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Experimental investigation of the effect of tebuconazole on three biomarkers of innate immunity in the house sparrow (*Passer domesticus*)

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Abstract
Triazoles are among the most widely used fungicides in the world due to their efficacy against fungal crop diseases and their broad spectrum of action. Intensive use of triazoles has resulted in residual contamination in different compartments of agroecosystems and exposes non-target species to potential sublethal effects. Triazoles are known to be immunomodulators in medicine and therapeutic treatments, but very little data is available on their potential effect on immune parameters of non-target vertebrate species living in agroecosystems. In this study, we experimentally examined the impact of tebuconazole on three immune biomarkers (haemagglutination titre (HA), haemolysis titre (HL), and haptoglobin concentration (Hp)), as well as on the body condition of house sparrows (*Passer domesticus*). Our results suggest that tebuconazole had very little, if any, effect on the studied immune parameters. However, further studies are needed to better assess the effect of tebuconazole on bird immunity because (1) experimental individuals were kept under optimal conditions and the impact of tebuconazole on immunity may occur under suboptimal conditions, (2) only one concentration of tebuconazole was tested and its effect could be dose-dependent and (3) other complementary immunological biomarkers should be studied, given the complexity of the vertebrate immune system. Current knowledge on the potential effects of triazoles on the immunity of wild farmland vertebrates is still largely insufficient. Further physiological and immune studies should be conducted to better understand the effect of triazole fungicides on farmland birds.

Keywords Pesticides · Sublethal effect · Immunity · Hemagglutination · Hemolysis · Haptoglobin

Introduction

During the 20th century, exponential human population growth has been accompanied by a dramatic shift in agricultural practices to ensure food security (Brown et al. 1995; Carvalho 2006). This shift has led to massive use of fertilizers and pesticides (Pimentel and Pimentel 1990, Pimentel 1996; Carvalho 2017), that are now known to have deleterious effects on environmental, animal and human health (Mahmood et al. 2016; Kim et al. 2017; Ali et al. 2021; Pesce et al. 2023). For instance, fungicides are used to circumvent fungi attacks, and among them, triazoles are broad-spectrum systemic fungicides, which are extensively used in agriculture. It is estimated that triazoles account for more than a quarter of global fungicide sales and their use has increased by 434% between 2006 and 2016 in the USA (Toda et al. 2021). They are also highly suspected of contaminating the environment (Kahle et al. 2008; Carvalho 2017; Rokbani et al. 2019; Pelosi et al. 2021). However, their impact on non-target species such as wildlife remains largely unknown (Lopez-Antia et al. 2013; Machado-Neves et al. 2018).

The main mode of action of triazole fungicides is to interfere with lanosterol-14α-demethylase, a cytochrome P450 (CYP 51) enzyme, which allows the formation of

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ergosterol, an essential compound for the integrity and fluidity of the fungal membrane (Peyton et al. 2015). However, triazole fungicide action is rather non-specific and could interfere with CYP 51 of non-fungal species, as well as with other cytochrome P450 enzymes (Zarn et al. 2003; Saxena et al. 2015) which could modulate many physiological functions (*e.g.,* Taxvig et al. 2007; Yu et al. 2013; Lv et al. 2017; Teng et al. 2018). For example, cytochromes are often involved in immune functions (Morfin 2002; Effner et al. 2017) and therefore, triazole fungicides may impair immunity by altering immune cell survival, proliferation and differentiation and affecting the associated signaling pathways (Mokarizadeh et al. 2015; Lee and Choi 2020). Importantly, some azole derivatives used in medicine are known to be immunomodulatory in humans (Yamaguchi et al.1993; Fringuelli et al. 2001; Kharb et al. 2011; Fidan et al. 2014; Naranjo et al. 2016). In addition, experimental studies have also demonstrated immunomodulatory effects of triazoles on aquatic model species (Mu et al. 2015; Teng et al. 2018; Liu et al. 2022). In the zebrafish, exposure to triazoles could lead to immunosuppression (Mu et al. 2015) and to a disruption of the cytokine-cytokine receptor interactions (Teng et al. 2018).

The immune system plays a key role in animals to fight pathogens, infectious diseases, parasites, cancers etc. (Wakelin 1996; Delves and Roitt 2000; Dhouib et al. 2016). In vertebrates, the immune system comprises an innate and an acquired arm (Delves and Roitt 2000). The innate immune response, highly conserved in the animal kingdom, encompasses elements that provide immediate defence for the host such as neutrophils, monocytes, macrophages, cytokines, and acute phase proteins (Parkin and Cohen 2001). This part of the immune system is effective because the immune response is rapid, but it can also be damaging to tissue because of its lack of specificity (Parkin and Cohen 2001). The acquired immune response is characterised by an improved response to repeated infections (Delves and Roitt 2000), which involves antigen-specific mechanisms. This part of the immune system allows to elicit a more specific response but is slower to develop than the innate response (Delves and Roitt 2000).

Among triazoles, tebuconazole is one of the most widely used, and it is frequently detected in most environmental compartments of agroecosystems (Berenzen et al. 2005; Kahle et al. 2008; Kalogridi et al. 2014; Désert et al. 2018; Toda et al. 2021). In addition, tebuconazole is the most frequently ingested pesticide by farmland birds (Lopez-Antia et al. 2016; Fernández-Vizcaíno et al. 2022), and these birds can be highly contaminated by tebuconazole (Angelier et al. 2023). Tebuconazole is suspected to act as an endocrine disruptor in birds, affecting reproductive parameters (Fernández-Vizcaíno et al. 2020; Lopez-Antia et al. 2021), survival (Ortiz-Santaliestra et al. 2020), and physiology (*e.g.,* metabolism Bellot et al. 2022). Although no data is currently available, studies of laboratory models suggest that triazoles, and more specifically, tebuconazole could affect immunity of vertebrates (Mu et al. 2015; Teng et al. 2018). Yet, available data about the potential impact of triazole fungicides on the immunity of wild vertebrates that live in agroecosystems, such as farmland birds, are very scarce.

In this study, we experimentally tested the impact of tebuconazole on three innate immune biomarkers in a bird species representative of agroecosystems, the house sparrow (*Passer domesticus*). We selected this species because house sparrows are sedentary birds, strongly linked to agricultural environments (feeding, nesting and breeding) and can be impacted by agricultural practices. Captive house sparrows were exposed to tebuconazole (exposed group) or not (control group) for three months and constitutive levels of three innate immune parameters were assessed before and after exposure, for both groups: haemagglutination titre (HA), haemolysis titre (HL) and haptoglobin concentration (Hp). Firstly, the haemagglutination-haemolysis test (HAHL) measures the ability of plasma to agglutinate and lyse an antigen with which the individual has never previously been in contact (Matson et al. 2005). Haemagglutination arises from the presence of natural antibodies (NAbs). These NAbs are multifunctional and circulate without any prior exposure to a specific antigen (Matson et al. 2005, Boes 2000, Ochsenbein and Zinkernagel 2000). Haemolysis is mediated by the interaction between NAbs and the complement system, which is composed by an enzyme cascade able to neutralise the antibody-antigen complexes and induces inflammatory responses (Janeway et al. 2001). The purpose of this measurement was to assess circulating NAbs levels and the activity of the complement system, which represent the first line of defence against pathogens (Matson et al. 2005). Secondly, haptoglobin (Hp) is an acute phase protein that usually circulates in the blood at a low concentration (baseline haptoglobin) but could widely increase in response to an acute infection, or an inflammation as the non-specific and adaptive immune responses (Millet et al. 2007; Quaye 2008; Matson et al. 2012). Hp helps minimizing the oxidative damages that are caused by the release of hemoglobin when red blood cells are lysed (Sadrzadeh et al. 1984; Alayash 2004; Quaye 2008; Andersen et al. 2017). Hp concentration should predict health status, physiological condition and immune responsiveness (Matson et al. 2012, Hõrak et al. 2002, 2003). To our knowledge, no data are available on the effect of tebuconazole on these immune parameters in wild vertebrates and it is therefore difficult to provide a directional hypothesis although we may predict that the three innate immune parameters measured may be altered following the exposure to tebuconazole.

Materials and Methods

Study species

This study was conducted on the colony of captive adult sparrows of the CEBC (*Passer domesticus*, Linnaeus, 1758). A total of 40 sparrows were used in this experiment ($N_{females} = 18$ and $N_{males} = 22$). All birds were kept under similar captive conditions. The sparrows were equitably distributed (aviary 1, $N = 11$; aviary 2, $N = 10$; aviary 3, $N = 9$; and aviary 4, $N = 10$) in 4 identical outdoor aviaries (length: 3 m, width: 2 m, height: 2 m) subject to natural seasonal temperature and light cycles. Birds were divided into aviaries by treatment type: two control aviaries (aviaries 2 and 4) and two exposed aviaries (aviaries 1 and 3) and the sex ratio was balanced between treatments (N_{female} controls = 10 and N_{female} exposed = 8; N_{male} controls = 10 and N_{male} exposed = 12). Each aviary was equipped with artificial shelter boxes (*i.e.*, one box per bird). The sparrows were fed *ad libitum* with a mixture of commercial seeds, fruit pate, millet sprays, grit, and salt/mineral blocks. In all aviaries, water was dispensed *ad libitum* from a water trough and was changed weekly.

Experimental design

In the two control aviaries, control individuals had access to regular tap drinking water without tebuconazole. In the two exposed aviaries, tebuconazole (Sigma-Aldrich, CAS. No: 107534-96-3, purity: ≥ 98.0%) was diluted with tap water using a precision balance (± 0.0001 g) to reach a concentration of 550 µg.L^{-1}. For a sparrow weighing about 25 g and based on daily water consumption (Bartholomew and Cade 1963), the amount of tebuconazole ingested daily is estimated to be about 4.1 µg, which corresponds to an exposure concentration of 0.164 mg kg-¹ bw d-¹ (*i.e.*, a concentration 36 times lower than the chronic NOEL for birds, Tebuconazole (Ref: HWG 1608) (herts.ac.uk)). In this study we did not measure the plasma concentration of tebuconazole in experimental birds, but this validation had already been carried out in the study by Bellot et al. (2022), which used the exact same exposure design. In this previous study, the experimental exposure to tebuconazole resulted in ecologically realistic contamination (*i.e.*, 59.7 pg/g plasma, Bellot et al. 2022) compared with the concentrations observed in free-ranging birds (*i.e.*, 70.7 pg/g plasma, see Angelier et al. 2023).

Exposure to tebuconazole started on 8 December 2020 until 23 February 2021 for a total exposure duration of 11 weeks, which is similar to the chronic exposure that can be found in agroecosystems (Angelier et al. 2023). Tebuconazole concentrations of exposed and control drinking water were assayed by an independent accredited analytical laboratory by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS, QUALYSE laboratory, La Rochelle, France). Tebuconazole was undetectable in control water while tebuconazole concentrations were 550 µg.L^{-1} in the water of exposed aviaries (only one measurement was made after dilution, as tebuconazole has stable physicochemical properties, http://sitem.herts.ac.uk/aeru/ppdb/en/Reports/610.htm).

Capture and blood sampling

Before the start of the exposure to tebuconazole (23rd of November 2020), blood samples (100–150 µL) were collected from control and exposed individuals ($N = 40$) by brachial vein puncture (T0) with heparinized capillaries. At the end of the period of exposure to tebuconazole (23rd of February 2021), all control and exposed individuals were captured ($N = 40$) and a second blood sample was collected (T1). All blood samples were centrifuged, red blood cells and plasma were separated and stored at −20 °C until immunological assays at the Centre d'Etudes Biologiques de Chizé (CEBC). At each capture (T0 and T1), all control and exposed birds were weighed with a digital scale (± 0.1 g). At T0, the tarsus of all birds was measured with a calliper (± 0.01 mm). A body condition index (BCI) then was calculated by extracting the residuals from the linear regression of individual body mass as a function of tarsus length ($F_{1,78} = 13.16$, $p = <0.001$). Control and exposed sparrows did not differ in body size (based on size measurements taken at time T0, tarsus, $F_{1,38} = 0.06$, $p = 0.814$) and in body condition (at T0, see Results).

Immune assays

In order to assess the innate immune function of our experimental house sparrows, we measured the constitutive levels of three immune biomarkers: haemagglutination (HA) and haemolysis (HL) titres, and haptoglobin (Hp) concentration.

Haemagglutination and haemolysis titres were assessed using a haemagglutination-haemolysis assay developed by Matson et al. (2005). In each couple of round-bottom 96-well plate ($n = 18$), 20 µl of seven plasma samples (rows A-G) and a positive control (plasma collected from a single duck in 2021, row H) were added to columns 1 and 2 of the first plate. From columns 2 to 23, samples were serially diluted using 20 µl of phosphate-buffered saline Dulbecco's solution (PBS 1X). Column 34 (column 12 of the second plate) only contained PBS, to be used as a negative control. 20 µl of a 1% rabbit blood cell (acting as the antigen) suspension was added to all wells. Plates were immediately incubated in a water bath at 37 °C for a duration of 90 min. After the incubation, the

plates were tilted at 45° from their long axis during 20 min to allow the haemagglutination visualisation. Right after, all plates were photographed to record haemagglutination and were kept for an additional 70 min before being photographed again to record haemolysis. From each picture, the eight rows were randomized and blindly scored by a single observer (A.Q.) to obtain HA and HL scores. HA score corresponded to the last plate row at which agglutination of the rabbit blood was detected. HL scores corresponded to the last plate row at which the complex NAbs-antigen was lysed. Higher scores mean higher haemagglutination or haemolysis titres. Because haemolysis was absent for 30% of our samples, we decided to transform HL titre into a binomial variable (no lysis: 0, lysis: 1). To control for initial plasma lysis (which could occur during blood sampling), all plasma samples were scored for redness (from 1 to 8) and this score was added as a covariate in the statistical models involving haemagglutination and haemolysis titres. Using the positive controls, we calculated an inter-plate coefficient of variation of 18% for haemagglutination and 10% for haemolysis.

Haptoglobin was quantified in 7.5 µl of plasma using a commercial assay (TP801, Tri-Delta Diagnostics), which colorimetrically measured the free heme-binding capacity of the plasma. We followed the manufacturer instructions with slight modification according to Matson et al. (2012). The standards (included in the two plates in duplicate) ranged from 2.5 to 0.039 mg/ml to allow the calculation of low concentration. A negative control (7.5 µl of diluent) was also included in duplicated in the two plates. We measured absorbance (Bio Tek - Synergy H1) at two wavelengths (425 and 600 nm) before adding the final reagent. Final absorbance values at 600 nm were corrected by the pre-scan absorbance values at 600 nm, in order to control for initial plasma colour and cloudiness (Matson et al. 2012). The pre-scan absorbance values at 425 nm were used to statistically correct for blood sample haemolysis in the model involving haptoglobin. The intra- and inter-plate coefficient of variations were 3% and 14%, respectively.

Statistical analysis

For all immune markers, we kept for statistical analysis only individuals which had data for both measurement times (before (T0) and after treatment (T1)). For some individuals, the amount of plasma required was not sufficient to perform all the 3 assays HA titre, HL titre and Hp concentration and our sample size therefore slightly differ between assays ($N_{HA} = 78$, $N_{HL} = 68$, $N_{Hp} = 78$).

All statistical analyses were carried out in Rstudio with "lme4" and "car" packages (R version 4.2.1). The functions "Anova", "lmer", "glmer" and "ggplot2" have been used. To test the effect of the experimental treatment (*i.e.*, control or exposed to tebuconazole) on the HA titre, Hp concentration and BCI, linear mixed models (LMMs) were used. We checked that all models met the assumptions of normality and homoscedasticity of residuals. Log-transformed functions were used for HA titre and Hp concentration to improve the distribution and homogeneity of the model residuals. To test the effect the experimental treatment on the HL titre, generalized linear mixed model (GLMER) was used because HL followed a binomial distribution (no lysis: 0 or lysis: 1). In all models, the aviary and the identity of the individuals was added as a random factor because individuals were measured twice. The deviance tables for each model have been summarised in a single table in the result section. When the effect of the treatment on a variable of interest was not significant but showed a visual trend for at least one of the sexes, we conducted a power analysis.

The HA titre was analysed using linear mixed models (LMM) with three explanatories factors (treatment: control or exposed; sex: female or male and time: before treatment (T0) and after treatment (T1)) and all their interactions (*i.e.*, second and third order interactions). Initial lysis and body condition of individuals were also added to the models as covariates. The HL titre was analysed using generalized linear mixed model (GLMER) with the same explanatory factors and co-variates as HA titre. The GLMER model was run with the HL titre as a binomial variable to discriminate between samples with no lysis (0) or with lysis (1). The Hp concentrations were analysed using LMM with three explanatories factors, (treatment; sex; time) and all their interactions. Absorbance values of the pre-scans at 425 nm and body condition of individuals were also added to the models as covariates. Specific comparisons were then conducted using contrast procedures with the Satterthwaite approximation to test the significance of the differences that were found between different groups of birds. A LMM with three fixed effects (treatment; sex; time) and all their interactions was carried out to test the effect of tebuconazole on BCI.

Results

Haemagglutination titre (HA)

The variations of HA titre were not explained by any of the explanatory factors included in our model (*i.e.*, treatment, time, sex, BCI and their interactions, Table 1; Fig. 1).

Haemolysis titre (HL)

The variations of HL titre were not explained by any of the explanatory variables included in our model (*i.e.*, treatment, time, sex, BCI and their interactions, Table 1).

Table 1 The deviance tables for each model have been summarised in a single table below

	HA titre (log-transformed) N = 78 measures from 39 individuals		HL titre N = 68 measures from 34 individuals		Hp concentration (log-transformed) N = 78 measures from 39 individuals		BCI N = 80 measures from 40 individuals	
Explanatory variables	F-value	Pr (> F)	F-value	Pr (> F)	F-value	Pr (> F)	F-value	Pr (> F)
Time	<0.10	0.955	1.57	0.210	2.22	0.136	**16.67**	**<0.001** ***
Treatment	1.42	0.233	0.14	0.706	< 0.10	0.934	0.16	0.689
Sex	0.82	0.366	0.02	0.877	**5.74**	**0.017** *	1.93	0.164
BCI	0.02	0.876	0.58	0.447	1.28	0.258		
Initial Lysis	**4.03**	**0.045** *	0.43	0.514				
Pre-scan absorbance					**169.87**	**<0.001** ***		
Time : Treatment	0.45	0.503	0.17	0.680	1.08	0.298	1.11	0.293
Time : Sex	0.23	0.630	<0.01	0.940	0.04	0.843	0.08	0.781
Treatment : Sex	0.12	0.733	2.23	0.135	0.21	0.645	0.28	0.596
Time : Treatment : Sex	0.34	0.561	0.04	0.836	**6.30**	**0.012** *	0.71	0.400

Effects of time of measurement (T0, before treatment and T1, after treatment), treatment (control or exposed to tebuconazole), sex (female or male) and their interactions on the HA titre (haemagglutination), HL titre (haemolysis), Hp concentration (haptoglobin) and BCI. A log-transformed function was applied to the HA titre and Hp concentration data in the statistical models. For the response variables HA and HL titres, initial lysis and BCI were added as co-variables while for Hp, pre-scan absorbance (at 425 nm) and BCI were added as co-variables. The cells were left empty when the explanatory factor was not present in the model. The aviaries and the individuals were added as random factors. Significant terms are indicated by the following codes: '***' < 0.001 '**' < 0.010 '*'. The abbreviations used are as follows: the Fisher statistic (F-value), the associated p-value (Pr (> F))

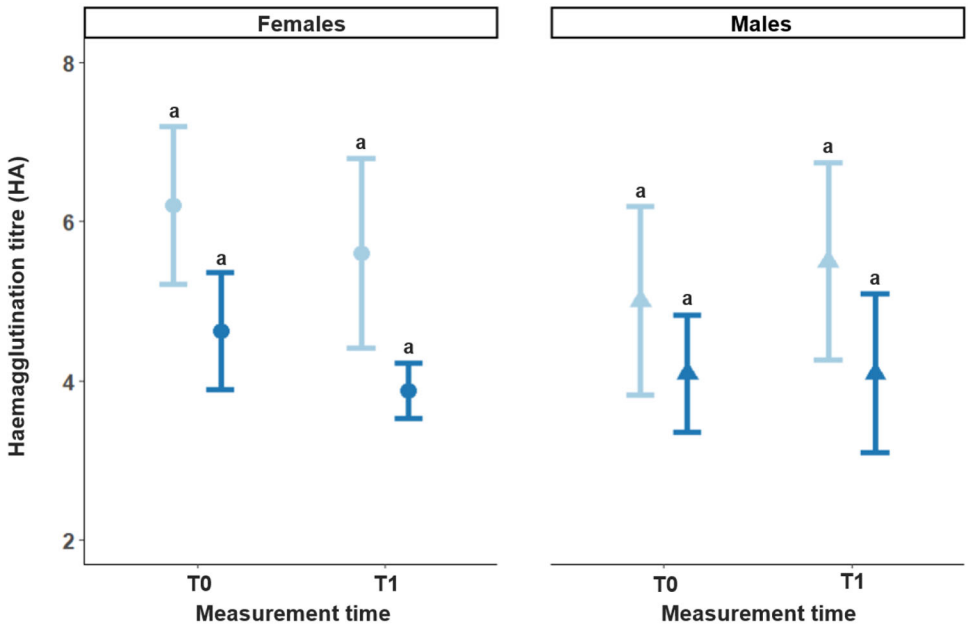

Fig. 1 Haemagglutination titre (HA) as a function of measurement time (T0, before treatment; T1, after treatment), and treatment (control or exposed to tebuconazole). Control and exposed individuals are shown in light and dark blue, respectively. Circles and triangles correspond to the means for females (left plots) and males (right plots), respectively. Significant terms are indicated by different letters ($\alpha < 0.05$). Means ± SE are presented

Haptoglobin (Hp) concentration

Hp concentration was higher in males than in females (Table 1, Fig. 2). Although Hp concentration was not affected by the treatment nor the exposure time (Table 1), the interaction between "Time", Treatment" and "Sex" was significant (Table 1). This indicates that the influence of the experimental treatment on the levels of Hp is complex and differs significantly depending on the time of measurement (*i.e.*, before or after the application of the treatment) and the sex of the individuals (Fig. 2). Specifically, the levels of Hp increased significantly in control females between T0 and

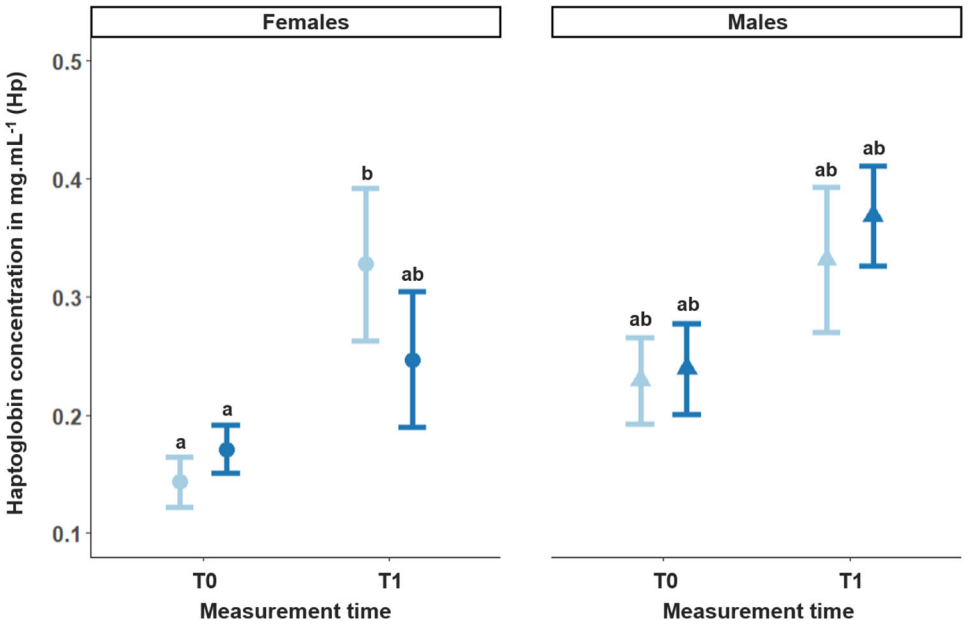

Fig. 2 Haptoglobin concentration in $mg.mL^{-1}$ (Hp) as a function of measurement time (T0, before treatment and T1, after treatment), and the treatment (control or exposed to tebuconazole). Control and exposed individuals are shown in light and dark blue respectively. Circles and triangles correspond to the means for females (left plots) and males (right plots), respectively. Significant terms are indicated by different letters ($\alpha < 0.05$). The means ± SE are presented

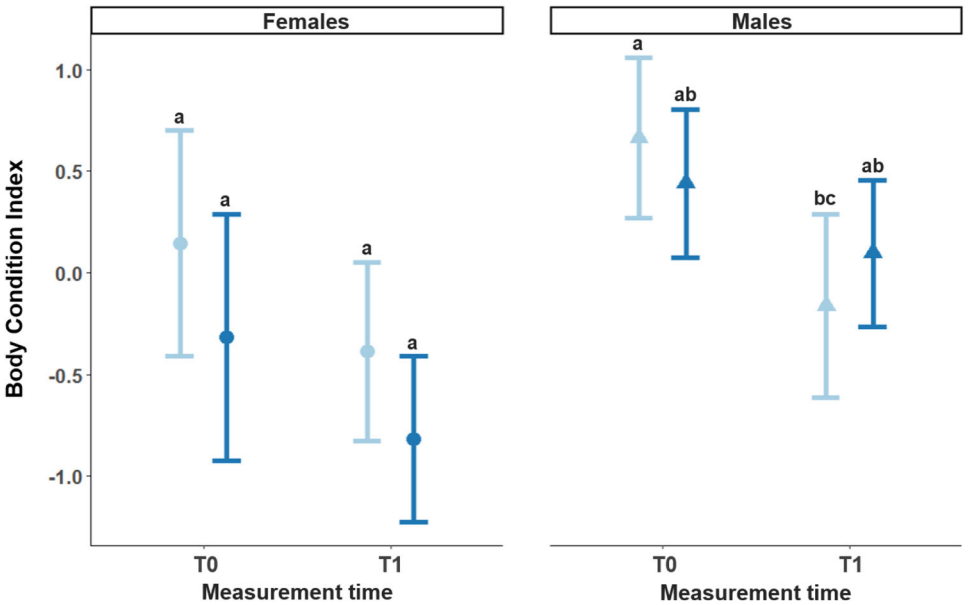

Fig. 3 Body condition index as a function of measurement time (T0, before treatment and T1, after treatment), and the treatment (control or exposed to tebuconazole). Control and exposed individuals are shown in light and dark blue respectively. Circles and triangles correspond to the means for females (left plots) and males (right plots), respectively. Significant terms are indicated by different letters ($\alpha < 0.05$). The means ± SE are presented

T1 (contrast: $t_{(66.6)} = -2.62$, $p = 0.011$, Fig. 2), but not in exposed females ($t_{(66.6)} = 0.98$, $p = 0.329$, Fig. 2). A power analysis revealed that, given our sample size, there is a probability of 0.32 to detect an effect of the treatment on changes in Hp levels between T0 and T1 in females. In males, the levels of Hp did not significantly differ between T0 and T1 (all p-values > 0.196, Fig. 2).

Body condition Index

BCI decreased over the study period (Table 1, contrast: $t_{(36)} = 4.10$, $p < 0.001$) and was not affected by any other factor included in the model (Table 1, Fig. 3).

Discussion

In this study, we investigated for the first time the effect of chronic exposure to a widely used triazole fungicide (tebuconazole) on the immunity of birds. We found no significant effect of such exposure to tebuconazole on HA and HL titres. However, the results obtained for HA titre should be taken with caution given that the two groups of females (control and exposed) tended to differ before the experimental phase (T0). Similarly, we found no significant effect of exposure to tebuconazole on Hp concentration in male sparrows. In female sparrows, Hp concentration increased during the experiment in controls females, but not

in exposed ones. However, after exposure, the Hp concentration did not differ between exposed females and controls, suggesting that the impact of exposure to tebuconazole on the dynamics of Hp levels is weak. Finally, the body condition of the sparrows was not affected by the treatment and did not appear to influence the immune biomarkers measured.

Overall, we found very little, if any, evidence for an impact of tebuconazole on the studied immune biomarkers in the house sparrow. This finding is supported by another recent study that reported no effect of tebuconazole on the cell-mediated immune response in red-legged partridges (Lopez-Antia et al. 2021). Importantly, Lopez-Antia et al. (2021) used a concentration of tebuconazole which was approximately 8 times higher than the concentration we used in our study (~1.10 mg kg^{-1} bw d^{-1} in Lopez-Antia et al. (2021) vs. ~0.164 mg kg^{-1} bw d^{-1} in the current study), further supporting the idea that sublethal doses of tebuconazole have little if any impact on some immune mechanisms in vertebrates. Accordingly, Moser et al. (2001) reported that high doses of tebuconazole (up to 60 mg kg^{-1} bw d^{-1}) had also no apparent effect on multiple immune mechanisms in developing rats. However, a few transcriptomic studies have reported that triazoles can alter some immune functions in aquatic laboratory models (Mu et al. 2015; Teng et al. 2018), suggesting that triazoles could have subtle effects on immunity. For example, exposure to difenoconazole at 0.5 to 1.0 mg/L was associated with the downregulation and the up-regulation of the transcription of multiple genes involved in immune and inflammatory responses in zebrafish, suggesting an immunosuppressive effect of triazoles (Mu et al. 2015).

In our study, we only examined three constitutive parameters of innate immunity, but it would be interesting to further examine the immune response to an experimental and standardized immune challenge. It would allow to assess whether tebuconazole might affect the ability of the immune system to cope with an infection. Furthermore, house sparrows were kept under optimal captive conditions (*i.e.*, water and food *ad libitum*), which may have affected our ability to detect an effect of tebuconazole exposure. Indeed, we cannot rule out that the effect of tebuconazole on immune functions may only become apparent when individuals face energetic challenges, such as those encountered in the wild. In addition, we have exposed house sparrows to a single concentration of tebuconazole, which was 36 times lower than the chronic NOEL for birds. This dose was selected to mimic the contamination that can be found in some agroecosystems (Bellot et al. 2022) but we cannot exclude that higher concentration may have had an effect on the immunity of house sparrows. Indeed, in our study, we exposed birds only by the oral route via drinking water. However, in addition to surface and ground water (Kahle et al. 2008; Huang et al. 2022), triazole fungicides, including tebuconazole, are found in the air (Désert et al. 2018, Angelier et al. 2023), coated seeds (Fernández-Vizcaíno et al. 2023), soil and earthworms (Pelosi et al. 2021). Therefore, birds in natural conditions could be exposed to multiple routes of exposure and therefore potentially to higher exposure concentrations than in our study. Furthermore, in this study, all experimental birds were exposed to the same concentration of tebuconazole, limiting certainly inter-individual variation in plasma tebuconazole contamination. In future studies, it would however be interesting to measure individual contamination precisely and to expose individuals to several concentrations of tebuconazole through contrasted sources of exposure to further test the impact of tebuconazole on these immune biomarkers. Finally, birds from agroecosystems are contaminated with several other azoles (Angelier et al. 2023) as well as other pesticides (e.g., Humann-Guilleminot et al. 2019) and it would therefore be relevant to test not only the impact of these other substances, but also their potential cumulative or interactive effects on immunity.

We did not find strong difference in Hp concentration between the exposed and the control groups. Specifically, we found that the Hp concentration of exposed sparrows did not differ from those of control sparrows after 11 weeks of exposure to tebuconazole. Hp is involved in scavenging heme from haemoglobin and aims to remove free hemoglobin from the circulation, and therefore limits oxidative damages caused by inflammation (Quaye 2008; Andersen et al. 2017). Our results suggest that exposure to tebuconazole for 11 weeks did not have any major impact on inflammation baseline in house sparrows.

However, we found that the Hp concentration increased between T0 and T1 only in the control females. Since Hp should be correlated with health status, physiological condition and immune responsiveness (Matson et al. 2012, Hõrak et al. 2002, 2003), this increase could indicate that controlled female condition improved during the experiment, which was not the case for exposed females. Unfortunately, this hypothesis cannot be confirmed by BCI variations. Another hypothesis is that tebuconazole may have induced some cellular haemolysis, which may have resulted in Hp depletion in exposed females, or that tebuconazole interfered with Hp synthesis. Indeed, exposure to tebuconazole can affect red blood cell parameters and result in increased hematocrit and hemoglobin concentration in the common carp, even if these effects are not maintained in the long-term (Lutnicka et al. 2016). In addition, tebuconazole and other triazoles have also been associated with increased oxidative damage and altered liver functioning in laboratory models (Souders et al. 2019; Tian et al. 2019; Valadas et al. 2019; Shen et al. 2021). Although our results might suggest that tebuconazole may affect birds in a sex-

dependent manner, strong evidence is still lacking, and we must remain cautious regarding our interpretation for several reasons. Firstly, Hp levels did not statistically differ between experimental females and controls at T1 (i.*e*., after exposure). Secondly, an additional analysis showed that the change in Hp levels between T0 and T1 did not statistically differ between exposed and control females despite a visual trend (Fig. 2). Finally, we did not find any effect of exposure to tebuconazole on the change in Hp levels between T0 and T1 in males. Our small sample size may have led to a lack of statistical power and to a non-significant effect of exposure to tebuconazole on Hp levels, especially in females (see Fig. 2). This non-significant result must therefore be interpreted with caution (as shown by the power analysis we conducted). Overall, we cannot conclude with certainty about the potential effect of tebuconazole on female Hp levels, although it appears to be relatively weak from our study. Hp levels are also known to vary not only through the life cycle of individuals but also with environmental constraints (Hegemann et al. 2012) and house sparrows were kept here under optimal captive conditions. Therefore, we cannot exclude that tebuconazole may have a stronger impact on inflammation status, and therefore Hp levels, when house sparrows face immune or energetic challenges. Because Hp levels classically increase in response to an infection, future studies should examine the influence of exposure to tebuconazole on the response of Hp levels to a standardized immune challenge (*e.g.,* LPS injection, Martin et al. 2010) in individuals kept under contrasted energetic situations. Although our experimental design provides a robust way to test our hypothesis, our sample size remains relatively small (40 individuals). Indeed, inter-individual variations in the studied immune parameters may be large and may have masked a potential effect of the treatment. Additional experiments with a larger sample size would therefore be necessary to confirm these results.

Overall, we did not find any major effects of chronic low dose of tebuconazole on the body condition of the sparrows. This result is in agreement with previous studies (Lopez-Antia et al. 2013, 2018; Fernández-Vizcaíno et al. 2020) that have reported no significant effect of triazole exposure on the body condition of individuals in other bird species. However, Bellot et al. (2022) recently found a sex-dependent effect of tebuconazole on body condition as tebuconazole induced a reduced metabolism and an increased BCI in female house sparrows. Importantly, the duration and the timing of exposure were different from those in the present study (duration: 7 months in the Bellot et al. 2022 study vs. 2.5 months in the present study; timing: start of breeding period in the Bellot et al. 2022 study vs. wintering period in the present study) and these differences may explain this discrepancy regarding the results on BCI. Indeed, Pandey and Mohanty (2015) found that another fungicide exposure had an impact of the condition of Red Munias (*Amandava amandava*), a passerine bird, but this effect was apparent during the breeding season only. This supports the idea that the impact of pesticides on physiological traits may vary between different phases of the life cycle and these impacts may be especially important during energetically demanding periods (*e.g.,* breeding period). Intriguingly, the BCI of the individuals decreased significantly between the beginning and the end of the experiment despite *ad libitum* food. This decrease could result from the specificity of the study period (*i.e*., early winter). Indeed, the onset of winter at the beginning of the experiment may have represented an important energy cost for the birds, and they may have relied on their body reserves to ensure metabolic, thermoregulatory, and immune processes (Swanson and Olmstead 1999; Angelier et al. 2011; Moreno-Rueda 2011). It is important to point out that weather and temperature conditions during the experiment may have acted as confounding factors on the effect of the treatment, whether in relation to immune parameters or the BCI. It would therefore be interesting to carry out the experiment at different seasons of the year or in indoor conditions (controlled temperature, humidity, and light).

Conclusion

Overall, our results suggest that tebuconazole had little if any effect on the studied innate immune biomarkers in captive house sparrows. However, our results should be taken with caution because (1) we focused on captive birds that were held in optimal conditions, (2) we exposed birds to a single low concentration of tebuconazole, and (3) we examined markers of innate immunity solely and without immune challenge. Future transcriptomics and eco-physiological studies are now warranted to test whether tebuconazole could affect other immune markers or pathways in farmland birds. Currently, there is too little knowledge to identify the potential impact of triazoles on the immunity of wild vertebrates and their long-term effects on survival, reproduction, and fitness.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Ethical approval All applicable institutional and/or national guidelines for the care and use of animals were followed. This work was approved by the French authorities (COMETHEA ethic committee and Ministère de L'Enseignement Superieur, de la Recherche et de L'innovation) under permit #APAFIS#12918–2018010515574796. Authorizations to capture and keep sparrows in captivity were provided to FA by the DREAL Nouvelle Aquitaine.

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