show means ± SE of the mean. Bars for panel (e) show the predicted means ± SE from the glmer model. Predicted means are plotted using the function cat_plot implemented in the "interactions" package.

and resistance to parasites (Arriero et al. 2018). Alternatively, longer incubation sessions by medicated females may be due to a negative effect of the medication, due to gastrointestinal disturbances and the potential development of anemia that primaquine may cause. However, those effects are dose-dependent (Mayorga et al. 1997b) and the use of a single sub-curative dose in our experiment probably avoids important secondary effects of medication. In fact, when we restricted analyses to females found infected, that is, those who clearly benefited from the antiparasitic effect of medication, we obtained similar results.

Consistent with the previous results, we found an effect of the reduction of infection on the proportion of time spent incubating, which decreases in the post-treatment phase in both control and medicated groups, but females in the medicated group showed a less pronounced reduction. In fact, taking into account

TABLE 6 | Model estimates of the effects of infection status (infected/uninfected) by treatment (control/primaquine) groups on the proportion of incubation time.

Variable	Estimate	SE	χ^2	df	*p* value
Intercept	1.78	0.19			
Phase (post-)	−0.19	0.01			
Group (uninfected-control)	−0.05	0.09			
Group (infected-medicated)	−0.01	0.08			
Group (uninfected-medicated)	−0.08	0.09			
Clutch size			0.15	1	0.690
Laying date	−0.01	0.00	7.38	1	**0.006**
Female weight at incubation			1.31	1	0.251
Female age			0.03	1	0.864
Phase (post-) × Group (uninfected-control)	0.11	0.01	186.82	3	**< 0.001**
Phase (post-) × Group (infected-medicated)	−0.05	0.01			
Phase (post-) × Group (uninfected-medicated)	0.09	0.01			

The values of *F*, df, and *p* are given for the variables before being removed from the full model. Estimates and SEs are given for a model containing only significant terms, including those belonging to significant interactions (shown in bold). Control group, pre-treatment phase, and infected group are, respectively, the treatment group, phase, and infection status of reference.

TABLE 7 | Post hoc Tukey tests for proportion of time spent incubating across infection status (infected/uninfected) by treatment (control/primaquine) groups.

Phase	Comparisons	Estimate	SE	*z*-ratio	*p* value
Pre	Medicated/infected—Control/infected	−0.02	0.009	−2.04	0.454
	Medicated/Uninfected—Control/Uninfected	0.02	0.010	2.00	0.480
Post	Medicated/infected—Control/infected	0.08	0.008	8.92	**< 0.001**
	Medicated/Uninfected—Control/Uninfected	0.02	0.010	2.32	0.283

p values < 0.05 are indicated in bold.

the infection status, infected females from the control group exhibited a more pronounced decrease in time spent incubating as compared to infected females from the primaquine group. That is, females decrease incubation time close to hatching (Heppner and Ouyang 2021) possibly due to improvement in environmental conditions as the season progressed (Amininasab et al. 2017). In other words, females have more time for feeding as the season progresses because temperature increases with the season and the eggs do not cool down as quickly during a recess at the end of the incubation period (Ar and Sidis 2002). In agreement with this result, the laying date was negatively related to the average length of incubation sessions. That is, the later the laying date (with higher temperatures), the shorter the average duration of the incubation sessions. However, reduction of incubation time is more marked in control females, probably due to their greater demands for self-maintenance and feeding provoked by infection. Furthermore, the mean number of recess sessions was negatively related to clutch size, implying that fewer eggs require less incubation effort.

It is well-established that the energy expended by female birds in parental care positively influences the growth and health status of pied flycatcher nestlings (Merino et al. 1996; Moreno et al. 1997). Previous studies have shown an increase in hatching success (Marzal et al. 2005) and in fledgling success (Merino et al. 2000; Marzal et al. 2005; Schoepf et al. 2022), as well as a decrease in nestling mortality (Merino et al. 2000) after reduction of hemoparasite infection by medication of adult birds. Furthermore, the reduction in parasite infection could also increase future reproductive success, since it has been seen that infection by hemoparasites is related to a decrease in the body mass of females (Merino et al. 2000) and to the survival of females until the next breeding season, with those with less infection by hemoparasites having a greater probability of reproducing again (Martínez-de la Puente et al. 2010). However, despite the effect on incubation patterns, our results did not show an impact of the reduction in blood parasite infections on the overall fitness. In fact, females from both groups reduced their body condition to a similar level. Female pied flycatchers usually lose mass

TABLE 8 | Model estimates of the effects of treatment groups on reproductive parameters.

Variable	Estimate	SE	*t* value	*p* value
(a) GLM for hatching success				
Intercept	2.80	1.01		
Treatment (medicated)			−0.88	0.383
Clutch size			0.59	0.555
Laying date			−1.00	0.317
(b) GLM for fledging success				
Intercept	10.82	2.99		
Treatment (medicated)			0.05	0.956
Clutch size			−1.93	0.056
Laying date	−0.09	0.02	−4.03	**<0.001**
(c) LM for nestling body condition				
Intercept	1.76	0.47	3.77	<0.001
Treatment (medicated)			1.16	0.248
Clutch size			1.29	0.199
Laying date			1.57	0.119

The values of *F*, df, and *p* are given for the variables before being removed from the full model. Estimates and SEs are given for a model containing only significant terms, including those belonging to significant interactions (shown in bold). The control group and pre-treatment phase are, respectively, the treatment group and the phase of reference.

across the breeding season, a fact interpreted as a facultative adjustment of females to different reproductive tasks (see Potti and Merino 1995; Slagsvold and Johansen 1998). Thus, our results imply that both groups of females lost body mass due to the incubation investment, but control females compensated for the cost of infection by increasing their feeding. Surprisingly, we did not find differences in hatching success, an effect that has previously been reported in our study population in pied flycatcher females infected by *Haemoproteus* parasites (Sanz et al. 2001). Furthermore, we did not find differences between fledgling success and the biometrics of nestlings from both treatments. This could also be because the primaquine treatment did not last the entire reproductive period, so that it was not excessively noticeable during the development of the nestlings. Alternatively, control females (or their male partners) may be able to adjust their parental effort during nestling development and compensate for the negative effect of infection.

Overall, to our knowledge, this is the first study showing changes in individual incubation patterns in response to parasites in a wild bird population. Therefore, our findings add to previous studies showing that hemoparasites have detrimental effects on reproductive success and support the importance of conducting experimental studies that verify the different negative effects of parasites on hosts. Furthermore, since no direct effects on the offspring have been found, it provides an opportunity for new experimental studies to look for potential behavioral changes in females to compensate for the effect of infection while rearing nestlings. That is, we can expect that infected birds modify their feeding patterns by increasing both the number of provisioning and self-maintenance periods to avoid the detrimental effects of parents' health on nestlings.

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Ethics Statement

This study complies with current European legislation on experimental procedures with animals (2013/53/UE) and was reviewed and approved by the "Dirección General de Agricultura, Ganaderia y Alimentación, Comunidad de Madrid" (Spain), Permission PROEX 125.1/23. Annual ringing permissions were provided by the Junta de Castilla y León.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Table S1 Average intensity of infection by different parasites (number of parasites per 2000 erythrocytes ±) in the 47 individuals separated by treatment and stages (pre- and post-treatment). **Table S2** Number of individuals infected by each parasite in the total number of individuals sampled by treatment and stages (pre- and post-treatment). **Table S3** Model estimates of the effects of the experiment on female body condition. **Table S4** Post hoc Tukey tests for average length of incubation sessions across phases and across treatment groups. **Table S5** Model estimates of the effects of treatment groups and phases on incubation variables including only females found infected. **Table S6** Model estimates of the effects of treatment groups and the proportion of incubation time including only females found infected. **Table S7** Post hoc Tukey tests on the average length of incubation sessions across phases for each infection status (infected/uninfected) by treatment (control/primaquine) groups. **Table S8** Model estimates of the effects of infection status (infected/uninfected) by treatment (control/primaquine) groups on reproductive parameters