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ENVIRONMENTAL OBSERVATION AND MONITORING THROUGH SPACE AND TIME: A CHALLENGE FOR ECOTOXICOLOGY

Chronic exposure to tebuconazole alters thyroid hormones and plumage quality in house sparrows (Passer domesticus)

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Abstract

Triazoles belong to a family of fungicides that are ubiquitous in agroecosystems due to their widespread use in crops. Despite their efficiency in controlling fungal diseases, triazoles are also suspected to affect non-target vertebrate species through the disruption of key physiological mechanisms. Most studies so far have focused on aquatic animal models, and the potential impact of triazoles on terrestrial vertebrates has been overlooked despite their relevance as sentinel species of contaminated agroecosystems. Here, we examined the impact of tebuconazole on the thyroid endocrine axis, associated phenotypic traits (plumage quality and body condition) and sperm quality in wild-caught house sparrows (*Passer domesticus*). We experimentally exposed house sparrows to realistic concentrations of tebuconazole under controlled conditions and tested the impact of this exposure on the levels of thyroid hormones (T3 and T4), feather quality (size and density), body condition and sperm morphology. We found that exposure to tebuconazole caused a significant decrease in T4 levels, suggesting that this azole affects the thyroid endocrine axis, although T3 levels did not differ between control and exposed sparrows. Importantly, we also found that exposed females had an altered plumage structure (larger but less dense feathers) relative to control females. The impact of tebuconazole on body condition was dependent on the duration of exposure and the sex of individuals. Finally, we did not show any effect of exposure to tebuconazole on sperm morphology. Our study demonstrates for the first time that exposure to tebuconazole can alter the thyroid axis of wild birds, impact their plumage quality and potentially affect their body condition. Further endocrine and transcriptomic studies are now needed not only to understand the underlying mechanistic effects of tebuconazole on these variables, but also to further investigate their ultimate consequences on performance (i.e. reproduction and survival).

Keywords Passerine birds · Agroecosystem · Fungicides · Sublethal effects · Thyroid hormones · Body condition · Sperm quality

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Introduction

It is estimated that, worldwide, only fourteen crops make up the majority of the human diet. However, all of these crops are susceptible to diseases while growing in the field or after the harvest (Strange and Scott 2005), and fungal diseases represent a major risk not only to crop yield (Fisher et al. 2012), but also to food safety (Strange and Scott 2005). Many pesticides, and more specifically fungicides, are massively used in agriculture to circumvent these problems and to reduce the economic costs and health issues of fungal pests. For example, fungicides accounted for more than 40% of total pesticide sales from 2011 to 2018 in the European Union (Zubrod et al. 2019). Fungicides can even represent up to 90% of the pesticides that are used for certain crops (e.g. orchards, vineyards) (Zubrod et al. 2019).

Triazoles are a large family of systemic, broad-spectrum fungicides used in agriculture since the 1970s–1980s (Ribas e Ribas et al. 2016). They are designed to interfere with lanosterol 14α-demethylase (CYP51), an enzyme of the cytochrome P450 monooxygenase (CYP) superfamily (Becher and Wirsel 2012). In fungi, CYP51 plays a key role in the biosynthesis of ergosterol, a sterol located in fungal membranes and involved in membrane permeability and fluidity (Daum et al. 1998). One of the main problems associated with agricultural triazoles is their potential effect on non-target species, through non-target physiological mechanisms (e.g. Fernández-Vizcaíno et al. 2020; Lopez-Antia et al. 2021; Bellot et al. 2022). In general, the effects of fungicides, and more particularly triazoles, are understudied in wildlife. Indeed, most studies focus on acute ecotoxicity tests and with model species commonly used in toxicology (e.g. zebrafish, earthworm) (Binev et al. 2005; Sancho et al. 2010; Andreu-Sánchez et al. 2012; Raby et al. 2019; Rico et al. 2016). Although these studies elucidate the biological targets of triazoles and their modes of action, they are not necessarily representative of the effects of triazoles at concentrations found in the environment. In addition, model species are often laboratory-raised species and, thus, are not always representative of the ecosystems where triazoles are used. For wildlife, acute exposures to high concentrations of triazoles are scarce and correspond to sporadic events, such as accidental contamination (e.g. Capel et al. 1988). Therefore, to better understand the sublethal effects that triazoles may have on non-target species, it is necessary to study the impact of long-term exposure of triazoles on wildlife, and especially vertebrate sentinel species of agroecosystems.

Among vertebrates, birds are particularly relevant to study this question because many bird species exploit or live near agricultural areas and are potentially exposed to triazoles through multiple routes of exposure (Robinson 2004; Lopez-Antia et al. 2016; Fernández-Vizcaíno et al. 2020, 2022; Ortiz-Santaliestra et al. 2020; Angelier et al. 2023). This contamination is likely associated with chronic exposure to low concentrations of triazoles, for example in water, soil, air and seeds (e.g. Kahle et al. 2008; Rokbani et al. 2019; Pelosi et al. 2021; Lopez-Antia et al. 2016, 2021). In this context, specific bird species (e.g. non-migratory species) can be relevant sentinel species of agroecosystems because they rely on this specific environment to complete their annual life cycle. As such, they should be chronically exposed to specific triazoles that are used for agricultural purposes and, thus, represent a powerful model to better understand the impact of environmental concentrations of triazoles on key organismal traits. Experimental studies have shown that chronic or subchronic exposure to triazoles can induce reprotoxic effects in birds (Grote et al. 2008; Lopez-Antia et al. 2018). These effects may arise from an interaction of triazoles with non-fungal sterol synthesis (Zarn et al. 2003). Thus, triazoles can act as major endocrine disruptors by modulating the expression and activity of enzymes such as cytochrome P450 aromatase (Goetz et al. 2007; Taxvig et al. 2007; Jacobsen et al. 2012; Warrilow et al. 2013; Yu et al. 2013) with potential implications for multiple metabolic pathways, and also for reproduction (Poulsen et al. 2015; Huang et al. 2022). Indeed, pesticides, and possibly triazoles, can have a direct impact on reproduction by causing testicular abnormalities that affect spermatogenesis and the mobility and quality of germ cells (carbamates and neonicotinoids (Tokumoto et al. 2013; Mohanty et al. 2017; Humann-Guilleminot et al. 2019a); triazoles (Machado-Neves et al. 2018)). For example, triazoles could alter the fertility of individuals, notably through a decrease in sperm quantity and quality (e.g. Taxvig et al. 2007; Grote et al. 2008; Pereira et al. 2019).

More recently, triazoles have also been suspected to affect key endocrine mechanisms, such as the hypothalamic-pituitary-thyroid (HPT) axis and the resulting regulation of thyroid hormones (THs). These hormones also play a key role in gonadal development and the regulation of seasonal reproduction in mammals and birds (McNabb 2007; Mohanty et al. 2017). The first evidence highlighting the effect of triazoles on thyroid disruption comes from the study of aquatic models (e.g. zebrafish) (Yu et al. 2013; Li et al. 2019). However, the potential effects of azole fungicides on thyroid functioning have been overlooked in non-aquatic vertebrates. In adult birds, the HPT axis is also a key physiological system, which is, for example, involved in (i) seasonal processes, notably the moulting process that allows birds to renew their plumage, and (ii) metabolism and thermoregulation (reviewed by McNabb 2007). An

increase in THs, such as triiodothyronine (T3) and thyroxine (T4), triggers the initiation of moulting (Groscolas and Leloup 1986; Groscolas and Cherel 1992; Jenni-Eiermann et al. 2002), as experimentally shown in the white-crowned sparrow (*Zonotrichia leucophrys gambelii*) (Pérez et al. 2018). Similarly, TH levels have been linked to metabolism in multiple bird species and increased TH levels are associated with increased maintenance costs (basal metabolic rate (Chastel et al. 2003; Elliott et al. 2013)).

In this study, we experimentally tested the impact of environmental concentration of tebuconazole on key traits of a sentinel bird species of agroecosystems, the house sparrow (*Passer domesticus*). Tebuconazole is the most widely used triazole fungicides in the world, and it is found in multiple agroecosystems, especially in air (Désert et al. 2018), soil (Kalogridi et al. 2014) and water (Kahle et al. 2008). To date, only a few studies have evaluated the impact of tebuconazole on terrestrial non-target vertebrate species: partridges (Fernández-Vizcaíno et al. 2020; Lopez-Antia et al. 2021), bats (Machado-Neves et al. 2018) and amphibians (Poulsen et al. 2015; Bernabò et al. 2020). However, none of these experimental studies has tested the impact of tebuconazole on the levels of TH and related physiological traits. Thus, the aim of this study was to experimentally test the impact of exposure to tebuconazole on the HPT axis in wild-caught house sparrows that were kept in captivity under controlled conditions. We also examined the impact of tebuconazole on plumage quality and body condition, two phenotypic traits that are closely related to the HPT axis, as well as on sperm quality.

To do this, we randomly chose half of our wild-caught captive house sparrows to be exposed to tebuconazole through their drinking water ('exposed sparrows'), while the other sparrows were not exposed ('control sparrows'). We then examined the impact of this experimental treatment on the (1) levels of THs (T3 and T4); (2) plumage quality, using the morphometric characteristics of feathers; (3) body condition (body mass corrected for body size: body condition index (BCI)); and (4) the morphology of the sperm. Plumage quality, body condition and sperm quality are closely related to individual performance and fitness. Indeed, feathers enable birds to perform vital functions such as flight, signalling and thermoregulation (Swaddle et al. 1996; Dawson et al. 2000; Jenni and Winkler 2020). Similarly, the BCI is a commonly used proxy of the physiological state of an individual, and disruption of metabolism usually translates into an abnormal BCI. Finally, sperm morphology may be an indicator of sperm quality and reproductive success (Helfenstein et al. 2010). We predicted that exposure to tebuconazole would (1) disrupt the HPT axis and result in reduced TH levels (T3 and T4), as previously shown in laboratory aquatic model species (Li et al. 2019); (2) affect moult, with a detrimental impact on plumage quality (smaller and less dense feathers in exposed sparrows); (3) result in an increased BCI, because tebuconazole should lower metabolism (Bellot et al. 2022), and thus maintenance costs, through its impact on TH levels (reduced TH levels, prediction 1); and (4) reduce sperm quality by altering sperm morphology.

Material and method

Study species

This study was conducted on captive adult's house sparrows (*Passer domesticus*, Linnaeus, 1758). House sparrows are an appropriate sentinel species for assessing the contamination of agroecosystems because they are sedentary, strongly linked to agricultural environments (feeding, nesting and breeding) and can be impacted by agricultural practices and landscapes (Swaileh and Sansur 2006; Humann-Guilleminot et al. 2019b). A total of 39 sparrows were used in this experiment ($n_{\text{females}} = 20$ and $n_{\text{males}} = 19$) that were captured in western France in 2019 (Secondigné-sur-Belle). Birds were acclimated to captivity for 2 weeks before the start of the experiment. All sparrows were maintained in captivity in 4 similar outdoor aviaries (length: 3 m, width: 2 m, height: 2 m). The birds were maintained in outdoor aviaries to keep them under natural seasonal temperature cycles. Birds were split in the aviaries by treatment type and sex: control birds were split into two aviaries for a total of 9 females and 9 males. Exposed birds were split into the two other aviaries for a total of 11 females and 10 males. As females and males were kept in separate aviaries for the duration of the experiment, no eggs were laid. These sample sizes differ slightly between the two treatments, as some of the birds did not adapt well to captivity and were released.

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, millet sprays, grit and salt/mineral blocks. In all aviaries, water was dispensed ad libitum through a drinking trough and was changed on a weekly basis.

Experimental design

Individuals were exposed to tebuconazole through their drinking water, a source of exposure that is thus far less studied in comparison to the ingestion of treated seeds (e.g. Lopez-Antia et al. 2013, 2018, 2021; Fernández-Vizcaíno et al. 2022). 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}. As in the control

group, the water was changed once per week. Based on the physico-chemical characteristics of tebuconazole (http://sitem.herts.ac.uk/aeru/ppdb/en/Reports/610.htm), it is considered that the weekly concentration in the drinking water should vary negligibly (i.e. non-photodegradable, non-volatile, thermostable and stable compound in water). Considering that a sparrow drinks, on average, the equivalent of 30% of its body weight per day (Bartholomew and Cade 1963), a 25-g individual would consume approximately 4.1 µg of tebuconazole per day via drinking water, corresponding to an exposure concentration of 0.164 mg kg^{-1} bw day^{-1}. Comparatively, the median lethal dose (LD_{50}) and the chronic (21 days) no-observed-effect level (NOEL) of tebuconazole reported in northern bobwhite (*Colinus virginianus*) were 1988 mg kg^{-1} and 5.8 mg kg^{-1} bw day^{-1}, respectively (http://sitem.herts.ac.uk/aeru/ppdb/en/Reports). Therefore, our exposure concentration is estimated to be 36 times lower than the NOEL. The plasma concentration of tebuconazole in the sparrows was checked analytically (see Bellot et al. 2022), and showed that the individuals from the control group were free of contamination and that the individuals from the exposed group had an average plasma concentration of 59.7 pg g^{-1} tebuconazole. Moreover, we chose this water concentration of tebuconazole because we have previously shown that it results in an environmentally realistic plasma contamination (Angelier et al. 2023). Exposure to tebuconazole started on 6 November 2019 and lasted until 29 June 2020 (i.e. until the end of sampling, see Fig. 1). We specifically decided to expose individuals to tebuconazole during 7 months to test the impact of a long-term exposure to environmental doses of tebuconazole on TH and key physiological parameters. Although the duration of exposure to tebuconazole is rather long in our experiment, such a long-term chronic exposure can be found in some agroecosystems that rely on multiple treatments of fungicides (e.g. vineyards where tebuconazole is used from April to August), and also in mixed landscapes with multiple crops that used triazoles during different periods (e.g. cereal crops in autumn and winter; orchards, vegetables and vineyards in spring and summer). In order to verify that there was no major difference in drinking behaviour due to treatment, we analysed the hydration state (plasma osmolarity, Vapro2 osmometer, Elitech Group, France) of several control and exposed sparrows (for individuals with sufficient plasma left, $n = 9$ for control sparrows and $n = 9$ for exposed sparrows). This parameter has been shown to vary according to sparrow drinking behaviour (Brischoux et al. 2020). We found no difference in plasma osmolarity ($F_{(1,16)} = 0.64$, $p = 0.436$), suggesting that there is no major difference in drinking behaviour between control and exposed sparrows. Tebuconazole concentrations of exposed and control drinking water were assayed by an independent accredited analytical laboratory (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 (by liquid chromatography tandem mass spectrometry (LC-MS/MS)).

Feather morphometry

We used a standardized protocol to study plumage quality of exposed and control sparrows. In April 2020, we plucked the two inner rectrices (central tail feathers) of all sparrows (Fig. 1). These plucked feathers had grown before birds were captured and before the experimental exposure to tebuconazole (i.e. in August/September 2019 during the annual post-nuptial moult), and they, therefore, did not allow us to test the impact of tebuconazole exposure on plumage quality. However, feather plucking stimulates the growth of new feathers, and all inner rectrices had regrown within a month in most of individuals ($n = 30$). Then, in May 2020, after 6 months of exposure to tebuconazole, we plucked these two inner rectrices that had regrown while birds were either exposed to tebuconazole or not in order to test the impact of tebuconazole exposure on plumage quality (Fig. 1). Five morphometric parameters were then assessed

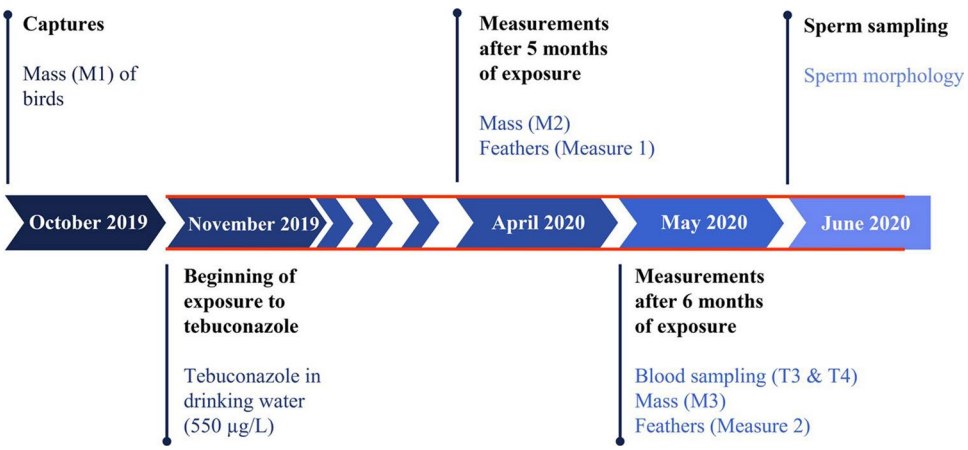

Fig. 1 Timeline describing the different steps of the experiments. The five dots highlight a key event during the experiment. The two red borders highlight the period of exposure of the experimental individuals to tebuconazole

on the plucked tail feathers of control and exposed house sparrows (initial and regrown). The morphometric parameters of both rectrices of each individual were averaged for each measurement timepoint. Only individuals with data for both measurement times (initial: measure 1, regrown: measure 2; Fig. 1) were considered for statistical analysis (n = 30 sparrows, with exposed and control individuals equally distributed). The first three morphometric parameters were measured using a high-resolution balance (feather mass, ±0.01 mg) and digital caliper (total feather length and diameter of the calamus ±0.01 mm). Standardized pictures of the feather were also taken, and feather area was calculated using a dedicated image software program (ImageJ, v1.53). Lastly, the density of feathers was calculated by dividing the mass of the feathers by their area.

Thyroid hormones

After 6 months of exposure, on 26 and 27 May, blood samples (100–150 µL) were successfully collected from all but one individual by puncture of the brachial vein to assess the effect of the treatment on total triiodothyronine (T3) and total thyroxine (T4) of the house sparrows (n = 37, Fig. 1). All blood samples were centrifuged, and red blood cells and plasma were separated and stored at − 20 °C until the assay. Plasma concentrations of total T3 and T4 were determined using radioimmunology at the Centre d'Études Biologiques de Chizé (CEBC) as previously described by Chastel et al. (2003). The lower limits of detection for T3 and T4 were 0.08 ng mL^{-1} and 0.602 ng mL^{-1}, respectively. Data below the detection limit were replaced by LOQ/sqrt(2) (De cock et al. 2014). For each hormone, all samples were run in duplicates in a single assay. The intra-assay CVs were 9.49% and 11.36% for T3 and T4, respectively.

Individual condition

To assess the effect of the treatment on the body condition of the sparrows, body mass was taken three times during the experiment (n = 38, Fig. 1): once at the start of the experiment (M1), a second time after 5 months (M2) and a third time after 6 months of experimentation (M3, Fig. 1). BCI was calculated by extracting the residuals from the linear regression of individual body mass as a function of tarsus length ($F_{(1,112)} = 44.28$, $p < 0.001$). Control and exposed sparrows did not differ in body size (tarsus, $F_{(1,36)} < 0.01$, $p = 0.932$).

Sperm morphology

In order to test the effect of the experimental treatment on the sperm morphology of the sparrows, we took a sperm sample during the breeding season (10 June 2020, Fig. 1). These sperm samples were obtained by faecal and abdominal massage described in detail by Girndt et al. (2017). This method allowed us to have a successful collection for 17 males including 8 males from the control group and 9 males from the exposure group. Each sperm sample was collected directly into a 0.5-mL microtube containing 200 µL of 5% formalin. At the laboratory, 10 µL of each sample was put on a slide to perform microscopic observation of sperm cells at ×400 magnification on a bright-field setting. For each individual, 100 photos of distinct intact sperm were taken (based on the method of Girndt et al. 2017, 2019). The different components of the sperm (i.e. head, including acrosome; flagellum, including midpiece) were measured by a single observer from digital images using ImageJ software (v1.53). Total sperm length was calculated as the sum of head and flagellum measurements (Girndt et al. 2017, 2019).

Statistical analysis

All statistical analyses were carried out in RStudio (R version 4.2.1). To test the effect of the experimental treatment (i.e. exposed to tebuconazole or control) on the levels of TH, feather morphometry, BCI and sperm length, linear mixed models (LMMs) and linear models (LMs) were used. All models were selected using the backward stepwise selection method by eliminating non-significant terms ($p > 0.100$). After conducting LMMs, specific comparisons were conducted using contrast procedures with the Satterthwaite approximation to describe the differences that were found between different groups of birds. The contrasts were made on the selected minimal model. We checked that all models met the assumptions of normality and homoscedasticity of residuals.

First, the plasma levels of T3 and T4 were analysed using LMs with two explanatory variables (treatment: control or exposed; sex: female or male) and their interaction. A log-transformed function was applied to the T4 thyroid hormone data to improve the distribution and homogeneity of the model residuals.

Second, because the morphometric parameters of the feathers were correlated, a principal component analysis (PCA) was performed. The first principal component (PC1) of the PCA explained 57.4% of the variation. PC1 was significantly and positively correlated with total feather length ($r = 0.92$, $p < 0.001$), feather mass ($r = 0.91$, $p < 0.001$), feather area ($r = 0.80$, $p < 0.001$), diameter of the calamus ($r = 0.60$, $p < 0.001$) and feather density ($r = 0.42$, $p < 0.001$). The second principal component (PC2) explained 28.9% of the variation. PC2 was significantly and positively correlated with feather density ($r = 0.87$, $p < 0.001$) and feather mass ($r = 0.38$, $p < 0.005$). Overall, PC1 represented the size of the feather while PC2 represented the density of the feather. For each individual, PC1 and PC2 values were extracted and

then analysed using an LMM. The initial model included 3 fixed factors (treatment: control or exposed; sex: female or male; and time: measurement 1 of initial feathers or measurement 2 of newly formed feathers; Fig. 1) and their interactions to test the effect of tebuconazole on feather morphometry (PC1 or PC2). The identity of individuals was added as a random effect in the models because individuals were sampled twice (measures 1 and 2).

In addition, an LMM with three fixed effects (treatment: control or exposed; sex: female or male; time of mass measurements: M1, M2 or M3; Fig. 1) and their interactions was carried out to test the effect of tebuconazole on BCI. Individual identity was added as a random effect in the model because the mass of individuals was measured three times.

Finally, the sperm length was analysed by LMMs with treatment as an explanatory variable (control or exposed). Specifically, three response variables were assessed: head length, flagellum length and total sperm length. The identity of the individual was added as a random effect in each model as 100 sperm samples were measured for each individual.

Table 1 Effect of treatment (control or exposed to tebuconazole) and sex (female or male) on the levels of T3 and T4 of house sparrows

Response variables	Explanatory factors	NumDF	DenDF	*F* value[a]	Pr (> *F*)[b]
T3	Treatment	1	34	1.00	0.324
	Sex	*1*	*35*	*5.16*	*0.029**
	Treatment:sex	1	33	0.09	0.770
T4	Treatment	*1*	*35*	*5.51*	*0.025**
	Sex	1	34	0.09	0.767
	Treatment:sex	1	33	0.04	0.838

A log-transformed function was applied to the T4 data in statistical models. All models were selected using the backward stepwise selection method by eliminating non-significant terms ($p > 0.100$)

NumDF degrees of freedom in numerator, *DenDF* degrees of freedom in denominator

*$p < 0.050$, significant

[a]Fisher's statistics

[b]p value associated

Results

Thyroid hormones (T3 and T4)

The "Treatment" factor did not significantly influence the T3 levels of sparrows (Table 1), whereas there was a significant effect of the "Sex" factor (Table 1), with higher T3 concentrations in females than in males (Fig. 2A). The treatment had a significant effect on the levels of T4 (Table 1). Individuals exposed to tebuconazole had significantly lower T4 levels than controls (Fig. 2B). T4 levels were not influenced by sex (Table 1). The "Treatment x Sex" interaction had no significant effect on T3 or T4 levels (Table 1), demonstrating that the influence of the experimental treatment on TH did not differ significantly between sexes (Fig. 2A, B).

Feather morphometrics

PC1 and PC2 scores significantly varied according to the "Time" factor (Table 2). Specifically, the regrown feathers were overall smaller and less dense relative to the initial feathers (Fig. 3).

Although there was no significant effect of either the "Sex" or "Treatment" factors on PC1 and PC2 scores (Table 2), there was a significant effect of multiple interactions on both PC1 and PC2 scores (Table 2), demonstrating that the influence of the treatment on PC1 and PC2 was complex and differed between sexes and groups.

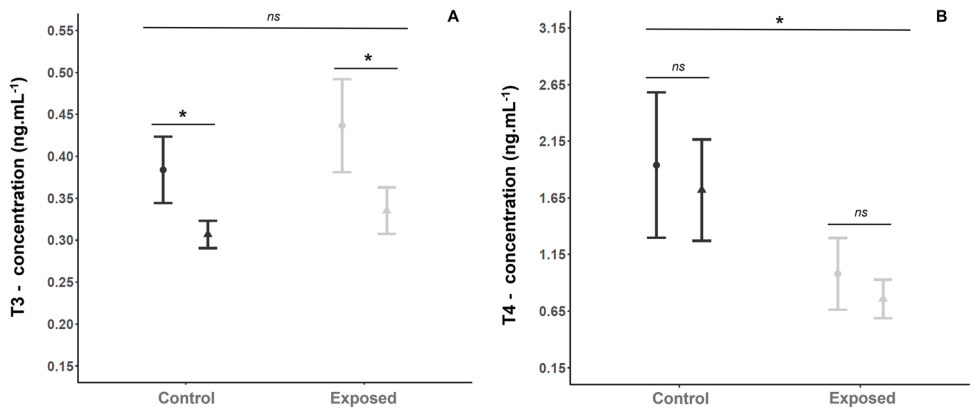

Fig. 2 Plasma concentrations of T3 (**A**) and T4 (**B**) in ng mL^{-1} according to treatment (control or exposed to tebuconazole) and sex (female or male). Control and exposed individuals are represented in black and grey, respectively. Females and males are represented by circles and triangles, respectively. The concentration of T3 did not differ significantly between exposed and control sparrows ('ns', $p > 0.100$), whereas the concentration of T4 was significantly lower for exposed sparrows compared to control sparrows (*$p < 0.050$). Means ± SE are presented

Table 2 Effects of time of measurement on feather morphometric parameters (initial or after regrowth), treatment (control or exposed to tebuconazole), sex (female or male) as well as their interactions on PC1 scores (feather size) and PC2 scores (feather density), respectively

Response variables	Explanatory factors	NumDF	DenDF	khi^2 value[a]	Pr (> khi^2)[b]
PC1 (~ length)	Time	*1*	26	*35.49*	*< 0.001****
	Treatment	1	26	2.50	0.114
	Sex	1	26	< 0.001	0.976
	Time:treatment	1	26	0.13	0.718
	Time:sex	*1*	26	*6.82*	*0.009***
	Treatment:sex	1	26	2.09	0.148
	Time:treatment:sex	*1*	26	*4.70*	*0.030**
PC2 (~ density)	Time	*1*	28	*43.43*	*< 0.001****
	Treatment	1	26	0.39	0.534
	Sex	1	26	2.53	0.112
	Time:treatment	*1*	28	*5.01*	*0.025**
	Time:sex	1	27	1.74	0.187
	Treatment:sex	*1*	26	*5.11*	*0.024**
	Time:treatment:sex	1	26	1.72	0.190

Individual identity was added as a random factor. All models were selected using the backward stepwise selection method by eliminating non-significant terms ($p > 0.100$)

NumDF degrees of freedom in numerator, *DenDF* degrees of freedom in denominator

***$p < 0.001$; **$p < 0.010$; *$p < 0.050$, significant

[a]Chi-square statistic

[b]p value associated

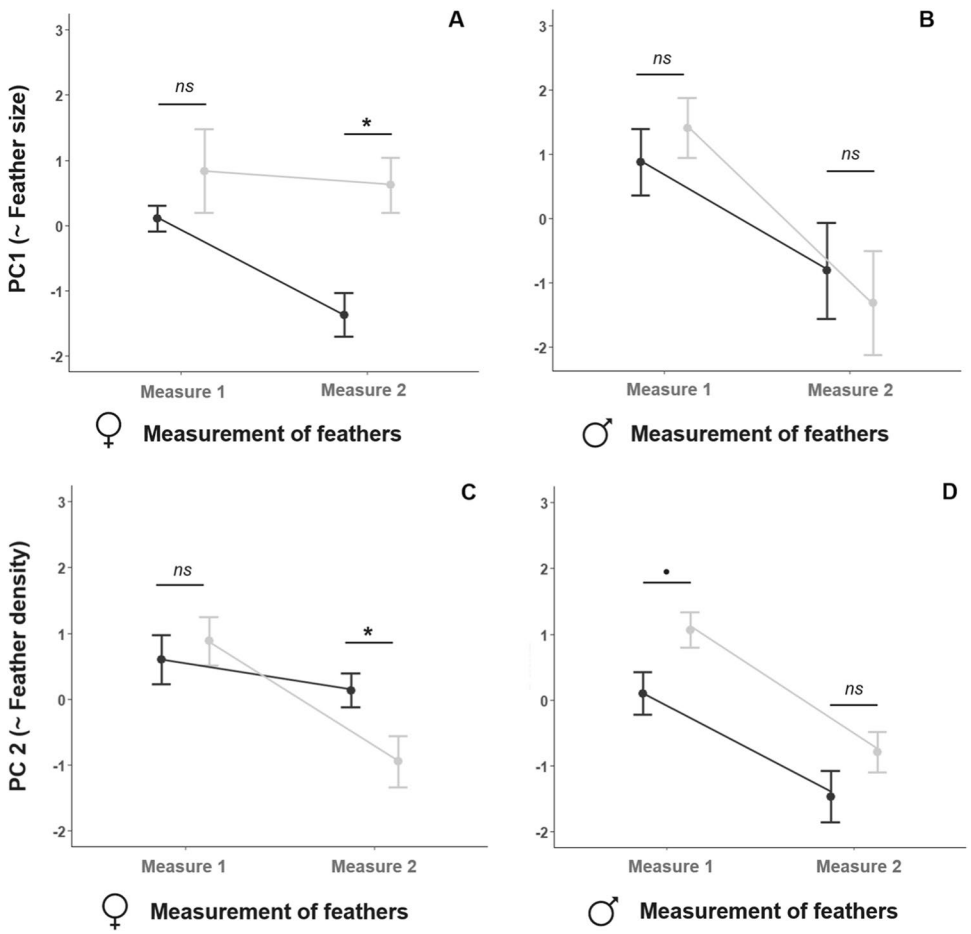

Fig. 3 PC1 scores (feather size, **A** and **B**) and PC2 scores (feather density, **C** and **D**) as a function of the time of measurement (initial feathers corresponding to measurement 1 and regrown feathers corresponding to measurement 2). Panels **A** and **C** show the results obtained for females, and panels **B** and **D** show the results obtained for males. Control and exposed individuals are represented in black and grey, respectively. Significant terms are indicated as follows: *$p < 0.050$, •$p < 0.100$ and $^{ns}p > 0.150$. The means ± SE are presented

Regarding PC1 scores, the "Time x Sex" and "Time x Treatment x Sex" interactions were significant (Table 2), demonstrating that the influence of the experimental treatment on this parameter differed significantly depending on the time of measurement (i.e. before or after feather regrowth) and the sex of the individuals (Fig. 3A, B). Specifically, the regrown feathers of exposed females were significantly larger than those of control females (measure 2, contrast: $t_{(41.2)} = -2.72$, $p = 0.009$; Fig. 3A), whereas this difference was not significant for the initial feathers (measure 1, contrast: $t_{(41.2)} = -1.00$, $p = 0.322$; Fig. 3A). For males, there was no difference in feather size between exposed and control individuals at any time (measure 2 [contrast: $t_{(41.2)} = 0.64$, $p = 0.524$] and measure 1 [contrast: $t_{(41.2)} = -0.68$, $p = 0.500$]; Fig. 3B).

Regarding PC2 scores, the "Treatment x Sex" and "Time x Treatment" interactions were significant (Table 2), demonstrating that the influence of the experimental treatment on this parameter differed significantly depending on the time of measurement (i.e. before or after feather regrowth) and the sex of the individuals (Fig. 3C, D). Interestingly the regrown feathers of exposed females (measure 2) were significantly less dense than those of control females (contrast: $t_{(48.5)} = 2.34$, $p = 0.024$), whereas this difference was not significant for the initial feathers (measure 1, contrast: $t_{(48.5)} = -0.60$, $p = 0.549$; Fig. 3C). The feathers of exposed males were overall denser than those of the control males (contrast: $t_{(26.0)} = -2.08$, $p = 0.048$; Fig. 3D). Specifically, feathers of exposed males were denser than those of control males before exposure to tebuconazole (contrast: $t_{(48.5)} = -1.94$, $p = 0.058$) but this difference was not significant for the regrown feathers, i.e. after exposure (contrast: $t_{(48.5)} = -1.37$, $p = 0.178$; Fig. 3D).

Body condition index

Although the factors "Sex", "Time" and "Treatment" did not significantly influence the BCI of individuals, the "Time x Treatment" and "Time x Treatment x Sex" interactions had significant effects on the BCI of the individuals (Table 3). This showed that the influence of the experimental treatment on the BCI differs significantly according to the sex of the individual and the time of the measurement (M1, M2 or M3; Fig. 4). Specifically, the BCI of control females varied over time, where they had a higher BCI at time M3 compared to that at time M1 (contrast: $t_{(68.0)} = -4.45$, $p < 0.001$; Fig. 4A). However, the BCI of exposed females did not vary over time (contrasts: all p values > 0.345 Fig. 4A). Moreover, exposed females had a greater BCI than control females at time M1, i.e. before exposure to tebuconazole (contrast: $t_{(59.1)} = -2.31$, $p = 0.024$) whereas this difference became marginal at time M2, i.e. after 5 months of exposure (contrast: $t_{(59.1)} = -1.97$, $p = 0.054$) and non-significant at time M3, i.e. after 6 months of exposure (contrast: $t_{(59.1)} = 1.20$, $p = 0.235$; Fig. 4A). In control and exposed males, there was no significant variation in BCI among times M1, M2 and M3 (contrasts: all p values > 0.089; Fig. 4B).

Sperm morphology

Sperm head length, flagellum length and total length were not significantly affected by treatment (control or exposed, Table 4). Sperm head length averaged as follows (mean ± SE): 14.85 µm ± 0.033 for controls and 14.68 µm ± 0.027 for exposed sparrows. The length of the sperm flagellum was on average as follows: 86.95 µm ± 0.15 for the controls and 87.09 ± 0.12 for the exposed sparrows. Finally, the total length of the spermatozoa was 101.80 µm ± 0.16 for the controls and 101.77 µm ± 0.12 for the exposed sparrows.

Table 3 Effects of the timing of mass measurement (M1, M2 or M3), treatment (control or exposed to tebuconazole), sex (female or male) and their interactions on the BCI of sparrows

Response variable	Explanatory factors	NumDF	DenDF	khi^2 value[a]	Pr (> khi^2)[b]
Body condition index	Treatment	*1*	*34*	*3.81*	*0.051*
	Sex	1	34	2.41	0.121
	Time	2	68	2.04	0.360
	Treatment:sex	1	34	0.10	0.748
	Treatment:time	*2*	*68*	*9.44*	*0.009***
	Sex:time	*2*	*68*	*4.70*	*0.095*
	Treatment:sex:time	*2*	*68*	*10.26*	*0.006***

Individual identity was added as a random factor. All models were selected using the backward stepwise selection method by eliminating non-significant terms ($p > 0.100$)

**$p < 0.010$, significant

NumDF degrees of freedom in numerator, *DenDF* degrees of freedom in denominator

[a]Chi-square statistic

[b]p value associated

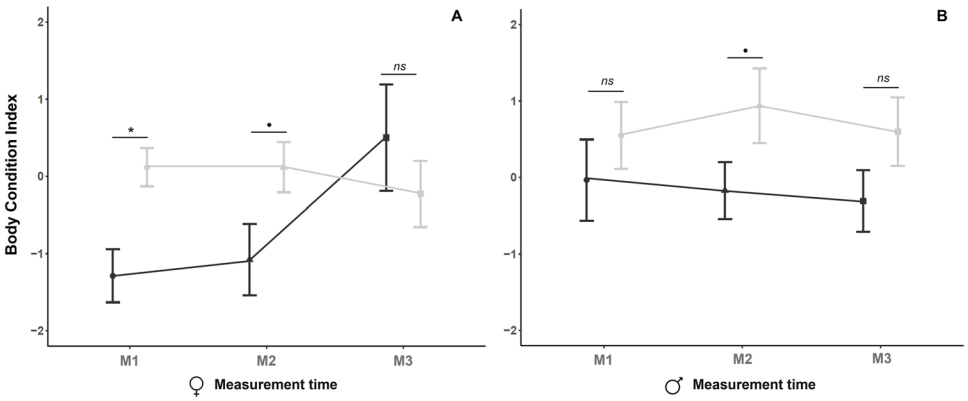

Fig. 4 Body condition index (BCI) of individuals as a function of the timing of mass measurement (M1, M2 or M3), treatment (control or exposed to tebuconazole) and the sex of individuals (**A** female or **B** male). Control and exposed individuals are represented in black and grey, respectively. Significant terms are indicated as follows: $^{*}p < 0.050$, $^{\bullet}p < 0.100$ and $^{ns}p > 0.150$. Means ± SE are presented

Table 4 Effects of treatment (control or exposed to tebuconazole) on the length of the different sperm components (head, flagellum and total length)

Response variables	Explanatory factors	NumDF	DenDF	*F* value[a]	Pr (> *F*)
Head length	Treatment	1	14.4	1.68	0.215
Flagellum length	Treatment	1	15.0	0.15	0.700
Total length	Treatment	1	15.0	0.04	0.844

Individual identity was added as a random factor

NumDF degrees of freedom in numerator, *DenDF* degrees of freedom in denominator

[a]Fisher's statistics

[b]*p* value associated

Discussion

In this study, we showed for the first time in wild-caught birds that chronic exposure to a widely used triazole fungicide (tebuconazole) could significantly reduce the levels of T4. However, we did not find any effect of such exposure to tebuconazole on the levels of T3. In female sparrows, exposure to tebuconazole also affected the structure of the feathers that grew during the experiment, with larger but less dense feathers for exposed individuals compared to controls. In addition, body condition did not vary considerably throughout the experiment, except for the control females that experienced a significant increase in their body condition towards the end of the experiment. Finally, sperm morphology was not affected by the treatment. Overall, we demonstrated sublethal effects of tebuconazole on key physiological parameters, such as the HPT axis and the other related physiological trait (plumage quality) in vertebrates that are commonly found in agroecosystems.

Thyroid hormones

Although T3 levels were not significantly affected by the chronic exposure to tebuconazole, our experimental treatment was associated with a significant decrease in circulating T4 levels. In agreement with our results, previous studies have shown that an exposure to tebuconazole could induce an alteration of the HPT axis and, notably, a decrease in T4 levels in laboratory model species (Yu et al. 2013; Li et al. 2019). Indeed, zebrafish showed a significant decrease in T4 levels when exposed to tebuconazole concentrations of 200 µg L^{-1} and 500 µg L^{-1} (Li et al. 2019). Similar effects were also found in zebrafish larvae, with a significant decrease in T4 levels when larvae were exposed to a tebuconazole concentration of 4 mg L^{-1} (Yu et al. 2013), although this effect was not found at lower concentrations (1 mg L^{-1} and 2 mg L^{-1}). Furthermore, and interestingly, Bernabò et al. (2016) found an effect of tebuconazole on the success and speed of metamorphosis in tadpoles, suggesting a disrupting effect of tebuconazole on the HPT axis.

Here, we showed for the first time that such dysregulation of the HPT axis may also occur in wild-caught terrestrial vertebrates. Importantly, the activity of the thyroid gland is under the control of the pituitary, which governs the biosynthesis and secretion of the thyroid-stimulating hormone (TSH, encoded by *tshα* and *tshβ*; Fig. 5) (Hall et al. 1970; Harris et al. 1978; Capen 1998; Stoker et al. 2006). When TH levels become insufficient, the levels of TSH increase in order to stimulate the secretion of THs by the

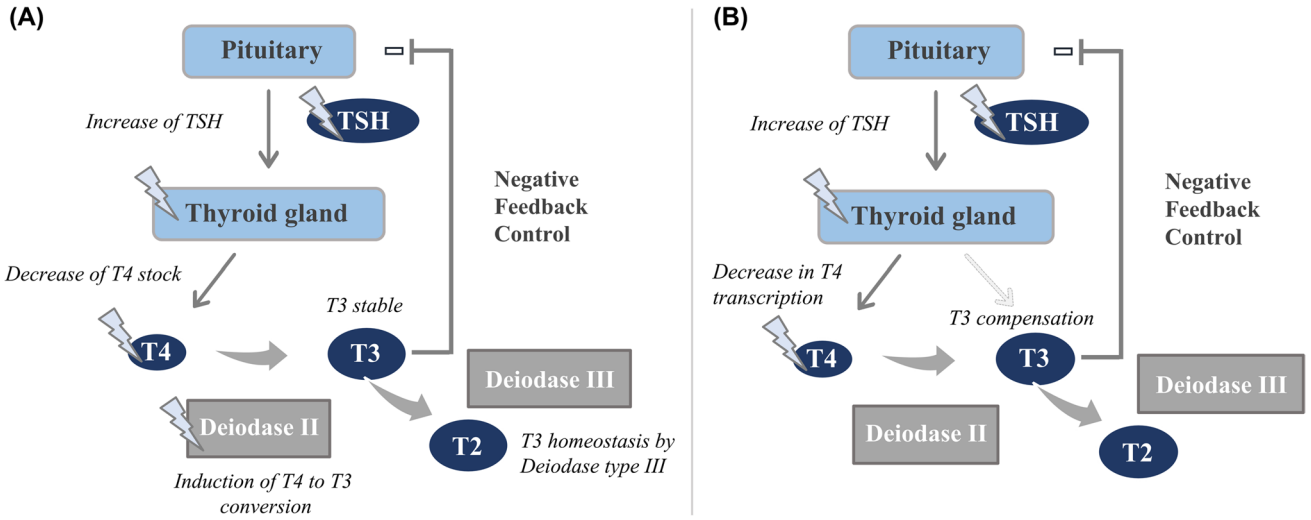

Fig. 5 Schematic representation of the different scenarios proposed for the mode of action of tebuconazole on the house sparrow endocrine system. **A** By induction of type II deiodase activity resulting in increased conversion of T4 to T3 to ensure production of active TH at the expense of a decrease in T4 stock. **B** By inhibition of T4 transcription or release leading to compensation of T3 production by a T4-independent biosynthetic pathway. These examples are not exhaustive, and hypotheses **A** and **B** are non-mutually exclusive. The flash symbol represents potential biological targets of tebuconazole in the negative feedback loop of thyroid hormones

thyroid and restore homeostasis. Some studies have shown that exposure to azoles (difenoconazole, hexaconazole and tebuconazole) (Liang et al. 2015; Yu et al. 2013) causes a significant increase in the transcription of genes related to TSH production and secretion (i.e. *tshβ*), which could result from a stimulation of the HPT axis in response to decreased T4 levels (Liang et al. 2015; Pirahanchi et al. 2018). To test this hypothesis, it would be interesting now to measure the transcriptomic activity of *tshβ* to know if it is inversely proportional to the decrease in T4 observed in the sparrows exposed to tebuconazole or not.

Contrary to our prediction, we did not find any effect of tebuconazole on the levels of T3. Li et al. (2019) showed that exposing zebrafish to tebuconazole caused a significant decrease in T3 levels (dose: 200 µg L^{-1} and 500 µg L^{-1}), but Yu et al. (2013) reported in another study that exposure to tebuconazole resulted in a significant increase in T3 levels in zebrafish larvae (4 mg L^{-1}). Interestingly, Liang et al. (2015) found similar results to our study. Indeed, after exposing zebrafish larvae to difenoconazole (another triazole), a significant decrease in T4 levels was observed while no significant effect was observed on T3 levels. Altogether, these results suggest that the effect of tebuconazole on T3 levels may depend on the dose of tebuconazole, life history stages and/or species.

At the mechanistic level, the decrease in T4 levels may be enhanced by an increased activation of type II deiodase (encoded by *dio2*, Fig. 5A). Type II deiodase is involved in the catalytic conversion of T4 to its more active form T3 (Bianco and Kim 2006), so its activation could lead to a decrease in the stock of T4 (as suggested by Pandey and Mohanty 2015; Fig. 5A). Supporting this idea, Liang et al. (2015) found that difenoconazole can cause an increase in *dio2* transcription. In agreement with the results of Liang et al. (2015), we did not observe any change in the levels of T3, although an increase in the conversion of T4 to T3 by type II deiodase should logically lead to an increase in T3 levels. However, the levels of T3 also depend on the activity of TH-degrading enzymes, such as type III deiodase (*dio3*, Bianco and Kim 2006), which activates the conversion of T3 into inactive forms (diiodothyronine (T2), Dentice and Salvatore 2011). Therefore, tebuconazole could affect the activation of these enzymes (*dio2* and *dio3*) and result in decreased T4 levels, but stable T3 levels (Dentice and Salvatore 2011; Pandey and Mohanty 2015). Alternatively, tebuconazole could also affect T4 transcription. If the transcriptomic activity of T4 is decreased but the levels of T3 are maintained, it is possible that exposed individuals show an increased biosynthesis of T3 independent of T4 in order to compensate for the decrease in T4 levels (Fig. 5B). Under normal circumstances, about 80% of T3 is formed from T4, while only 20% is produced directly by the thyroid (Pirahanchi et al. 2018). Thus, as suggested by Pandey and Mohanty (2015), the induction of a T4-independent T3 biosynthetic pathway could explain why T4 levels decrease but T3 levels are maintained (Fig. 5B).

Therefore, there is no clear consensus on how tebuconazole affects the thyroid system. Our study shows that tebuconazole seems to disrupt the HPT axis in wild terrestrial vertebrates, as previously found in aquatic models (Yu

et al. 2013; Liang et al. 2015; Li et al. 2019). However, its mode of action could be complex and may involve several metabolic pathways (Fig. 5; see also Kjærstad et al. 2007; Yang et al. 2013; Yu et al. 2013; Liang et al. 2015; Li et al. 2019; Zhang et al. 2019). To further understand the impact of tebuconazole on the HPT axis, it would be relevant to use a transcriptomic approach to identify the biological targets responsible for this disruption of the thyroid axis.

Feather quality

After exposure to tebuconazole, exposed individuals had larger but less dense feathers than the controls, but this effect was only found in females. To our knowledge, our study is the first to provide evidence for an effect of triazoles on plumage quality. Although we could not demonstrate that these effects were linked to a disruption of the HPT axis, this is plausible because the relationship between TH and the moulting process is well established (Davis and Davis 1954; Wilson and Farner 1960; Groscolas and Cherel 1992; Cherel et al. 2004; McNabb 2007). Previous studies have shown that moulting can be induced by the administration of exogenous TH, while the removal of the thyroid gland leads to the inhibition of moult or regrowth of artificially plucked feathers (Voitkevich 1966; Hahn et al. 1992; Kuenzel 2003; Pérez et al. 2018).

We found that tebuconazole caused a decrease in circulating T4 levels in exposed sparrows and several studies have demonstrated that T4 plays a key role in the moulting process of birds (Groscolas and Cherel 1992; Cherel et al. 2004; McNabb 2007). For example, Groscolas and Leloup (1986) showed a positive correlation between peak plasma T4 and the onset of moult in birds, suggesting a more important role of T4 than that of T3 in this process. These findings are supported by several studies in multiple bird species (Decuypere and Verheyen 1986; Cherel et al. 1988, 2004; Jenni-Eiermann et al. 2002; Vézina et al. 2009). According to these studies and the results that we obtained on T4, we can hypothesise that the interference of tebuconazole with the HPT axis may have caused a change in the quality of the feathers that regrew during the phase of exposure to tebuconazole. However, it is important to note that we are not aware of any study that has linked circulating TH levels with feather quality because most studies focused on the onset or the rate of moulting rather than on the quality of the feathers produced (e.g. Decuypere, 1986; Cherel et al. 2004; Pérez et al. 2018). In addition, we found an effect of tebuconazole on feather quality mainly in females, while the T4 levels of both males and females were affected by tebuconazole. This suggests that the observed effects on feather quality could also be related to the effect of tebuconazole on other mechanisms. For example, feather quality and moult processes have been also linked to levels of glucocorticoids (corticosterone in birds) and testosterone (Nolan et al. 1992; Stoehr and Hill 2001; DesRochers et al. 2009; Siefferman et al. 2013; Jenni-Eiermann et al. 2015). Previous studies have found that triazoles can also affect the synthesis of these two steroids in vertebrates through their action on cytochrome P450 enzymes (Goetz et al. 2007; Taxvig et al. 2007; Yang et al. 2018; Draskau et al. 2019). Therefore, disruption of sex hormones, such as testosterone, could potentially explain the sex-dependent effect of tebuconazole on feather quality. Further studies of these endocrine axes are therefore required to better understand the impact of triazoles on moult processes and feather quality.

The regrown feathers of the exposed females were overall larger and less dense relative to control females. This structural modification of feathers can potentially have implications for individual performance (i.e. survival and reproduction) through an impact on flight quality, the ability of individuals to thermoregulate or the ability to express secondary sexual signals through plumage (Swaddle et al. 1996; Dawson et al. 2000; Jenni and Winkler 2020). Future studies should now examine the impact of tebuconazole on these feather-related traits.

Body condition index

Contrary to our prediction, we did not find any major effects of tebuconazole on the body condition of individuals. Most individuals maintained their body condition throughout the experiment, independent of the treatment. The only significant effect was an increase in BCI for control females towards the end of the experiment (M3), whereas this increase was not observed in exposed females or control and exposed males. This puzzling result may be an artefact, although our sample size is quite robust for such an experimental study. Indeed, most previous studies have not reported any significant effect of exposure to triazoles on the body condition of birds (Lopez-Antia et al. 2013, 2018; Pandey and Mohanty 2015; Fernández-Vizcaíno et al. 2020). However, it could also be linked to a biological process: control females may have accumulated body reserves at the end of the experiment in preparation for breeding. Indeed, females usually increase their body mass a few days/weeks before laying their eggs (e.g. Kvalnes et al. 2013; Hennin et al. 2015) while this phenomenon is not detectable in males. Tebuconazole exposure may have delayed or even suppressed the initiation of reproduction in females by interfering with seasonal endocrine processes, explaining then this absence of body weight gain in exposed females. Accordingly, studies have reported that triazoles may impair reproduction in birds (e.g. Fernández-Vizcaíno et al. 2020; Lopez-Antia et al. 2021). Moreover, triazoles are also known to interfere with cytochrome P450 enzymes, which are associated with the regulation of sex hormones (e.g. CYP19, which is involved in the conversion

of androgens to estrogens) (Poulsen et al. 2015; Saxena et al. 2015). Since estrogens (such as estradiol) are known to control feeding behaviour, fat deposition and body condition in vertebrates, it is possible that deregulation of the steroid axis during the reproductive period could result in a sex-dependent effect of treatment on body condition. Interestingly, Pandey and Mohanty (2015) found a significant decrease in the mass of exposed birds in response to fungicides, but only during the breeding phase, suggesting that the effects of fungicides on the body condition of individuals may be more apparent during the energetically demanding reproductive stage. We do not know the exact age of our adult sparrows as they were caught in the wild, but it would also be interesting to see if age could influence the response to tebuconazole exposure. We must, however, remain cautious because exposed birds and controls unexpectedly differed in body condition at the beginning of the experiment, making it difficult to draw strong conclusions. Moreover, the sparrows had access to food ad libitum in our experiment and captivity limits mobility and, therefore, energy expenditure. These ideal nutritional conditions may have masked a potential impact of tebuconazole on metabolism and/or food consumption, and individuals may have been able to maintain their BCI despite the tebuconazole treatment. Finally, it would be interesting to further test how tebuconazole may affect body condition. For example, it is possible that tebuconazole has an impact on food intake, water consumption, metabolism or/and activity, all of which influence body condition. However, Lopez-Antia et al. (2021) showed no effect of tebuconazole on food intake in red-legged partridges, suggesting that there is no effect on palatability or hunger. Future studies should therefore examine the impact of tebuconazole on body mass under realistic food situations and test whether exposure to tebuconazole affects food consumption, activity and reproduction.

Sperm morphology

Chronic exposure to tebuconazole prior to the breeding season did not result in any significant effect on sperm length in exposed sparrows. This result is contrary to our prediction as tebuconazole is known to interfere with steroid hormones and catalase and to have a negative impact on the reproduction of individuals (Poulsen et al. 2015; Lopez-Antia et al. 2021; Huang et al. 2022). In addition, some studies have shown that pesticides can affect male germ cells, such as causing a decrease in the quantity and density of these cells, a reduction in testicular mass and an interruption of spermatogenesis (Tokumoto et al. 2013; Mohanty et al. 2017; Humann-Guilleminot et al. 2019a Machado-Neves et al. (2018) also showed that bats treated with 1 mL L^{-1} of tebuconazole for 30 days had morphometric changes in their testes and epididymis, suggesting a potential effect of tebuconazole on the gonadal axis. Importantly, it would be interesting to complement our measurements with an assessment of sperm motility, sperm count, abnormalities and oxidative damage, because fungicides may affect these variables despite no effect on sperm morphology. Therefore, further studies would be needed to understand how tebuconazole impacts fertility and reproductive success in wildlife.

Conclusion

Overall, our results showed physiological effects of tebuconazole on a sentinel species of agroecosystems, the house sparrow. These effects include a significant decrease in T4 levels, and an alteration of feather structure, which altogether suggests a dysregulation of thyroid homeostasis by tebuconazole. Therefore, our study highlights the importance of assessing the potential risks of chronic exposure to tebuconazole for wild vertebrates living in agroecosystems.

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Author contributions Pauline Bellot: data collection, data curation, formal analysis, writing of original draft and visualization. François Brischoux: conceptualization, data collection, validation, writing including review and editing and funding acquisition. Hélène Budzinski: data collection. Sophie Dupont: writing including review and editing. Clémentine Fritsch: conceptualization, writing including review and editing and funding acquisition. Sydney Hope: data collection and writing including review and editing. Bruno Michaud, Marie Pallud, Charline Parenteau and Louise Prouteau: data collection and data curation. Steffi Rocchi: writing including review and editing. Frédéric Angelier: conceptualization, data collection, data curation, validation, resources, writing including review and editing, supervision and funding acquisition.

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Data availability Data available on request from the authors.

Declarations

Ethical approval and consent to participate 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 ethics committee and Ministère de L'Enseignement Superieur, de la Recherche et de L'innovation), under permit #APAFIS#12918–2018010515574796. Consent to participate is not applicable.

Consent for publication Not applicable

Competing interests The authors declare no competing interests.

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