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Behavioural and fitness effects of experimental immune activation during incubation in a wild passerine

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Incubation is vital for avian embryo development but demands significant parental investment, potentially at the cost of self-maintenance and immune function. For example, the acute phase response (APR), a systemic reaction against inflammation, infection or tissue injury, can trigger sickness behaviours such as reduced activity and foraging. At the same time, immune activation may serve as an internal cue that prompts birds to prioritize current reproduction over potential future reproductive opportunities. In this study, we experimentally exposed wild European Pied Flycatcher *Ficedula hypoleuca* females to an immune challenge through lipopolysaccharide (LPS) injection to investigate the effects of immune activation on female incubation behaviour and its reproductive consequences. We found a clear reduction in the average duration of incubation and recess sessions in those birds administered with LPS, as well as a tendency to increase the number of incubation sessions, while other behaviours remained unaffected. These changes had no apparent effects on fitness-related traits of females, but they were related to fledging success, which was higher in females administered with LPS. These findings suggest that females can maintain sufficient incubation conditions while simultaneously sustaining their own fitness during immune activation, as well as a compensation for potential future reproductive failures by the prioritization of immediate reproduction.

Keywords: immune function, incubation cost, life history, terminal investment.

Incubation is a critical period of the breeding cycle in birds (Tatner & Bryant 1993), and parental effort during this period can have substantial consequences for the reproductive fitness of the pair and offspring (Deeming & Gray 2016). Regulation of egg temperature by incubating parents is vital to both the survival and proper development of avian embryos (Webb 1987), as eggs must be maintained at relatively high temperatures (36–40°C) for optimal embryonic development (DuRant *et al.* 2013). Incubation may reduce the time that a parent bird can devote to foraging for self-maintenance at the expense of egg care (Stearns 1989, Reznick *et al.* 2000, Zera & Harshman 2001), a particularly relevant challenge in species where one parent is solely responsible for this task (Deeming 2002). The resource-limitation hypothesis suggests that resource allocation involves trade-offs between competing functions (Sheldon & Verhulst 1996). For example, investment in immune activation may reduce the resources available for other biological functions such as reproduction (Raberg *et al.* 1998). Therefore, it has been proposed that a resource shift away from the immune system may have reproductive costs and vice versa (Sheldon & Verhulst 1996, Gustafsson *et al.* 1997, Raberg *et al.* 1998).

Physiological trade-offs are, however, not the only explanation for the altered reproductive

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behaviour that follows immune activation. An alternative hypothesis posits that behavioural changes during infection could result from adaptive plasticity, where individuals temporarily reduce reproductive investment in order to promote survival and preserve future reproductive opportunities (Lochmiller & Deerenberg 2000). Conversely, a third hypothesis proposes a 'terminal investment' in which individuals facing infection may increase reproductive effort if future breeding opportunities become uncertain (Clutton-Brock 1984, Velando *et al.* 2006).

When infected, organisms may use multiple strategies to avoid or combat disease, one of which is the activation of the immune system (Schreier & Grindstaff 2020). Upon exposure to a pathogen, the innate immune system is activated as an organism's first line of physiological defence. Activating the immune system can involve fitness costs and affect reproduction, as it requires diverting energy and resources from other functions (Burness *et al.* 2010, Munoz *et al.* 2010, Hasselquist & Nilsson 2012). However, immune system activation can sometimes act as an internal cue signalling potential mortality (Schaller & Park 2011). In this scenario, some organisms facing reduced chances of future survival may increase their current reproductive effort at the expense of their own condition and increasingly uncertain future reproductive success (Clutton-Brock 1984).

The acute phase response (APR) occurs at the onset of infection. It is part of the innate immune system and represents an early and highly complex reaction of the organism to various injuries such as parasitic, bacterial or viral infections (Van Miert 1990). The APR typically involves adjustments in metabolism, including thermoregulation and sickness behaviours (Owen-Ashley & Wingfield 2007, Burness *et al.* 2010). Anorexia (loss of appetite), adipsia (absence of thirst), decreased activity and body-mass loss are common sickness behaviours, adaptations that are believed to lower energy expenditure on activities that are not immediately essential to survival (Hart 1988).

Lipopolysaccharide (LPS) is the major biologically active component of endotoxin, a potent inducer of inflammation (Hewett & Roth 1993) found in the cell wall of Gram-negative bacteria such as *Escherichia coli* (Raetz & Whitfield 2002). LPS has been used for many years as a tool to induce and study inflammatory responses, giving rise to the APR (Roth *et al.* 1997). The administration of LPS enables the study of an individual's behavioural and metabolic responses to a simulated infection, regardless of the specific harm inflicted by a pathogen (Owen-Ashley & Wingfield 2007). The LPS model has advantages over the bacterial infection model, as it is more standardized and reproducible, less expensive to develop and validate, and more accessible.

Avian behaviour during APR has been widely assessed in captive birds and chickens (Teemer & Hawley 2024). Stereotypical behavioural changes in passerines during APR include a reduction in grooming, loss of appetite, adipsia, weight loss, reduced activity and changes in social behaviour (Owen-Ashley & Wingfield 2007). Captive Zebra Finches *Taeniopygia guttata* challenged with LPS presented a decrease in activity and a short-term reduction or cessation in food consumption (Owen-Ashley & Wingfield 2007, Sköld-Chiriac *et al.* 2014). In captive House Finches *Haemorhous mexicanus*, APR induced the expression of sickness behaviours such as overall reduced activity, a decrease in foraging and an increase in resting (Teemer & Hawley 2024). However, studies evaluating APR in wild birds are still scarce. Owen-Ashley and Wingfield (2006) described how LPS reduced territorial aggression and song in Gambel's White-crowned Sparrow *Zonotrichia leucophrys gambelii*. Other reproductive patterns assessed in House Sparrows *Passer domesticus*, such as parental care and nestling feeding rates, have been reduced after LPS treatment (Bonneaud *et al.* 2003). However, fever responses during APR in birds are ambiguous; passerines sometimes respond with fever and sometimes with hypothermia (Sköld-Chiriac *et al.* 2015).

The impact of APR during incubation and immediate consequences for both incubation behaviour and reproductive performance remain poorly understood. In this study, wild European Pied Flycatcher *Ficedula hypoleuca* (henceforth 'Pied Flycatcher') females were exposed to an immune challenge through LPS injection at the end of the incubation period. We tested whether immune activation affected incubation behaviour, and whether this effect was consistent with resource trade-offs, adaptive plasticity or other regulatory mechanisms. At the same time, we evaluated the effect of immune activation on reproductive success-associated parameters. We predicted that females exposed to an immune challenge would change their incubation behaviour, but we also considered the possibility that birds may maintain or alter incubation effort based on perceived future reproductive value, which could have a possible effect on reproductive parameters.

METHODS

Study area and species

The study was conducted in the spring of 2024 in a montane deciduous forest of Pyrenean oak *Quercus pyrenaica* in Valsaín, central Spain (40°54′N, 04°01′W; 1200-m elevation). In the study area, there are 300 nestboxes occupied by Pied Flycatchers and other cavity-nesting birds, such as Great Tits *Parus major*, Eurasian Blue Tits *Cyanistes caeruleus*, Eurasian Nuthatches *Sitta europaea* and Rock Sparrows *Petronia petronia*.

We followed breeding activities from the early stages of nest construction to fledging in nestboxes occupied by Pied Flycatchers. The breeding period for this species runs from mid-April, when the first males return after migration, until early July, when most nestlings have fledged. Egg-laying in this population usually begins around mid-May (Cantarero *et al.* 2013a), with a modal clutch size of six eggs (Lundberg & Alatalo 1992). Pied Flycatchers are typically single-brooded with biparental care. Polygamous relationships occur frequently in this species (Alatalo *et al.* 1981). The female incubates alone for approximately 14 days, being partially fed by her mate (Cantarero *et al.* 2014). The female also broods the nestlings until the age of 7 days (Sanz & Moreno 1995), but both parents usually contribute to feeding the nestlings after hatching (Cantarero *et al.* 2014). Nestlings usually fledge at the age of 17 days.

Experimental design

Forty-six female Pied Flycatchers with laying dates between 36 and 46 (1 April = Day 1) were selected for the experiment. Breeding activities were followed up by daily visits to determine the laying date and clutch size. Females were sequentially assigned to either the control or the LPS treatment group as they started incubation. This random allocation ensured that potential confounding factors such as laying date and clutch size were evenly distributed between treatments. On day 11 of incubation, females were hand-captured in the nestbox in the early morning, as they are unafraid and rarely leave the nest at this stage. The exact age (number of years) of the females older than 1 year was established if ringed as nestlings in the study area or, for unringed birds, classified as yearlings or older based on the colour and wear of the inner great wing coverts according to Svensson (1984) and Jenni *et al.* (2011). Following Alonso-Alvarez *et al.* (2004), females belonging to the experimental group ($N = 24$) received an immune challenge to mimic a bacterial infection via subcutaneous injection into the back of bacterial LPS (LPS serotype O55:B5; Sigma-Aldrich) at a concentration of 0.01 mg of LPS diluted in 0.1 mL of phosphate-buffered saline (PBS). Females assigned to the control group were similarly injected with the same quantity of PBS ($N = 22$). Because we wished to study the short-term impact of LPS and minimize any adverse effects, we opted for a low-concentration single dose. In addition, females were individually identified based on their existing metal rings or, if needed, newly ringed using numbered metal rings, and body-mass was weighed to the nearest 0.01 g with a digital balance. Handling time was minimized to avoid unnecessary stress. We decided to carry out this immune activation at the end of incubation to avoid potential desertions, as females are more likely to desert their nests when handled at the beginning of incubation (Criscuolo 2001, Johnston 2011). In this study, no female deserted after manipulation.

The number of nestlings was recorded 3 days after the observed hatching date, by which time all eggs should have hatched. Hatching success was calculated as the proportion of eggs that successfully hatched. Seven days after the hatching date, adult birds were captured when entering to feed the nestlings, by using a passive trap placed inside their nestbox. The trap was left for a maximum of 1 h and was removed once both adults were captured. Adult birds were identified from their rings or assigned new numbered rings. Tarsus-length was measured with a digital calliper to the nearest 0.01 mm; wing length was measured with a stopped ruler to the nearest 0.1 mm, and their body-mass was measured as stated above. Nestlings were ringed and measured at 13 days old in the same way as adults. The body condition of both the parents and nestlings was evaluated by dividing body-mass by (tarsus-length)^3, following Griggio and Hoi (2010). Fledging success was calculated as the proportion of hatched nestlings that successfully fledged.

Video recordings

On day 10 (pretreatment) and 12 (post-treatment) of incubation, we recorded nest activity of each nest for about 80 min (79.14 ± 2 min se, $N = 46$) with digital video cameras (Sony Dcr-sr190, with an extra Sony NP-FH100 battery). Cameras were placed 20 m away from the nestbox tree to minimize any potential disturbance to the incubating female and recorded an area of approximately 1 m around the nestbox. All of the video recordings were performed in a maximum of two daily rounds using seven cameras simultaneously, which were deployed at approximately the same time. The use of video cameras to record incubation patterns is a widely used method in behavioural ecology (Kovařík *et al.* 2009, Amininasab *et al.* 2016a) and has demonstrated a high correlation with the incubation patterns obtained with other methods, such as passive integrated transponder (PIT) tagging (Bueno-Enciso *et al.* 2017, Bambini *et al.* 2018, Capilla-Lasheras 2018, Arct *et al.* 2022). We selected an 80-min observation period as this is sufficient to yield valuable insights into incubation behaviours, and this encompasses several on- and off-bouts (Nord & Nilsson 2012, Bueno-Enciso *et al.* 2017, Diez-Méndez *et al.* 2021). All videos were recorded between 08:00 AM and 01:00 PM.

Behavioural data analysis

VLC Media Player software (VideoLan 2006) was used for the visualization of recordings. We calculated the average duration (in min) of incubation sessions and recess sessions, the hourly number of incubation and recess sessions, as well as the 'egg attendance' or proportion of time spent by the female inside the nestbox. This activity includes the period dedicated almost exclusively to incubating and rotating the eggs (Kovařík *et al.* 2009, Cantarero *et al.* 2013b, Amininasab *et al.* 2016a, 2016b), where other activities unrelated to incubation, such as grooming or nest sanitation, represent a negligible part (barely 1%) of the total time spent on the main activity (Cantarero *et al.* 2013a). The hourly frequency of male provisioning visits (hourly male feeds) was also determined in each recording.

'Length of incubation sessions' was defined as the total time (in min) that a female spent inside the nestbox between entering and leaving it, excluding sessions where the female was already inside at the start or still inside at the end of the recording. 'Length of recess sessions' was defined as the total time (in min) a female spent outside the nestbox between leaving and re-entering it, excluding cases where the female was already outside at the start or remained outside at the end of the recording.

Data analysis

In order to ensure that treatment groups were balanced, we first used Student's *t*-tests to control for the effects of any confounding variables between experimental groups, such as laying date, clutch size, brood size, female body condition, female age, and dates and hours of recording.

Four different models were used to investigate behavioural changes in incubation patterns owing to LPS treatment, as they allowed us to control for more variables compared to other models (see Table 1). Female age (quadratic) was also included as an explanatory variable to explore possible non-linear distributions, which are common in reproductive parameters of small passerines (Fay *et al.* 2021, Martyka *et al.* 2023). 'Female ID' was included as a random factor to account for repeated measures. We found a strong association between the hourly number of incubation and recess sessions ($r = 0.9371$; $P < 0.001$) and thus restricted the model to only the first of these two variables. The dependent variables in these models were log-transformed to improve the normality and homoscedasticity of model residuals. To further investigate whether LPS treatment affected incubation behaviour, we conducted an additional model on the proportion of total time spent incubating (time spent incubating/total time recorded). Here, the 'proportion of time spent incubating' was modelled using the same fixed and random structure as the previous models (Table 1).

Lastly, we explored whether LPS treatment affected hourly male feeds during days 10 and 12 of incubation, as well as hatching success (proportion of eggs that successfully hatched), fledging success (proportion of hatched nestlings that fledged) and nestling body condition at fledging (Table 1). For hatching success, we ran a generalized linear model

Table 1. Summary of statistical models and variables used to evaluate the effects of LPS treatment on incubation behaviour, parental investment and reproductive success.

Research goal/dependent variable	Model type	Distribution/link function	Fixed factors and covariates	Random effects
Group balancing (female condition-g/mm^3, tarsus-length-mm, wing length-mm, laying date-Day)	LM	Gaussian/ identity	Treatment groups	N/A
Group balancing (age-years and clutch size)	GLM	Poisson/log	Treatment groups	N/A
Overall treatment effect on female body condition (g/mm^3)	LMM	Gaussian/ identity	Treatment, Stage, Treatment × Stage	Female ID
Incubation patterns (session length-min, and hourly session number for both incubation and recess)	LMM (4 models)	Gaussian/ identity (log-transformed)	Treatment, Phase, Treatment × Phase; Covariates: clutch size, laying date, female body condition, female age	Female ID
Proportion of time spent incubating (% time inside nestbox)	GLMM	Quasibinomial/ logit	Same as for incubation patterns (above)	Female ID
Hourly male feeds (days 10 and 12)	GLMM	nbinom1/log	Treatment, Phase, Treatment × Phase; Covariates: clutch size, laying date, female body condition, female age	Female ID
Hatching success (% eggs that hatched)	GLM	Quasibinomial/ logit	Treatment, clutch size, laying date	N/A
Fledging success (% hatched nestlings that fledged)	GLM	Quasibinomial/ logit	Treatment, clutch size, laying date	N/A
Nestling body condition at fledging (g/mm^3)	LMM	Gaussian/ identity	Treatment, clutch size, laying date	Female ID
Within-brood CV (nestling condition-g/mm^3)	Student's *t*-test	N/A	Treatment	N/A

(GLM); for fledging success, we ran two GLMs. After comparing both, we retained the model without the interaction with female age, as it explained more variation in fledging success, and offered a more parsimonious and better-fitting representation of the data. Differences in means and standared error of 'clutch size' and 'brood size' between groups were tested to make sure the factors were unbiased (all *P*-values >0.05). We calculated 'nestling body condition' at fledging and we used a separate linear mixed model (LMM) (Table 1). The within-brood coefficient of variation (CV) for nestling condition was also calculated (CV = (sd/mean) × 100%) and statistical differences between groups were assessed using a Student's *t*-test. All of the statistical models and variables used to evaluate the effects of LPS treatment on incubation behaviour, parental investment and reproductive success are specified in Table 1.

We performed all LMMs with the *lmer* function in the 'lme4' package (Bates *et al.* 2015). For GLMs we used the glm() function and for generalized linear mixed models (GLMMs) we used the 'glmmTMB' package (Brooks *et al.* 2017) in the R environment (R Core Team 2017). To test the significance of 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 & Højsgaard 2014). We carried out planned contrast tests using the *emmeans* function in the 'emmeans' package (Lenth 2020). The significance of the fixed effects in the GLMM was estimated with the function *Anova*, implemented by the 'car' package. Overdispersion and zero-inflation in the GLMs and GLMMs were assessed using the 'performance' package (Lüdecke *et al.* 2021). Significance was taken at $\alpha = 0.05$. We assessed collinearity between covariates (clutch size, laying date, female weight and female age) in the models using the 'car' package (Bates *et al.* 2007) and found no evident correlation among them. Model residuals were tested for normality and homoscedasticity, also using the 'car' package.

RESULTS

LPS treatment and female variables

We found no differences in any female attributes (weight at incubation, tarsus length and wing length) across the different treatment groups before the experiment (all *P*-values >0.05). As expected, female body condition decreased significantly between the incubation (day 11) and the chick feeding stage (day 7 of nestling age; $F_{1,43} = 8.46$, $P = 0.006$), being affected by neither the experimental treatment ($F_{1,43} = 1.01$, $P = 0.321$) nor the interaction between stage and treatment ($F_{1,43} = 0.997$, $P = 0.324$). Student's *t*-tests showed no significant differences in experimental groups for laying date, clutch size, brood size, female body condition, and dates and hours of recording of the first and second video (Table 2). However, female age differed significantly between groups, with control females being slightly older than LPS females (Table 2).

Incubation patterns

We found a significant interaction between treatment and phase (pre- vs post-treatment) of the average length of incubation sessions (Table 3a). Specifically, *post hoc* tests showed that the average length of incubation sessions differed between phases in the LPS group ($t = 3.52$, $P = 0.005$; Fig. 1a) being shorter in the post-treatment phase, but not for the control group ($t = 0.62$, $P = 0.925$; Fig. 1a).

The hourly number of incubation sessions was significantly affected by phase (Fig. 1b; Table 3b). The interaction between treatment and phase approached statistical significance (Table 3b) and *post hoc* tests indicated that the hourly number of incubation sessions significantly increased in the post-treatment phase only in the LPS group ($t = -3.86$, $P = 0.002$; Fig. 1b).

Table 3. Model estimates of the effects of treatment groups, phases (pre- vs post-treatment) and breeding variables on the length and frequency of incubation and recess sessions. *P*-values <0.05 are indicated in bold.

Variable	*F*-estimate	df	df (KR)	*P*
(a) LMM for length of incubation sessions				
Phase (Post-) × Treatment (Control)	7.34	1	439	**0.007**
Phase (Post-)	13.67	1	438	**<0.001**
Treatment (Control)	0.96	1	39	0.334
Clutch size	0.14	1	38	0.706
Laying date	0.36	1	38	0.550
Female body condition (incubation)	0.17	1	38	0.678
Female age	0.04	1	40	0.850
Female age^2	0.04	1	40	0.845
(b) LMM for hourly number of incubation sessions				
Phase (Post-) × Treatment (Control)	3.77	1	43	0.059
Phase (Post-)	11.94	1	43	**0.001**
Treatment (Control)	0.61	1	38	0.439
Clutch size	0.03	1	38	0.861
Laying date	0.28	1	38	0.598
Female body condition (incubation)	0.03	1	38	0.858
Female age	0.01	1	38	0.924
Female age^2	0.07	1	38	0.934
(c) LMM for length of recess sessions				
Phase (Post-) × Treatment (Control)	3.99	1	457	**0.046**
Phase (Post-)	0.09	1	456	0.767
Treatment (Control)	0.42	1	39	0.519
Clutch size	3.91	1	38	0.055
Laying date	14.43	1	38	**<0.001**
Female body condition (incubation)	0.24	1	38	0.628
Female age	0.28	1	39	0.599
Female age^2	0.41	1	39	0.525

Table 2. Mean ± se values and differences between treatment groups in breeding, female and video recording variables. *P*-values <0.05 are indicated in bold.

Variable	Control	Experimental	*t*-value	*P*
Laying date	41.09 ± 0.61	41.74 ± 0.62	0.76	0.454
Clutch size	5.95 ± 0.10	5.91 ± 0.13	−0.26	0.799
Brood size	5.27 ± 0.20	5.17 ± 0.27	−0.30	0.767
Female age	3.63 ± 0.29	2.48 ± 0.20	−3.29	**0.002**
Female body condition (incubation)	0.003 ± 0.00	0.003 ± 0.00	0.53	0.599
Date of recording of video 1	55.00 ± 0.58	55.61 ± 0.61	0.73	0.470
Hour of recording of video 1	09:27:03 ± 11:23	09:53:00 ± 12:29	1.55	0.128
Date of recording of video 2	57.05 ± 0.59	57.61 ± 0.61	0.67	0.505
Hour of recording of video 2	09:48:05 ± 10:40	09:41:39 ± 10:32	−0.43	0.666

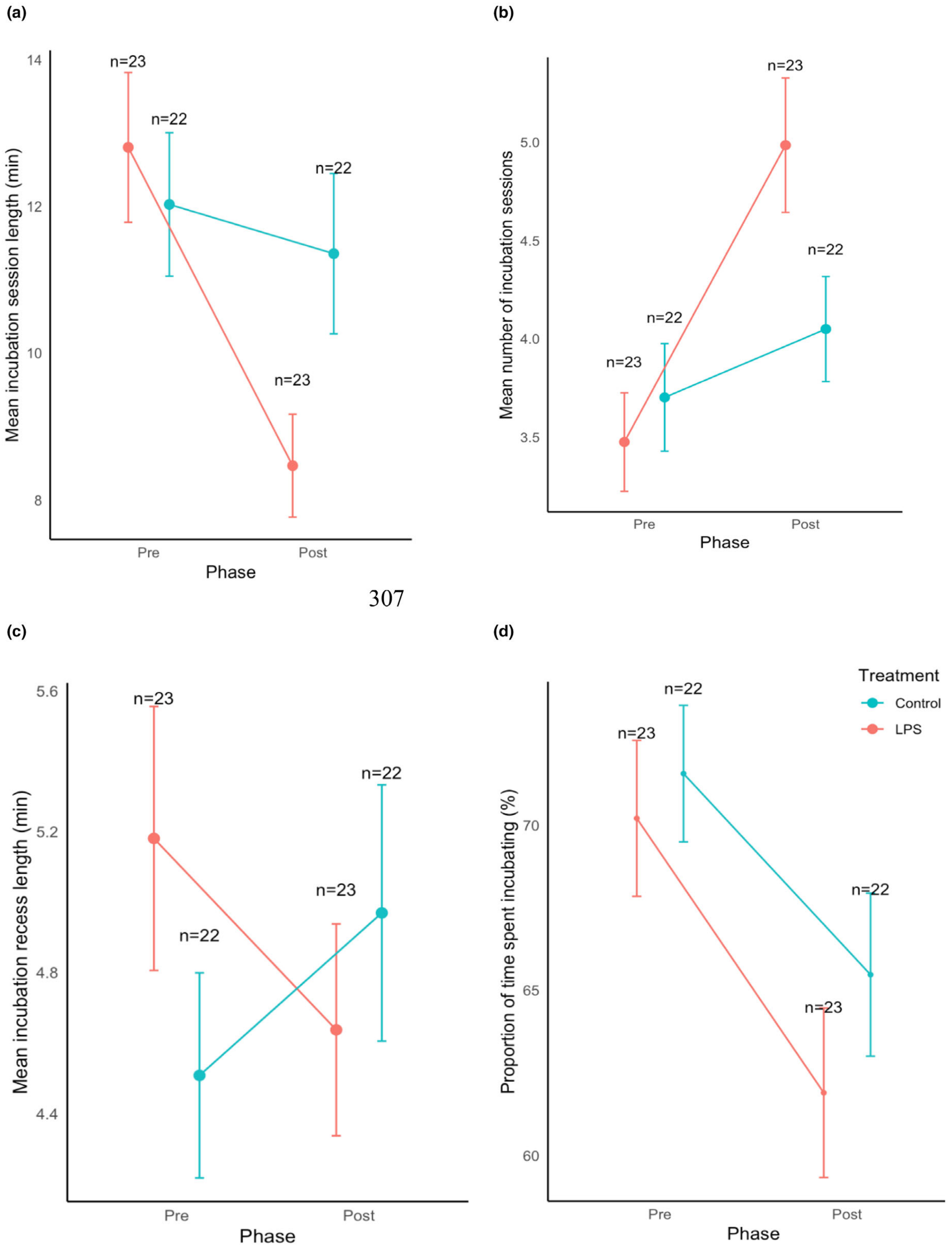

Figure 1. (a–d) Differences in four incubation variables for Pied Flycatcher pairs for which females were (LPS) or were not (control) exposed to an immune challenge, before and after exposure (phase). Bars show means ± se (raw data).

The average length of recess sessions also varied across treatment groups and phases (Fig. 1c; Table 3c), and was positively related to laying date, that is, earlier laying dates had shorter recess sessions (Table 3c).

The percentage of time incubating was affected by phase, showing a general decrease after treatment (Fig. 1d; Table 4), but there was no effect of the treatment (Table 4). Laying date negatively affected the percentage of time spent incubating; females that laid earlier in the season spent more time incubating (Table 4).

LPS treatment of females did not have an overall effect on hourly male feeds, but laying date did have a significant effect (Table 5), with earlier laying dates having a higher frequency of hourly male feeds.

We found no significant relationships between behavioural variables and time of day ($P > 0.05$ in all tests).

LPS treatment and reproductive success

The difference in within-brood CV for nestling condition between LPS broods (CV = 18.21%) and control broods (CV = 21.09%) was not significant ($t = -0.48$, df = 41, $P < 0.632$). Hatching success and nestling body condition at fledging were unaffected by treatment (Table 6a,c). However, fledging success was affected by clutch size, laying date and treatment (Table 6b), resulting in higher fledging success in LPS broods than control broods (Fig. 2).

Table 4. Model estimates of the effects of treatment groups, phases (pre- vs post-treatment) and breeding variables on the proportion of time spent incubating. *P*-values <0.05 are indicated in bold.

GLMM for the proportion of time spent incubating	β-estimate	se	*z*-value	*P*
Intercept	5.07	1.13	4.48	<**0.001**
Phase (Post-)	0.16	0.05	3.35	**0.002**
Treatment (Control)	−0.03	0.06	−0.49	0.628
Clutch size	−0.11	0.10	−1.15	0.259
Laying date	−0.07	0.02	−3.36	**0.002**
Female body condition (incubation)	−134.77	233.00	−0.58	0.566
Female age	0.28	0.29	−0.98	0.334
Female age^2	0.04	0.04	1.00	0.319
Phase (Post-) × Treatment (Control)	0.02	0.05	0.49	0.625

Table 5. Model estimates of the effects of treatment groups, phases (pre- vs post-treatment) and breeding variables on number of male feeds per hour. *P*-values <0.05 are indicated in bold.

Variable	β-estimate	se	*z*-value	*P*
GLMM for hourly male feeds				
Intercept	2.88	2.28	1.26	0.207
Phase (Post-)	0.04	0.09	0.44	0.661
Treatment (Control)	0.06	0.10	0.62	0.534
Clutch size	0.01	0.19	0.06	0.949
Laying date	−0.07	0.02	−3.22	**0.001**
Female body condition (incubation)	−0.36	1.18	−0.30	0.764
Female age	0.36	0.68	0.53	0.595
Female age^2	−0.06	0.08	−0.76	0.449
Phase (Post-) × Treatment (Control)	−0.10	0.11	−0.87	0.382

DISCUSSION

The immune system is a key defence mechanism to combat parasites and disease, and infection is traditionally expected to divert resources away from costly reproductive behaviours towards self-maintenance. However, recent studies have demonstrated that under low-dose infections some organisms increase their current reproductive effort over future reproductive opportunities (Hudson *et al.* 2020). Consistent with this

Table 6. Model estimates of the effects of treatment groups and breeding variables on hatching and fledging success, and nestling condition. *P*-values <0.05 are indicated in bold.

Variable	*F*-estimate	df	df (res)	*P*
(a) GLM for hatching success				
Treatment	0.00	1	207	0.949
Clutch size	0.00	1	207	0.962
Laying date	0.06	1	207	0.814
(b) GLM for fledging success				
Treatment	5.41	1	137	**0.021**
Clutch size	5.61	1	137	**0.019**
Laying date	7.07	1	137	**0.008**
(c) LMM for nestling body condition				
Treatment	0.42	1	39	0.523
Clutch size	0.91	1	42	0.346
Laying date	0.93	1	39	0.341

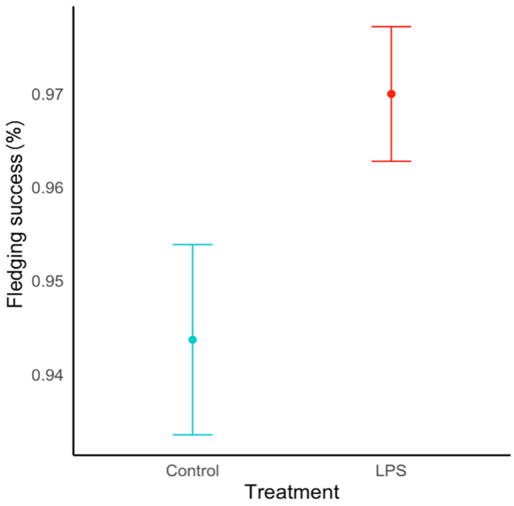

Figure 2. Differences in fledging success (% hatched nestlings that fledged) of Pied Flycatcher pairs for which females were (LPS) or were not (control) exposed to an immune challenge. Bars show means ± se (raw data).

framework, our field study demonstrates that immune activation in female Pied Flycatchers induced behavioural changes in their incubation patterns and an increase in their reproductive success. There was a reduction in the average duration of incubation and recess sessions in those birds administered with LPS, and a tendency towards increasing the number of incubation sessions, while the body condition of females did not vary. Despite these behavioural adjustments, neither the proportion of time spent incubating nor the hourly rate of male feeds differed between treatments, allowing females to maintain proper incubation conditions for the developing embryos, and therefore resulted in similar rates of hatching success between groups. Moreover, LPS-treated females may have exhibited behavioural changes not only during incubation, but also during the nestling feeding stage (Burness *et al.* 2018), which could help explain the higher fledging success observed in their broods.

LPS administration is known to trigger an APR, a complex systemic early-defence system that causes behavioural changes such as inactivity, lethargy and anorexia (Hart 1988, Parejo-Pulido *et al.* 2024), with a peak occurring from 3 to 24 h after infection and lasting no more than 48 h (Kozak *et al.* 1994, Parejo-Pulido *et al.* 2024). In addition, LPS effects are documented to also occur beyond the APR (Sandell *et al.* 2009, Grindstaff *et al.* 2012, Serra *et al.* 2012, Parejo-Pulido *et al.* 2025). In our study, we found that LPS-treated females modified their incubation behaviour after the treatment, by shortening their incubation and recess sessions in the post-treatment phase and increasing their number.

Rather than increasing energy intake, the higher frequency of shorter foraging trips observed in immune-challenged females is likely to reflect their elevated energy expenditure and the need to regularly return to warm the eggs (Zanette *et al.* 2011). Given the energetic cost of travel between the nest and feeding sites, this behaviour may reduce foraging efficiency and contribute to greater physical strain (Poulin 1994, Moreno & Møller 2011). Hence, the change in incubation pattern appears to be a consequence of increased energetic demands imposed by infection, rather than an adaptive strategy aimed at maximizing energy intake. Studies have found that higher habitat quality, characterized by increased caterpillar availability, allows Pied Flycatcher females to reduce the number of off-bouts and increase nest attendance (Koski *et al.* 2020), and birds in poorer condition leave the nest more often to forage (Martin 1987, Webb 1987, Sanz 1996). Under this scenario, females treated with LPS may need to break incubation more frequently to feed more often in response to activation of the immune system in response to infection (García-del Río *et al.* 2026). The expression of sickness behaviours in passerines appears to modulate reproductive effort in both wild and captive populations (Adelman *et al.* 2010, Andreasson *et al.* 2025). Generally, stereotypical behavioural changes in passerines in response to infections include a reduction in grooming, loss of appetite, absence of thirst, weight loss, reduced activity and changes in activity (Owen-Ashley & Wingfield 2007).

Specifically, LPS-induced changes in behaviour have been described in numerous studies in Zebra Finches (Grindstaff *et al.* 2012, Sköld-Chiriac *et al.* 2014, Love *et al.* 2024), House Finches (Teemer & Hawley 2024) and nestling Spotless Starlings *Sturnus unicolor* (Parejo-Pulido *et al.* 2024). Therefore, alteration of incubation patterns in response to immune challenge is an expected result. It should be noted that all of these experiments were conducted under captive conditions, where the birds were provided with food *ad libitum* (Zebra Finches and House Finches) or under strictly controlled conditions (Spotless Starlings).

This could explain why, even while experiencing an immune challenge, female Flycatchers apparently increased their activity, as they had to offset the effects of greater energy expenditure by increasing their feeding rates. In contrast, studies examining the effects of LPS-induced APR in wild birds are scarce. Reproductive patterns assessed in House Sparrows, such as parental care and nestling feeding rates, have been described as reduced after LPS treatment (Bonneaud *et al.* 2003). White-crowned Sparrows exhibited abnormal songs, elevated alarm behaviour and slower singing rates after short-term immune challenges (Munoz *et al.* 2010).

We cannot exclude that LPS females may have experienced some degree of body temperature increase (fever) in response to the infection (Møller 2010, Sköld-Chiriac *et al.* 2015). Although immune activation can also be induced by other substances, the induction of the APR by LPS injection has been described in numerous studies (Bonneaud *et al.* 2003, Bowers *et al.* 2012, 2015). In fact, LPS has been shown to induce fever in many laboratory animals (e.g. guinea pigs, rabbits, rats), reaching peaks from a few hours to 24 h post-injection (Kozak *et al.* 1994, Møller 2010, Sköld-Chiriac *et al.* 2015). In passerine birds, the optimal temperature for embryo development is 36–40°C, and prolonged exposure to temperatures above 40.5°C is usually fatal (Griffith *et al.* 2016). An increase in body temperature might expose clutches to temperatures outside this optimal range, which could damage developing embryos. Thus, incubating birds should optimize their incubation patterns within limits imposed by the onset of developmental abnormalities and foraging needs. The reduction of the length of the incubation sessions in combination with a larger number of sessions could serve as a strategy to avoid the overheating of the eggs, resulting in smaller temperature fluctuations for the developing eggs (Arct *et al.* 2022). However, this hypothesis would need specific testing. We did not measure the temperature of females during the APR, as it would have required a second capture of the females in a very short time, possibly causing some nests to be abandoned and complicating the study of behavioural responses after infection. Thus, it cannot be assumed that females experienced an increase in body temperature because fever during an APR in passerines is not always present (Sköld-Chiriac *et al.* 2015). Andreasson *et al.* (2025) described that the appearance of fever during an immune challenge in Eurasian Blue Tit females was strongly related to brood size, and some infected females experienced hypothermia. Additionally, the ambient temperature of the nestbox was not recorded, and this could have contributed to explaining the results (Conway & Martin 2000, Ardia *et al.* 2006, Bleu *et al.* 2017). Secondly, if there was an increase in body temperature resulting from LPS, females should have experienced a decrease in activity to lower energy expenditure (Hart 1988), which was not the case.

Recess duration also varied with laying date, with shorter recesses early in the season, which is likely to reflect lower ambient temperatures and increased demands for nest temperature maintenance (Conway & Martin 2000, Amininasab *et al.* 2016b).

It was anticipated that the duration of the immune challenge may have been too short to induce changes in hourly male feeding rates between treatment and control groups, which has been seen in other studies when the time elapsed between female manipulation and the behavioural measure duration is longer (Cantarero *et al.* 2014). This lack of compensation by the males may further explain why females had to break incubation more frequently to feed. In addition, LPS-induced loss of appetite could reduce the frequency of food-begging behaviour in females (Parejo-Pulido *et al.* 2024). The effect of laying date on the hourly male feeds could be explained by the anomalous freezing ambient temperatures experienced during mid-April, which reduced the availability of food for insectivorous birds and forced males to work harder where laying dates were earlier.

The proportion of time spent incubating declined in both groups as hatching approached, consistent with previous studies showing reduced incubation requirements late in development (Bueno-Enciso *et al.* 2017, García-del Río *et al.* 2026), as the incubation time requirement of the eggs decreases at this stage of the incubation phase (Heppner & Ouyang 2021). We can hypothesize that conducting the experiment at earlier stages of incubation might have resulted in a higher proportion of incubation time in both groups (Conway & Martin 2000, Ardia *et al.* 2006). As there were no differences in the proportion of incubation time between groups, we can assume that LPS-induced females were able to maintain proper conditions for the developing embryos. Alternatively, the dose might have not been strong enough to induce differences, as effects of LPS in behaviour could be dose-dependent (Armour *et al.* 2020, Butler *et al.* 2021).

There were no significant differences in hatching success and nestling body condition between groups. This outcome could be explained by the behavioural adjustments made by females that could allow maximized egg performance, helping to mitigate the detrimental effects of the APR on incubation and, consequently, on the clutch. Some studies have shown that females adjust their incubation behaviour to reduce temperature variation without compromising egg viability (Cooper & Voss 2013). In addition, experimental studies evaluating the effects of cold stress have found that intermittent cooling does not increase embryonic mortality, suggesting that even if LPS females altered their behaviour, this would not necessarily affect hatching success (Suarez *et al.* 1996). Also, the short duration of the immune challenge might have been insufficient to impact these two reproductive parameters, at least in comparison with the effect expected from a real, long-lasting infection. The timing of the experiment could be considered a limitation too, as behavioural changes during the later stages of incubation, when embryos are at an advanced stage of development, are expected to have a lesser impact compared to earlier stages, when embryos are more sensitive to fluctuations of the nest environment (Khalil *et al.* 2024). When analysing the within-brood CV for nestling condition, we also found that there was a larger variance in body condition in the control broods compared to the LPS broods, although this difference was not significant.

Female behavioural adjustments were more consistent with the finding that fledging success was higher for LPS than for control females. Higher rates of fledging success in infected (Bowers *et al.* 2012) or LPS-induced (Sanz & Moreno 1995, Williams *et al.* 1999, Sköld-Chiriac *et al.* 2019) female passerines have been observed in some previous studies, aligning with predictions of terminal investment theory (Clutton-Brock 1984). When future survival prospects are reduced, individuals may increase current reproductive effort (Clutton-Brock 1984, Bowers *et al.* 2012, 2015, Schulz *et al.* 2023).

LPS injections have been demonstrated to increase plasma corticosterone in birds (Johnson *et al.* 1993). Fluctuations in corticosterone levels in animals have been found to promote the preservation of energy for survival rather than reproductive investment (Wingfield & Sapolsky 2003, Angelier *et al.* 2009, Romero *et al.* 2009, Lothery *et al.* 2014). However, in short-lived species with limited reproductive opportunities during their lifespan, moderate increases in glucocorticoid production are predicted to enhance rather than suppress reproductive effort, encouraging parents to increase their foraging and feeding behaviour (Bowers *et al.* 2015, Sapolsky 2021). The terminal investment theory has been supported across multiple studies in birds and insects (Bonneaud *et al.* 2004, Sköld-Chiriac *et al.* 2019, Hudson *et al.* 2020). For example, House Sparrows subjected to immune system activation showed increased breeding effort, with induced females laying more replacement clutches than controls (Bonneaud *et al.* 2004). Similarly, in House Wrens *Troglodytes aedon*, maternal immune stimulation led to heightened reproductive investment, mediated by increased glucocorticoid production (Bowers *et al.* 2015). In Common Eiders *Somateria mollissima*, immune-challenged females had longer incubation periods and greater body-mass loss, indicating a shift in reproductive priorities consistent with reduced survival expectations (Hanssen 2006). In Zebra Finches, immune-challenged females increased clutch size by 20%, showing that terminal investment can occur early, during egg laying, without compromising body condition (Sköld-Chiriac *et al.* 2019). In addition, our results align with those of Burness *et al.* (2018), who found an increase in fledging success in Tree Swallow *Tachycineta bicolor* females when exposed to LPS. Unexpectedly, LPS-treated females in our study were younger than controls yet achieved higher fledging success. As reproductive performance typically increases with age in Pied Flycatchers up to approximately 3.5 years (Sanz & Moreno 2000, Sendecka *et al.* 2007, Fay *et al.* 2021), this age bias strengthens rather than weakens the inference that immune activation enhanced reproductive output. Nevertheless, this result should be interpreted with caution, as the age imbalance between groups could have influenced reproductive performance independently of the experimental treatment. Consistent with this, we found an interaction between age and treatment in the model explaining fledging success. In addition, other studies have shown that immune activation can lead to a decrease in parental feeding effort, resulting in lower reproductive output for immune challenged birds (Ilmonen *et al*. 2000, Råberg *et al*. 2000).

For LPS-treated females, it is possible that the simulated infection triggered a shift in their reproductive strategy, leading them to invest more in current offspring rather than future survival. This could explain why we observed higher fledging success in LPS females as compared to controls. Females exposed to an immune challenge might be compensating for potential future reproductive failures by prioritizing immediate reproduction, which resulted in more fledging success. We acknowledge that other factors not studied here, such as feeding rates, food quality, differential allocation of resources among nestlings or potential male compensation, could have contributed to the higher fledging success observed in the LPS group. A higher fledging success in treated broods may also have been enhanced by increased male provisioning during the nestling period. A few studies have demonstrated that mates of LPS-treated parents increased their feeding rates, suggesting parental care compensation (Schoepf *et al*. 2022, Martínez-Flores *et al*. 2024). Investigating these mechanisms would provide additional insight into the extent of female reproductive effort.

CONCLUSIONS

Our study demonstrates that immune activation during late incubation induces changes in incubation behaviour by wild female Pied Flycatchers without compromising hatching success or female body condition. The increased fledging success observed in the broods of LPS-treated females suggests that immune activation may trigger a terminal investment strategy, prompting greater reproductive effort when future survival is uncertain. This behavioural flexibility highlights the adaptive value of plastic responses to physiological stressors in wild birds and underscores the importance of considering immune-related trade-offs in the study of avian life-history strategies. Further studies using an integrative approach could shed more light on the underlying processes regulating reproductive effort in passerines during an infection.

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ETHICAL NOTE

The work was approved by Consejería de Medio Ambiente de la Comunidad de Madrid (approval ref. PROEX 164.5/24).

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CONFLICT OF INTEREST

We declare we have no competing interests.

AUTHOR CONTRIBUTIONS

Isabel Barreda: Methodology; writing – original draft. **Marina García-del Río:** Methodology; formal analysis; writing – review and editing; software. **Santiago Merino:** Methodology; writing – review and editing; supervision. **Manuel Fuertes-Recuero:** Methodology. **Alejandro Cantarero:** Writing – review and editing; project administration; supervision; resources; methodology; funding acquisition; conceptualization.

Data Availability Statement

Data are available from the Digital CSIC Repository: https://doi.org/10.20350/digitalCSIC/17887.

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