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Experimental Exposure to Tebuconazole Affects Metabolism and Body Condition in a Passerine Bird, the House Sparrow (*Passer domesticus*)

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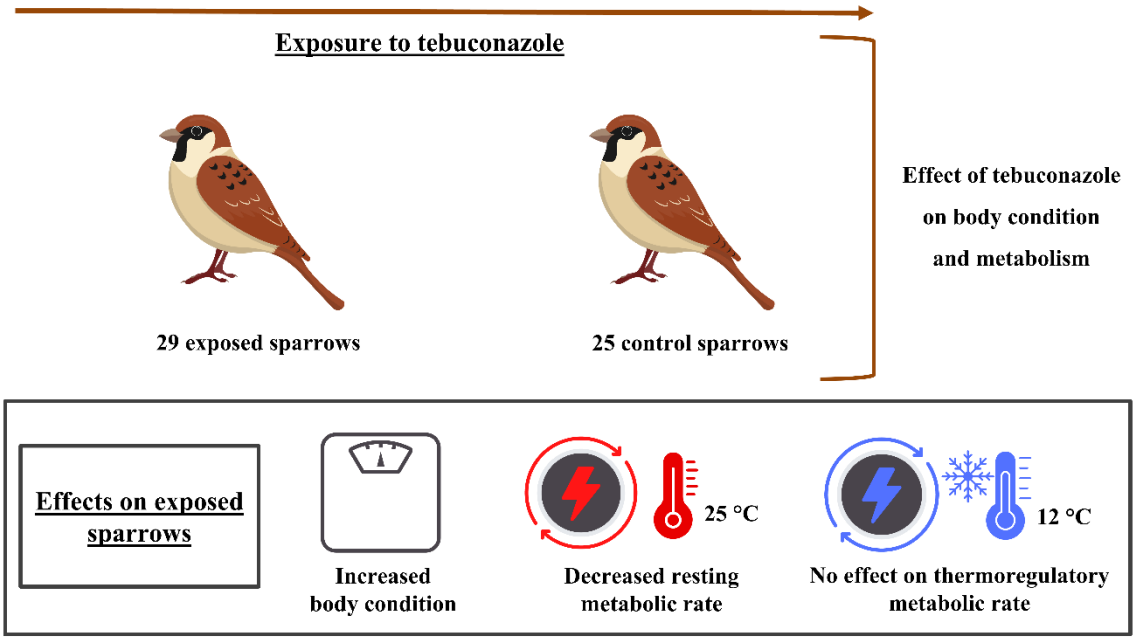
Graphical Abstract



ABSTRACT

Triazole compounds are among the most widely used fungicides in agroecosystems to protect crops from potential fungal diseases. Triazoles are suspected to have an impact on non-target species due to their interactions with non-fungal sterol synthesis and wild birds are likely to be contaminated by triazoles fungicides as many of them live in agroecosystems. We experimentally tested whether exposure to environmental concentrations of a triazole could alter key integrative traits (metabolic rates and body condition) of an agroecosystem sentinel species, the house sparrow (*Passer domesticus*). Wild-caught adult sparrows were maintained in captivity and exposed (exposed group) or not (control group) for seven continuous months to tebuconazole through drinking water. The metabolic rates of exposed and control sparrows were then measured at two different temperatures (*i.e.,* 12°C and 25°C), which correspond respectively to the thermoregulation and thermoneutrality temperatures of this species. We found that exposed sparrows had lower resting metabolic rates (*i.e.,* measured at thermoneutrality, 25°C) than controls. However, the thermoregulatory metabolic rates (*i.e.,* measured at 12°C) did not differ between exposed and control sparrows. Although the body mass and condition were not measured at the beginning of the exposure, sparrows at the time of the metabolic measurements seven months after the onset of such exposure had a higher body condition than controls, supporting further the idea that tebuconazole affects metabolic functions. Our study demonstrates for the first time that the use of tebuconazole can alter metabolism and could potentially lead to adverse effects in birds.

INTRODUCTION

Whole-organism metabolism is a key physiological trait because it is involved in multiple biological processes (e.g., foraging, reproductive behavior; Biro & Stamps, 2010; Bryant, 1997; Careau et al., 2011; Daan et al., 1990; McNab, 2002). Whole-organism metabolic rates are measured as a function of the rate at which an organism oxidizes substrates to produce energy (Careau et al., 2008). Classically, they are determined by the sum of the energy cost of self- maintenance (resting metabolic rate [RMR]; Bryant, 1997; Daan et al., 1990; McNab, 2002), and also the energy output that is available to the organism to perform additional daily or seasonal physiological and behavioral processes (Biro & Stamps, 2010). Resting metabolic rate is defined as the metabolic rate of an adult animal at rest in its thermoneutral zone and in a postabsorptive state (Hulbert & Else, 2004). Importantly, metabolic rates often show strong interindividual variations within species (Burton et al., 2011; Konarzewski & Książek, 2013), and this variability has previously been linked to different individual energetic strategies (Biro & Stamps, 2010; Burton et al., 2011; Careau et al., 2008; Nilsson, 2002; Rønning et al., 2016). In birds, metabolic rates are known to be dependent on many biotic and abiotic factors such as individual mass, age, hormones, and ambient temperature (e.g., Bouwhuis et al., 2011; Broggi et al., 2007; Burton et al., 2011; Chappell et al., 1999; Chastel et al., 2003; Dupont et al., 2019; Konarzewski & Książek, 2013; Nzama et al., 2010) and, importantly, metabolic rates can also be affected by exposure to environmental contaminants. For example, Verreault et al. (2007) demonstrated that organohalogen contaminants could lead to a decrease in RMR in glaucous gulls.

Such contaminant-related alterations of metabolic rates may have detrimental impacts on fitness as a result of pathologies (e.g., Quagliariello et al., 2017) and/or a mismatch between energy availability and energy demands. In vertebrates, metabolism is under the control of the hypothalamus–pituitary–thyroid (HPT) axis and the resulting regulation of circulating thyroid hormones (TH) such as thyroxine (T4) and triiodothyronine (T3; Danforth & Burger, 1984; Ortiga-Carvalho et al., 2011). For example, plasma concentrations of TH have been functionally related to RMR in humans and wild vertebrates (Chastel et al., 2003; Elliott et al., 2013; Hulbert & Else, 2004; Kim, 2008; Welcker et al., 2013). In that context, the metabolic function of vertebrates is likely to be altered by pollutants which target the functioning of the HPT axis (i.e., endocrine disruptors, e.g., Ask et al., 2021; Jugan et al., 2010; Li et al., 2019; Sebastiano et al., 2021; Yu et al., 2013).

Several studies have demonstrated that contaminants can alter the functioning of the HPT axis and the metabolism of vertebrates (e.g., Braham & Neal, 1974), but most of these studies have focused on polychlorinated biphenyls (French et al., 2001; Smits et al., 2002; Tori & Mayer, 1981; Verreault et al., 2007; Voltura & French, 2000), organochlorine pesticides (Blévin et al., 2017), polybrominated diphenyl ethers (Allen et al., 2016; Lema Sean et al., 2008), per- and polyfluoroalkyl compounds (Ask et al., 2021; Blévin et al., 2017; Sebastiano et al., 2021), and neonicotinoids (Zgirski et al., 2021). The potential impact of endocrine disruptors on metabolism remains neglected, especially regarding contaminants of emerging interest such as azoles. To comprehensively assess the potential actions of endocrine disruptors on the metabolism of organisms, it is essential to widen the panel of studied contaminants (including overlooked emerging, albeit widespread, molecules).

Among the known endocrine disruptors, many pesticides are attracting increasing attention because of their massive release in the environment (Mahmood et al., 2016). Fungicides are widely used pesticides that are increasingly employed in many crops, sometimes in very large quantities (Zhang, 2018). Azoles compounds are currently the most used fungicides worldwide and they are applied to a wide range of crops, including vegetables, orchards, vineyards, field crops, and ornamental cultures (Souders et al., 2020). This widespread and intensive production of azole compounds has led to environmental contamination of surface water (Kahle et al., 2008), soil (Pelosi et al., 2021), and air (Rokbani et al., 2019). As a result, residual contamination is also found in food (European Food Safety Authority, 2016), invertebrates, wild vertebrates, and humans (Daniele et al., 2018; Pelosi et al., 2021; Polledri et al., 2019).

One concern with the release of azoles in the environment is their potential impact on nontarget species such as wild vertebrates (fish, birds, mammals, reptiles, or amphibians), mainly because azoles may affect multiple endocrine mechanisms (Zarn et al., 2003). For example, recent studies have demonstrated that birds show a disruption of their physiology and a reduced breeding output when they are exposed to triazoles through their diet (Fernández-Vizcaíno et al., 2020; Lopez-Antia et al., 2018, 2021). In addition, experimental studies in aquatic models have shown that triazoles can modify several physiological and behavioral components, such as glucose and protein regulation, mitochondrial function, endocrine mechanisms, and locomotor activity (Cao et al., 2019; Souders et al., 2020; Toni et al., 2011). Interestingly, few studies have shown that triazoles can affect the HPT axis and circulating TH levels in laboratory fish (Li et al., 2019; Yu et al., 2013). Despite these significant advances,

the effects of low environmental concentrations of triazoles on physiological processes of wild terrestrial vertebrates is overall overlooked because most of the literature deals with acute ecotoxicity tests (e.g., median lethal dose; LC50) using aquatic vertebrate or invertebrate model species (such as zebrafish and earthworms; Andreu-Sánchez et al., 2012; Binev et al., 2005; Raby et al., 2019; Rico et al., 2016; Sancho et al., 2010).

In the present study, we experimentally tested the impact of tebuconazole on metabolic rates and condition (body mass and body condition) in a wild bird species that inhabits agricultural environments, the house sparrow (*Passer domesticus*). Tebuconazole is currently one of the most used triazole in crops (Berenzen et al., 2005; Kahle et al., 2008), and it is suspected to have detrimental effects on wild birds (e.g., Fernández-Vizcaíno et al., 2020; Lopez-Antia et al., 2021). Although several studies suggest that triazole compounds may interfere with metabolic rates, to our knowledge no study has experimentally tested the impact of tebuconazole on metabolic rates in wildlife. In the present study, we experimentally contaminated captive wild- caught house sparrows with tebuconazole and mimicked a realistic environmental exposition to tebuconazole. We then determined the impact of a chronic exposure to tebuconazole (~7 months) on the RMR (i.e., at 25 °C, which corresponds to thermoneutrality for house sparrows) and on the thermoregulatory metabolic rate (TMR; i.e., at 12 °C when sparrows have to spend additional energy to actively thermoregulate; Dupont et al., 2019) of sparrows. Metabolic rates were measured at these two temperatures to test if the potential effect of tebuconazole on metabolism may be exacerbated under energetically constraining conditions (i.e., at 12 °C). We also studied the effect of exposure to tebuconazole (exposed vs. control) on sparrow body mass and body condition (i.e., the mass corrected by the size of the birds) because these traits are tightly linked to energy balance, body reserves, and metabolic rates. In addition, we measured the overnight body mass loss of all individuals (see Dupont et al., 2019) as a measure of energy depletion during the overnight fast. Although no data are available regarding the impact of tebuconazole on metabolic rates in terrestrial vertebrates, we predicted that exposed sparrows would show reduced metabolic rates relative to controls because of the previously reported effect of triazoles on metabolic rates and TH levels in fish (Cao et al., 2019; Li et al., 2019; Souders et al., 2019; Yu et al., 2013). We also predicted that these effects of tebuconazole on metabolic rates may be exacerbated at lower temperature (TMR, 12 °C) compared to thermoneutrality (RMR, 25 °C) because altered metabolic rates may be more apparent when individuals are energetically constrained (i.e., thermoregulating). Finally, we predicted that exposed birds may also show higher body mass

and body condition, and a reduced overnight body mass loss relative to controls if tebuconazole exposure is associated with reduced metabolic rates. Alternatively, tebuconazole could lead to a reduction in the body mass and body condition of individuals due to altered health status (e.g., reduced energy reserves).

MATERIAL AND METHODS

Study species and experimental design

The present study was conducted with captive wild-caught adult house sparrows. A total of 54 adult sparrows were used in this experiment (Nfemales = 31 and Nmales = 23). All sparrows were maintained in captivity in four 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. Each aviary was equipped with artificial shelter boxes (one box per bird). The sparrows were fed ad libitum with a mixture of commercial seeds, millet on the stack, sand, and salt/minerals blocks. In all aviaries, water was dispensed through a drinking trough. Of the four aviaries, two of them formed the group of control individuals and the other two formed the group of individuals exposed to tebuconazole. Birds were split in the aviaries by treatment type and sex (Aviary 1: exposed females, N = 17; Aviary 2: control females, N = 14; Aviary 3: exposed males, N = 12; Aviary 4: control males, N = 11). This gives a total of 29 exposed and 25 control individuals. Over the total duration of the experiment, there was one death in each group (one in the control group and one in the exposed group). These individuals are not included in the numbers reported above. Sample sizes slightly differed between categories because a few of these wild-caught birds did not adjust to captivity and were released. Exposed individuals were treated with tebuconazole through their drinking water. Although other routes of exposure are classically used for farmland granivorous species (consumption of treated seeds; Lopez-Antia et al., 2016, 2018, 2021), we chose a drinking water exposure to mimic an oral contamination potentially present in natura due to the presence of trace triazoles in surface water (Kahle et al., 2008). In the two exposed aviaries, tebuconazole (CAS. No 107534-96-3, purity ≥98.0%; Sigma- Aldrich) was diluted with tap water using a precision balance (±0.0001 g) to reach a concentration of 550 µg L^{-1} . Considering that the average sparrow drinks the equivalent of 30% of its body weight per day (Bartholomew & Cade, 1963), a 25-g individual would consume approximately 4.1 µg of tebuconazole per day via drinking water. Comparatively, the LD50 of tebuconazole reported in the Northern Bobwhite was 1988 mg kg^{-1} (Pesticide Properties Database, 2022). In the two control aviaries, control individuals had

access to regular tap drinking water without tebuconazole. The concentration of tebuconazole in the drinking water of the control and exposed groups was analyzed to confirm an exposure concentration of 550 µg L−1 in the exposed individuals and tebuconazole-free water for the control individuals (by liquid chromatography tandem mass spectrometry [LC-MS/MS]; Qualyse Laboratory). Water was changed every week for both control (tap water) and exposed sparrows (tap water with tebuconazole). Exposure to tebuconazole began on 6 November 2019 until 29 June 2020, immediately after the metabolic rates measurements (see below). We specifically decided to expose individuals during several months to the exposed or control treatments to test the impact of a prolonged exposure to tebuconazole on metabolic rates ("exposed sparrows" vs. "control sparrows"). Accordingly, in vineyards, the period of spraying of pesticides occurs from the beginning of March to the end of August (~6 months). After 7 months, four exposed and four control sparrows were captured and bled to obtain plasma to measure their degree of contamination by tebuconazole. Plasma levels of azoles were determined using LC-MS/MS methodology at the LPTC-EPOC (L'équipe de Physico et Toxico Chimie de l'environnement - Environnements et Paléoenvironnements Océaniques et Continentaux) laboratory (Prouteau, 2021). Briefly, 25 µl of sparrow plasma was weighed to which 30 µl of Isotope-Labelled Internal Standards (ILISs) solution was gravimetrically added. A chicken quality control plasma was used. Then, a precipitation solvent (acetonitrile) was added to the mixture to obtain a total volume of 150 µl. After extraction and centrifugation of the samples, the supernatant was transferred to an injection vial prefilled with 1050 µl of Milli-Q water. Each extract was then injected into the analytical system composed of a Poroshell 120 SB C18 guard column (Agilent) used as on-line SPE cartridge. Target compound separation was achieved on a Poroshell Phenyl- Hexyl column (Agilent). The quantification of azoles was performed by using a 6490 Triple Quad mass spectrometer (Agilent) in positive electrospray mode in dynamic multiple reactions monitoring. Two high-resolution transitions were chosen for the analyte: one for quantification and the second for confirmation. Finally, we checked the analytical sequence with quality controls, and we used internal standard ILIS to calculate the concentration. Injection and handling blanks were used to confirm the accuracy of the quantification. The limit of quantification of tebuconazole was 25 pg g−1 of plasma (see Prouteau [2021] for more details on the methodology). To have enough plasma to assay tebuconazole and reduce the amount of blood that was drawn from each bird, samples from the four exposed sparrows and the four control sparrows, respectively, had to be pooled. The concentration of tebuconazole in the pooled plasma of exposed sparrows was 59.7 pg g−1 , which is similar to the concentrations that are found in the plasma of birds living in conventional

agroecosystems (in June/July 2019, 34 individuals were caught in the Cognac vineyard area, France, mean ± SD: 70.7 ± 84.9, range 26.0 [min], 222.2 [max]). Tebuconazole was not detected in the plasma pool of control sparrows.

Metabolism measurements

In the present study, the main variables of interest were TMR and RMR. During the treatment period, the birds were kept in outdoor aviaries and were subject to the natural climatic cycle before being transferred to temperature-controlled respirometry chambers. Metabolic rate was measured by multi-channel continuous flow through respirometry (RM8 Multiplexer; Sable Systems International) following previously described protocols (Dupont et al., 2019). Metabolic rates were measured for each individual at two ambient temperatures (Ta), (i) in colder thermal conditions, Ta = 12 °C (TMR), and (ii) in the thermoneutral zone for this species, Ta = 25 °C (RMR) using a temperature-controlled climatic chamber (±1 °C; Cryosystem, air-conditioned system Carel Master Cella). The multichannel open-circuit respirometer includes seven test chambers and an empty chamber considered as a control (3000 ml). This system enabled the metabolic rates of seven individuals to be analyzed simultaneously. The outside air was supplied at a constant flow rate (~450 ml min−1) to each chamber via an air pump (Bioblock Scientific 551) and a mass flow controller (FlowBar-8; Sable Systems). To obtain the target humidity of the supply air, ambient air was first saturated by bubbling it through two serially connected, heated water columns and then though an empty column, all within a temperature-controlled chamber (400 DG; LMS) set at 4 °C. Sequential samples were taken from the air expelled from each chamber via a gas flow switch (RM- Multiplexer; Sable Systems). Then the expelled air was passed through a hygrometer (RH 300; Sable Systems) and the water was extracted using Drierite. Finally, dried air samples were sent to a CO2 analyzer (CA10A; Sable Systems) and then to an O2 analyzer (FC10A; Sable Systems).

Measurements of TMR and RMR were carried out over a period from 3 June 2020 to 28 June 2020. The two measurements were carried out over two experimental sessions per individual, each 10 days apart. For these measurements, the order of passage of the birds was randomly chosen. Each bird was randomly assigned to a specific temperature of measurement for the first session (TMR at 12 °C or RMR at 25 °C) and was then assigned to the other temperature of measurement for the second session so that all birds were tested at both temperatures (TMR and RMR). The effects of the session did not significantly affect metabolic rates (linear module [LM], F(0.08) = 0.776). On the day of the measurement, at 18:00 hours, food was removed

from the aviaries so that the individuals could fast before the TMR and RMR measurements. At 20:00 hours, the sparrows were weighed with an electronic scale (initial mass), their tarsus was measured with a caliper (±0.01 mm) to assess body size, and the birds were placed in the dark glass chamber. The Ta of the climatic chambers was set at 12 °C or 25 °C depending on the session of measurement (TMR or RMR). The metabolic rates were measured six times per sparrow per session for 10 h. These measurements were averaged for each individual to give an average TMR and RMR. The oxygen level was calculated following Dupont et al. (2019). The next morning (at 6:00 AM), the sparrows were removed from the metabolic analysis boxes and weighed (final mass) before being released in their aviary of origin. The TMR and RMR were corrected by the mass of each individual (ml h^{-1} g^{-1}).

Individual body mass and body condition index

The initial body mass (before the metabolism measurement) and the final body mass (after the metabolism measurement) were used to assess the effect of the treatment on the body condition of the sparrows. The tarsus of each individual was also measured with a caliper (±0.01 mm) before the metabolism measurement. The body condition index (BCI) was calculated from the extraction of residuals from the linear regression of individual body mass as a function of tarsus length (LM, $F_{(1,214)} = 165.5$, $p \leq 0.001$).

Statistical analysis

All the statistical analyses were carried out on Rstudio (R Ver 4.0.3) with the packages lm4, lmerTest, AICcmodavg, car, MuMIn, and ggplot2. To test the effect of the experimental treatment on metabolic rates (TMR 12 °C and RMR 25 °C) and body condition of the sparrows, linear mixed-effect models (LMMs) were applied. Our variables of interest (dependent variables) were metabolic rates (TMR and RMR) and body condition. Both TMR and RMR were measured for all individuals, and they were statistically analysed together by including the temperature of metabolic measurements variable (TMR 12 °C, RMR 25 °C). We checked that all models met the assumptions of normality and homoscedasticity of residuals. First, an LMM with three fixed-effect variables (sex, temperature of metabolic measurements [TMR 12 °C or RMR 25 °C], treatment [control or exposed]) and their interactions were carried out to test the effect of treatment on the metabolic rates of sparrows (TMR and RMR). Individual identity was added as a random effect in the models because each metabolic measurement was performed twice (for TMR 12 °C and RMR 25 °C). The models were selected using the

corrected Akaike information criterion selection (AICc) method. After selection of the best model, specific comparisons were conducted by using contrast procedures to better describe the differences that were found between different groups of birds. Second, an LMM with four fixed effects (sex, temperature of metabolic measurements [TMR 12 °C or RMR 25 °C]), treatment [control or exposed], time of mass measurements [initial or final], and their interactions) were carried out to test the effect of tebuconazole on BCI and the body mass of individuals. Individual identity was added as a random effect in the models because the mass of individuals was measured twice (initial and final body mass) for each session (TMR 12 °C and RMR 25 °C). Model selection was done as stated above, using the AICc selection method. After LMM, specific comparisons were conducted by using contrast procedures to better describe the differences that were found between different groups of birds. Because the results on body mass and body condition were very similar (same significant and nonsignificant terms), we only present the results on body condition in the results section. Indeed, body mass and body condition were highly correlated (Pearson correlation, $r = 0.75$, $t_{(1,214)} = 16.64$, $p \leq 0.001$). The results on body mass are presented as an annex (see Supporting Information).

RESULTS

TMR and RMR

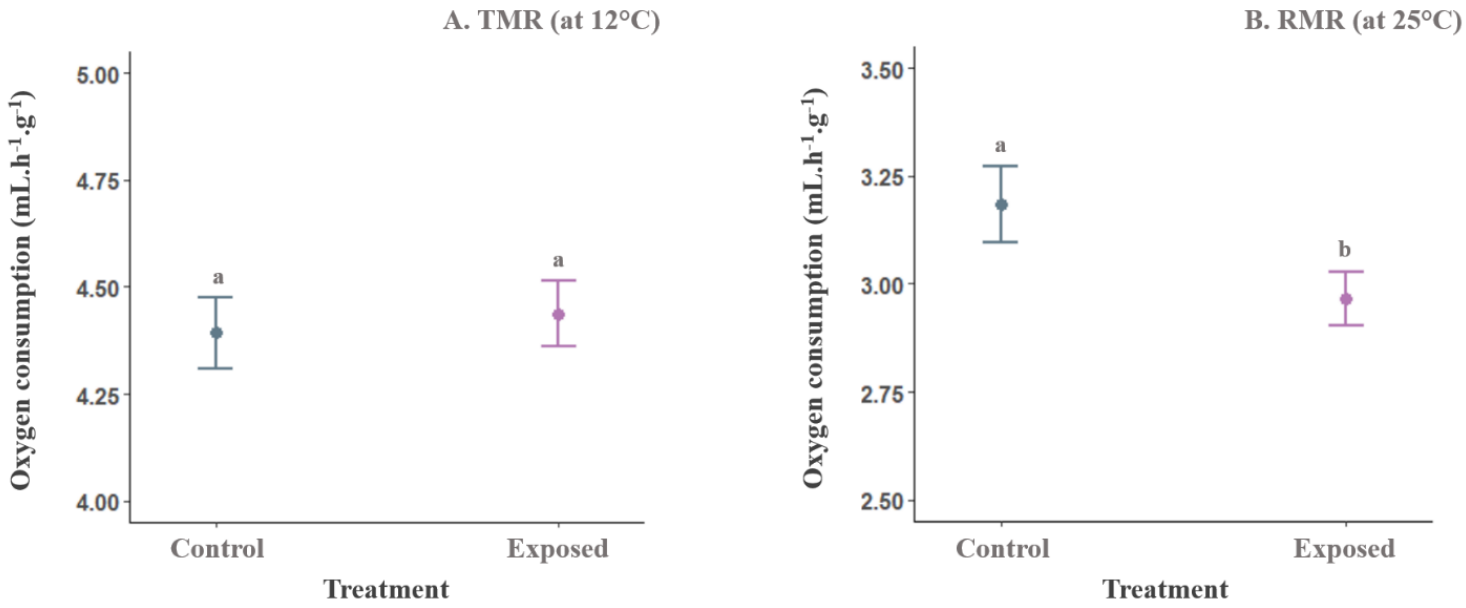
As expected, there was a very strong and significant effect of temperature on the metabolic rates of sparrows (Table 1 and Figure 1). Specifically, TMR (12 °C) was significantly higher than RMR (25 °C; contrast: $t_{(1,52)} = 22.11$, $p < 0.001$). We found a significant "Temperature × Treatment" interaction on metabolism (Table 1 and Figure 1), showing that the influence of the experimental treatment on metabolism differed significantly depending on the temperature of the metabolic measurements (i.e., 12 °C vs. 25 °C). Indeed, at 12 °C the TMR of the exposed group was not significantly different from the control group (contrast: $t_{(1,90.6)} = -0.40$, $p = 0.694$; Figure 1A). However, at 25 °C, the RMR of exposed individuals was significantly lower than that of controls (contrast: $t_{(1,90.6)} = 2.00$, $p = 0.049$; Figure 1B). Sex did not significantly influence the metabolism of individuals ($t_{(1,52)} = 1.11$, $p = 0.298$). Moreover, all the interactions related to sex were nonsignificant (all p-value > 0.207), suggesting that sex did not influence the TMR and RMR of the control and exposed sparrows (Table 1).

TABLE 1: Effects of temperature of metabolic measurements (thermoregulatory metabolic rate 12 °C or resting metabolic rate 25 °C), treatment (control or exposed to tebuconazole), and their interaction on the metabolism of individuals (rate of oxygen consumption, VO2, ml h−1 g−1).

Response variable	Explanatory variables	NumDF	DenDF	F-value	*p-value*
Oxygen Consumption	**Temperature**	**1**	**52**	**488.89**	**<0.001 *****
	Treatment	1	52	0.92	0.341
	Temperature x Treatment	**1**	**52**	**4.65**	**0.036 ***

The identity of the individuals was added as a random factor. A linear mixed-effects model was used. Significant terms are indicated with the following codes: ***

FIGURE 1: Rate of oxygen consumption (VO2, ml h−1 g−1) according to the temperature of the metabolic measurement: at 12 °C (A, thermoregulatory metabolic rate [TMR]) and at 25 °C (B, resting metabolic rate [RMR]) as a function of treatment (control or exposed to tebuconazole).



The gray and purple colors represent the metabolic values of control and exposed individuals, respectively. Resting metabolic rate was significantly lower in exposed sparrows compared to control sparrows, whereas TMR did not differ statistically between exposed and control sparrows. Means ± SE are presented. The different letters indicate significant differences between treatments (controls or exposed to tebuconazole).

Body condition index

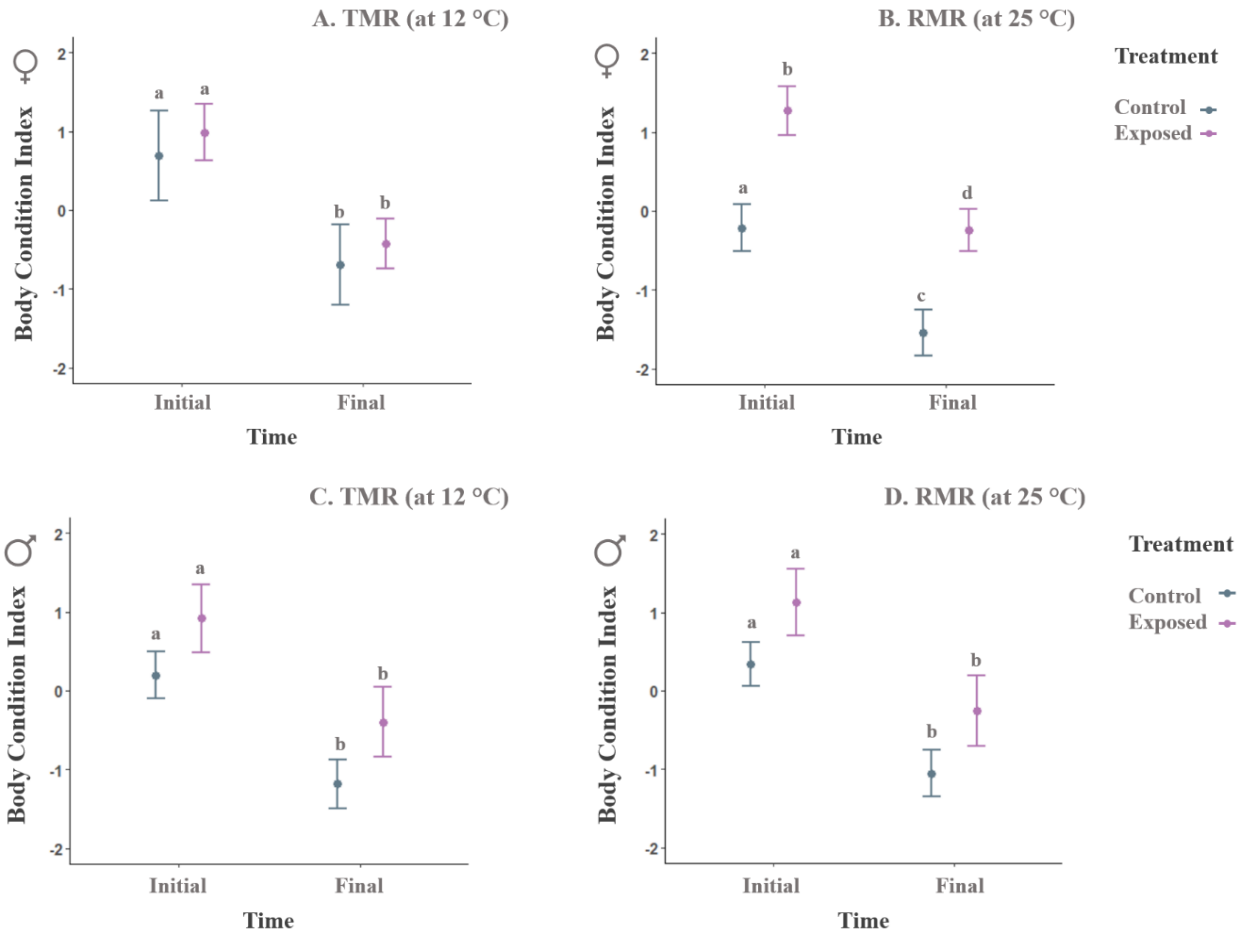
Control and exposed sparrows did not differ in initial body size (i.e., tarsus length before the metabolism measurement, LM, $F(1, 52) = 0.05$, $p = 0.830$). As expected, the initial BCI of the sparrows was higher than their final BCI, because the explanatory variable "Time" was highly significant (Table 2), showing that all birds lost weight overnight (Figure 2). Importantly, there was no significant effect of the "Treatment × Time", "Sex × Time", or "Sex × Time × Treatment" interactions on BCI (Table 2), demonstrating that the overnight weight loss did not differ between sexes or between exposed and control sparrow. Sex did not significantly influence the BCI of individuals (Table 2) although there was an effect of the "Treatment" on BCI (Table 2) with an overall higher BCI for exposed birds relative to control ones (contrast: $t(1,50) = -2.40$, $p = 0.020$). Interestingly, the "Temperature × Sex," "Temperature × Treatment", and "Temperature × Treatment × Sex" interactions had significant effects on BCI (Table 2), demonstrating that the influence of the experimental treatment on BCI significantly differs depending on the temperature of metabolic measurements (i.e., TMR 12 °C vs. RMR 25 °C) and the sex of the individual (Figure 2). Specifically, exposed females had a higher BCI than control females at 25 °C (contrast: $t(1,60.2) = -3.03$, $p = 0.004$; Figure 2B), but not at 12 °C (contrast: $t(1,60.2) = -0.62$, $p = 0.540$; Figure 2A). In males, this effect was not significant at both 25 °C (contrast: $t(1,60.2) = -1.50$, $p = 0.140$; Figure 2D) and 12 °C (contrast: $t(1,60.2) = -1.42$, $p = 0.162$; Figure 2C) despite a trend toward a higher BCI in exposed males relative to controls (Figure 2C,D).

TABLE 2: Effects of temperature of metabolic measurements (12 °C or 25 °C), time of measurement (initial: before measuring metabolism or final: after measuring metabolism), treatment (control or exposed to tebuconazole), sex (female or male), and their interaction on the body condition index according to linear mixed-effects modelling.

Response variable	Explanatory variables	NumDF	DenDF	F-value	p-value
Body Condition Index	Temperature	1	157	0.65	0.421
	Time	1	157	180.32	<0.001 ***
	Treatment	1	50	5.78	0.020 *
	Sex	1	50	<0.01	0.958
	Temperature x Treatment	1	157	7.51	0.007 **
	Temperature x Sex	1	157	5.12	0.025 *
	Treatment x Sex	1	50	<0.01	0.924
	Temperature x Treatment x Sex	1	157	6.43	0.012 *

The identity of the individuals was added as a random factor. Significant terms are indicated with the following codes: ***

FIGURE 2: Variation in body condition index (BCI) according to the temperature of the metabolic measurement: at 12 °C (A and C) and 25 °C (B and D) as a function of treatment (control or exposed to tebuconazole) and time of measurement (initial: before measuring metabolism or final: after measuring metabolism). (A) and (B) correspond to the BCI of females and (C) and (D) correspond to the BCI of males.



The gray and purple colors represent the BCI of control and exposed individuals, respectively. Means ± SE are presented. The different letters indicate significant differences between treatments (controls or exposed to tebuconazole) and time of measurement (initial: before measuring metabolism or final: after measuring metabolism).

DISCUSSION

In the present study, we showed for the first time in wild bird species that long-term exposure to a widely used azole (tebuconazole) can significantly reduce the RMR (i.e., measured at thermoneutrality, 25 °C) of exposed individuals although their TMR (i.e., 12 °C) was not affected by the experimental treatment. In addition, exposed individuals had a higher body mass and BCI than controls, although overnight body mass loss did not differ between the two groups.

Metabolic rates

Our study demonstrates for the first time that the use of tebuconazole can alter metabolic rates and may have a detrimental impact on birds. Overall, tebuconazole exposure was associated with a significant decrease in RMR (25 °C). Consistent with our results, previous studies have shown that exposure to propiconazole and cyproconazole in zebrafish embryos caused up to a 50% decrease in RMR, as well as an overall alteration in mitochondrial bioenergetics (Cao et al., 2019; Souders et al., 2019). Similarly, Teng et al. (2018) and Perez-Rodriguez et al. (2019) found that difenoconazole and tebuconazole, respectively, had similar effects in developing zebrafish. In the present study, we demonstrated for the first time that environmental concentrations of tebuconazole can have qualitatively similar effects in nonaquatic vertebrates (i.e., adult wild birds). This shows that different azole fungicides are able to alter, and more specifically to reduce, metabolic rates in birds, probably through a modification of mitochondrial processes (Cao et al., 2019; Souders et al., 2019). However, and interestingly, Souders et al. (2020) did not report any effect of triticonazole on the metabolic rates of zebrafish embryos, even after increasing the maximum test concentration (from 100 to 250 μM) and extending the exposure time (from 24 to 48 h). Therefore, the mode of action of azole compounds on metabolic rates may be specific to the compound, the duration of exposure or the stage of development. Importantly, we used much lower tebuconazole concentration in our study relative to previous studies (e.g., Andreu-Sánchez et al., 2012; Raby et al., 2019; Toni et al., 2011). Nonetheless, based on preliminary field data (see Methods), our concentrations gave plasma tebuconazole levels similar to those found in wild birds living in agroecosystems. Despite the use of these lower, but environmental, concentrations, we found a significant impact of tebuconazole exposure on the RMR of sparrows. This suggests that environmental fungicide applications are sufficient to cause metabolic disruption in nontarget bird species, such as those

living in agroecosystems. As a result, our study demonstrates that the use of tebuconazole can alter a key physiological trait and may have a detrimental impact on wild birds.

Contrary to our prediction, the impact of tebuconazole exposure on metabolic rates was not exacerbated when the birds had to thermoregulate (i.e., for the TMR measurement at 12 °C). Indeed, we did not detect any impact of tebuconazole on this metabolic measurement (TMR). We predicted that tebuconazole would have a stronger impact on TMR because mitochondrial activity is increased when individuals have to thermoregulate (i.e., 12 °C in our study; Mozo et al., 2005) and previous studies have suggested that azole compounds could alter the functioning of mitochondria (Souders et al., 2019). However, it is likely that the energetic costs of thermoregulation vary dramatically between individuals depending on their insulation properties (feather density and quality, fat content, etc.). This interindividual variability could have masked a potential effect of tebuconazole on TMR. In addition, it is also possible that the impact of tebuconazole on metabolic rates is reduced when the activity of the cellular machinery is important, as is the case at 12 °C (TMR) relative to 25 °C (RMR). To better understand how tebuconazole can affect metabolic rates of wild birds, it appears now crucial to study the impact of environmental concentrations of tebuconazole on the mitochondrial functioning and bioenergetics of terrestrial birds. It would also be relevant to study the effect of tebuconazole on metabolic rates along a wide range of ambient temperature to better understand if the impact of tebuconazole on metabolism may be exacerbated at more challenging temperatures. One of the potential targets of tebuconazole responsible for the alteration of RMR in exposed individuals could be the HPT axis. Indeed, in vertebrates metabolism is under the control of this neuroendocrine pathway. Therefore, the impact of azoles on RMR could be related to a potential disruption of this axis (Liu et al., 2011; Yu et al., 2013). Specifically, it has been shown that the thyroid hormones are key drivers of tissue oxygen consumption and metabolic activity in endotherms (Bobek et al., 1977; Danforth & Burger, 1984; Freake & Oppenheimer, 1995; Hulbert, 2000). Moreover, T3 levels are positively associated with RMR in multiple bird species (Elliott et al., 2013; Welcker et al., 2013; Zheng et al., 2013), including house sparrows (Chastel et al., 2003). Several studies have shown that triazoles are potential endocrine disruptors of the HPT axis in model species and exposure to tebuconazole has been associated with alteration of T3 levels and with numerous dysregulations of the genes involved in the HPT axis (Li et al., 2019; Yu et al., 2013). Therefore, the reduction of RMR in exposed individuals could result from an endocrine disruption of the HPT axis and more specifically from a reduction of T3 levels in exposed individuals. To our knowledge, the impact of environmental

concentrations of tebuconazole or azole compounds on T3 levels has never been examined in wild terrestrial vertebrates. In addition, tebuconazole may also indirectly affect RMR through its impact on other mechanisms of actions that are tightly connected to metabolism (e.g., oxidative stress, blood biochemistry, immunity; Lopez-Antia et al., 2018). Future studies should now examine the response of the HPT axis, and T3 levels, to environmental concentrations of tebuconazole to better understand how this fungicide can alter metabolic rates.

Body mass and body condition

As predicted, the body mass and BCI of exposed individuals were significantly higher than those of controls. However, body mass was measured only after several months of exposure to tebuconazole, and we must therefore remain cautious regarding this result. Additional experiments examining the long- term impact of exposure to tebuconazole on body condition are necessary to confirm the results from our study. This effect was surprisingly dependent on the temperature of measurement. Despite this intriguing temperature-dependent effect, this result is in line with the lower RMR of exposed birds because metabolic rates are positively correlated with energy mobilization and the depletion of body reserves in vertebrates (Nagy et al., 1999; Peters & Peters, 1986), including the house sparrow (Dupont et al., 2019). Although our results were only apparent at one temperature and in females only, they suggest that environmental concentrations of tebuconazole can have a strong impact on energy regulation in wild birds, as previously suggested by Lopez-Antia et al. (2021). Interestingly, we further found that tebuconazole had a significant impact on the body condition of females, and a similar (but not significant) trend was observed in males. This suggests that females may be more sensitive to the impact of environmental concentrations of tebuconazole on energy regulation. At the proximate level, such a sex-dependent effect may be explained by sexual differences in the metabolic and endocrine pathways that are disrupted by tebuconazole. Indeed, azole compounds such as tebuconazole are known to readily interfere with cytochrome P450 enzymes, which are involved in the synthesis and regulation of sexual steroid hormones such as oestradiol (Taxvig et al., 2007; Vinggaard et al., 2000; Yang et al., 2018; Zarn et al., 2003; Zhang et al., 2002). Importantly, oestradiol is known to affect energy regulation, fat deposition, and body condition in vertebrates, including humans (Brown & Clegg, 2010). Dysregulation of oestradiol levels by chronic exposure to tebuconazole could explain the higher body condition of exposed females. Supporting this hypothesis, Lopez- Antia et al. (2021) found that tebuconazole can reduce circulating cholesterol levels in red-legged partridges (Alectoris rufa)

and cholesterol is a precursor of sexual hormones (Brown & Clegg, 2010). This effect was also found with exposure to another azole on the same species (Lopez-Antia et al., 2018). In addition, Fernández-Vizcaíno et al. (2020) even found that exposure to tebuconazole was associated with reduced oestradiol levels in the same species.

Surprisingly, and despite these effects of tebuconazole on blood biochemistry (Fernández-Vizcaíno et al., 2020; Lopez- Antia et al., 2021), these studies did not report a significant impact of tebuconazole on body mass or body condition in red- legged partridges (Fernández-Vizcaíno et al., 2020; Lopez- Antia et al., 2021). Similarly, a previous study did not find any effect of another triazole compound on the body condition of the same species (difenoconazole; Lopez-Antia et al., 2013). However, and importantly, the impact of tebuconazole on body condition has to our knowledge never been tested in other bird species. Red-legged partridge and house sparrows have different body size, body mass, fat reserves, and mass-specific metabolic rates. These differences are associated with different fasting capacities (Rodríguez et al., 2005) that could explain this discrepancy between studies. In addition, most of these previous studies have exposed individuals to tebuconazole during short periods (i.e., less than a month), although the impact of tebuconazole on body condition could appear progressively and become apparent after several weeks or months of exposure. Although exposure to plant protection products is multifactorial and chronic, the duration of exposure in our experiment is probably in the upper range of what can be found in agroecosystems. Thus, it is possible that the effects of tebuconazole varied over the time of exposure, particularly if the birds up regulated their detoxification system. Therefore, it would be relevant in future studies to test how metabolism and body condition change during chronic exposure to triazole.

Despite a strong effect of tebuconazole on RMR, overnight body mass loss surprisingly did not differ between exposed and control sparrows. However, we would expect overnight body mass loss to be reduced in exposed sparrows because of their lower RMR. Such a counterintuitive result may be explained by different body composition, such as fat content, between exposed and control sparrows. Indeed, lipid catabolism is associated with a much higher energetic yield (energy produced per gram of tissue) than protein catabolism in birds (Jenni & Jenni-Eiermann, 1998) and consequently with a lower body mass loss. Body composition was not available in our study, but other experimental studies reported that exposure to tebuconazole or other triazoles can affect circulating blood metabolites, such as triglycerides and cholesterol, in the red- legged partridge (Lopez-Antia et al., 2013, 2021; Fernández- Vizcaíno et al., 2020) suggesting a strong impact on energy regulation and metabolic pathways in terrestrial birds.

In addition to potential dysregulation of lipid metabolism, tebuconazole could also affect the body mass of sparrows by modifying feeding behaviour (e.g., hyperphagia). In our study, we did not measure the feeding behavior of exposed and control sparrows. This potential effect of tebuconazole on feeding behavior could be mediated by changes in the hypothalamic–pituitary–adrenal (HPA) axis. Indeed, this endocrine axis plays a key role in the control of appetite in vertebrates (Grün & Blumberg, 2009; Suzuki et al., 2010), including birds (Astheimer et al., 1992). Such an effect could also be mediated by the disruption of other endocrine axes, such as the hypothalamic–pituitary–gonads (HPG) axis. Indeed, tebuconazole is known to affect steroidogenesis in nontarget species by interfering with aromatase (CYP19), which is responsible for the conversion of androgens to estrogens (Poulsen et al., 2015; Saxena et al., 2015). This may cause a dysregulation of estrogen levels (such as oestradiol), and estrogens are known to have an inhibitory action on food intake (Asarian & Geary, 2006; Butera, 2010; Hirschberg, 2012). Accordingly, some studies have experimentally demonstrated that estrogen deficiency via aromatase elimination causes obesity in mice (e.g., Misso et al., 2003; Takeda et al., 2003). Fernández- Vizcaíno et al. (2020) found that exposure to tebuconazole is associated with reduced oestradiol levels in the red-legged partridge, suggesting that a disruption of steroid hormone homeostasis could affect feeding behavior and explain why exposed individuals had a higher BCI/mass than controls. However, Lopez-Antia et al. (2021) also reported that exposure to tebuconazole did not have any significant effect on feeding behavior in the same species, suggesting that increased body condition in exposed sparrows may be mainly explained by the impact of tebuconazole on metabolic rates and energy expenditure. Further studies are now necessary to better understand how tebuconazole affects the condition of sparrows and to test whether these effects are mediated by the disruption of several endocrine axes (HPA, HPG, HPT).

CONCLUSION

Taken together, our results showed physiological effects of tebuconazole on house sparrows, including a significant decrease in RMR (e.g., 25 °C) and a significantly higher mass and BCI in exposed individuals. These first results on terrestrial vertebrates show the importance of evaluating the potential risks associated with chronic exposure to tebuconazole in agroecosystems, including for humans. It now seems relevant to investigate what the underlying effects of a decrease in RMR might be on the fitness of the species (reproduction and survival) and more broadly in vertebrates. Indeed, a reduction in RMR could result in a decrease in the energy devoted to self- maintenance and lead to an alteration in the ability of individuals to

reproduce and survive (Blévin et al., 2016; Burton et al., 2011; Goutte et al., 2015). Recent studies have demonstrated impaired reproductive performance in red-legged partridges following exposure to triazole compounds (Fernández-Vizcaíno et al., 2020; Lopez-Antia et al., 2018, 2021). Thus, these first results involving the effect of triazole compounds on vertebrates living in agroecosystems stimulate further studies to fill the existing gaps on the toxicity of these phytosanitary products widely used throughout the world.

Supporting Information

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Conflict of Interest

The authors declare no conflict of interest. Author Contributions Statement—Pauline Bellot: Data curation; Formal analysis; Writing—original draft; Visualization. Sophie Marie Dupont: Investigation; Data curation; Writing— review & editing. François Brischoux: Conceptualization; Validation; Writing—review & editing. Hélène Budzinski: Funding acquisition; Investigation; Resources; Data curation; Writing—review & editing. Olivier Chastel: Resources; Writing—review & editing; Funding acquisition. Clémentine Fritsch: Writing—review & editing; Funding acquisition. Olivier Lourdais: Methodology; Resources; Writing—review & editing. Louise Prouteau: Investigation; Resources; Data curation; Writing—review & editing. Steffi Rocchi: Writing— review & editing. Frédéric Angelier: Conceptualization; Validation; Resources; Writing—original draft; Writing— review & editing; Supervision; Funding acquisition

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ADDITIONAL MATERIALS

Table A : Model selection regarding the influence of our variables of interest (temperature, treatment, sex) and their interactions on metabolic rates (TMR: 12°C, RMR: 25°C). AICc and weight of the selected, full, and null models are presented. Models ranked by AICc.

Model selection table	df	Log-Likelihood	AICc	Δ AICc	weight
M1 Selected model	**6**	**- 48.08**	**109.0**	**0.00**	**0.964**
M2 Full model	10	- 46.65	115.6	6.58	0.036
M3 Null model	3	- 127.13	260.5	151.50	0.000

Structures of the different models M1, M2, and M3 :
M1 : Temperature + Treatment + (**Temperature : Treatment**)
M2 : **Temperature** + Treatment + Sex + (**Temperature : Treatment**) + (Temperature : Sex) + (Sex : Treatment) + (Temperature : Treatment x Sex)
Random terms (all models): '1 | ID'

Table B : Summary of the model selected by AICc regarding the effects of temperature of metabolic measurements (TMR, 12°C or RMR, 25°C), treatment (control or exposed to tebuconazole), and their interaction on the metabolism of individuals (rate of oxygen consumption, VO_2, $mL.h^{-1}.g^{-1}$). The identity of the individuals was added as a random factor. A linear mixed effects model was used. Significant terms are indicated with the following codes: '***' < 0.001 '**' < 0.01 '*' < 0.05 '.' < 0.1.

Fixed effects :	Estimate	Std. Error	df	t value	p-value
Intercept	4.39	0.08	90.59	54.84	<0.001 ***
Temperature 25	**- 1.21**	**0.09**	**52.00**	**- 13.62**	**<0.001** ***
Treatment Exposed	0.04	0.11	90.59	0.40	0.694
Temperature 25 : Treatment Exposed	**- 0.26**	**0.12**	**52.00**	**- 2.16**	**0.036** *

Figure A : Variation in body mass according to the temperature of the metabolic measurement: at 12°C (A. and C.) and 25°C (B. and D.), as a function of treatment (control or exposed to tebuconazole), and time of measurement (initial : before measuring metabolism or final: after measuring metabolism). Figures 2.A and 2.B correspond to the body mass of females and figures 2.C and 2.D correspond to the body mass of males. The grey and purple colours represent the BCI of control and exposed individuals, respectively. Means ± SE are presented.

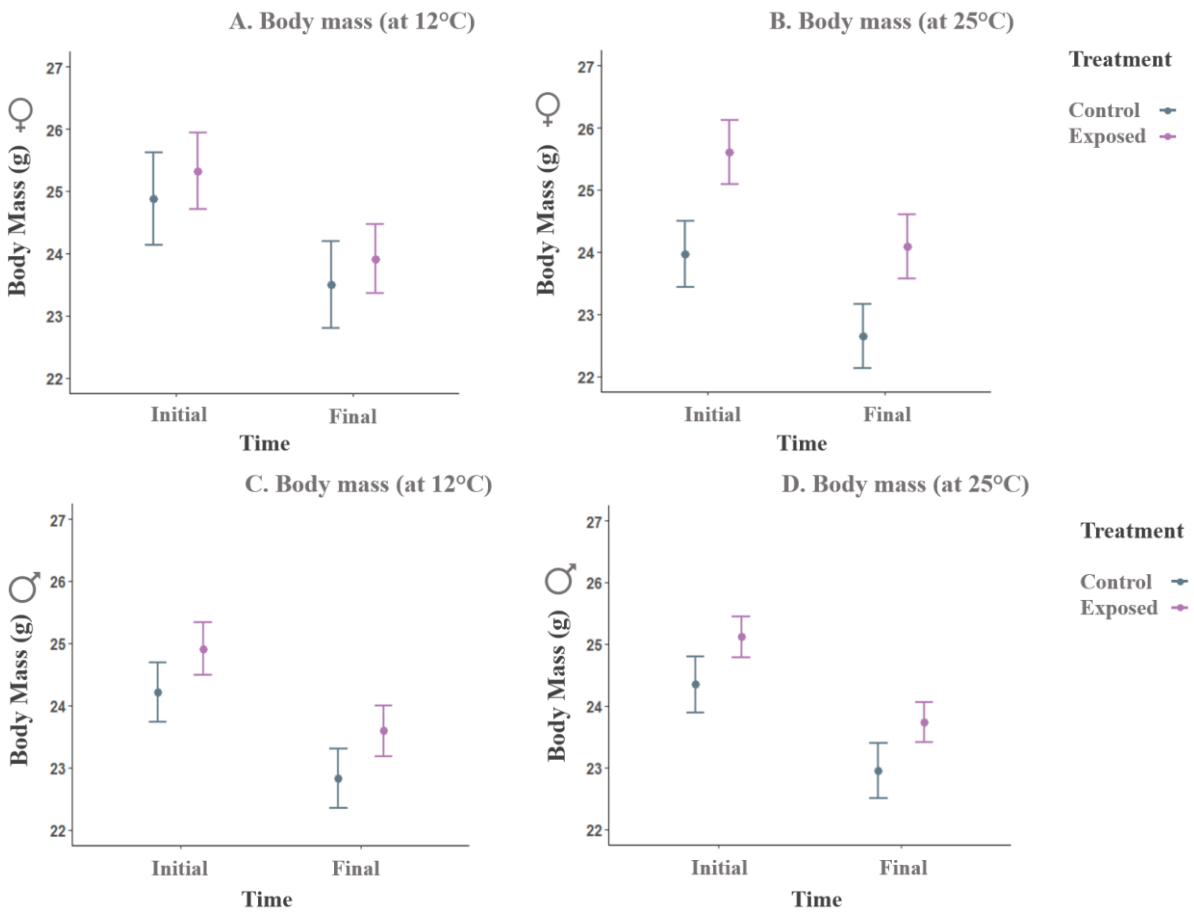

Table C : Model selection regarding the influence of our variables of interest (temperature, treatment, sex, time) and their interactions on the body mass. AICc and weight of the selected, full, and null models are presented. Models ranked by AICc.

Model selection table	df	Log-Likelihood	AICc	Δ AICc	weight
M1 Selected model	**11**	**- 329.33**	**681.9**	**0.00**	**1.000**
M2 Full model	18	- 329.16	697.8	15.84	0.000
M3 Null model	3	- 397.98	802.1	120.13	0.000

Structures of the different models M1, M2, and M3 :

M1 : Temperature + **Treatment** + Sex + **Time** + **(Temperature : Treatment)** + **(Temperature : Sex)** + (Treatment : Sex) + **(Temperature : Treatment : Sex)**

M2 : Temperature + **Treatment** + Sex + **Time + (Temperature : Treatment) + (Temperature : Sex)** + (Treatment : Sex) + (Temperature : Time) + (Treatment : Time) + (Sex : Time) + **(Temperature : Treatment : Sex)** + (Temperature : Treatment : Time) + (Temperature : Sex : Time) + (Treatment : Sex : Time) + (Temperature : Treatment : Sex : Time)

Random terms (all models): ‘1 | ID’

Table D : Summary of the model selected by AICc on the effects of temperature of metabolic measurements (12°C or 25°C), time of measurement (initial : before measuring metabolism or final: after measuring metabolism), treatment (control or exposed to tebuconazole), sex (female or male) and their interaction on the body mass according to linear mixed effects modelling. The identity of the individuals was added as a random factor. Significant terms are indicated with the following codes: '***' < 0.001 '**' < 0.01 '*' < 0.05 '.' < 0.1.

Fixed effects :	Estimate	Std. Error	df	t value	p-value
Intercept	24.88	0.51	55.33	48.51	<0.001 ***
Temperature 25	**- 0.88**	**0.20**	**157.00**	**- 4.31**	**<0.001** ***
Treatment Exposed	0.43	0.69	54.22	0.63	0.533
Sex M	- 0.67	0.77	54.22	-0.87	0.389
Time 2	**- 1.39**	**0.10**	**157.00**	**- 13.43**	**<0.001** ***
Temperature 25 x Sex M	**1.01**	**0.31**	**157.00**	**3.29**	**0.001** **
Treatment Exposed x Sex M	0.30	1.05	54.22	0.29	0.777
Temperature 25 x Treatment Exposed	**1.11**	**0.28**	**157.00**	**4.03**	**<0.001** ***
Temperature 25 x Treatment Exposed x Sex M	**-1.07**	**0.42**	**157.00**	**- 2.54**	**0.012** *

Table E : Model selection regarding the influence of our variables of interest (temperature, treatment, sex, time) and their interactions on the BCI. AICc and weight of the selected, full, and null models are presented. Models ranked by AICc.

Model selection table	df	Log-Likelihood	AICc	Δ AICc	weight
M1 Selected model	**11**	**- 306.03**	**635.4**	**0.00**	**1.000**
M2 Full model	18	- 305.86	651.2	15.84	0.000
M3 Null model	3	- 375.951	758.0	122.66	0.000

Structures of the different models M1, M2, and M3 :

M1 : Temperature + **Treatment** + Sex + **Time** + **(Temperature : Treatment)** + **(Temperature : Sex)** + (Treatment : Sex) + **(Temperature : Treatment : Sex)**

M2 : Temperature + **Treatment** + Sex + **Time + (Temperature : Treatment) + (Temperature : Sex)** + (Treatment : Sex) + (Temperature : Time) + (Treatment : Time) + (Sex : Time) + **(Temperature : Treatment : Sex)** + (Temperature : Treatment : Time) + (Temperature : Sex : Time) + (Treatment : Sex : Time) + (Temperature : Treatment : Sex : Time)

Random terms (all models): '1 | ID'

Table F : Summary of the model selected by AICc on the effects of temperature of metabolic measurements (12°C or 25°C), time of measurement (initial or final), treatment (control or exposed to tebuconazole), sex (female or male) and their interaction on the BCI according to linear mixed effects modelling. The identity of the individuals was added as a random factor. Significant terms are indicated with the following codes: '***' < 0.001 '**' < 0.01 '*' < 0.05 '.' < 0.1.

Fixed effects :	Estimate	Std. Error	df	t value	p-value
Intercept	0.69	0.34	62.87	2.01	0.048 *
Temperature 25	**- 0.88**	**0.20**	**157.00**	**- 4.31**	**<0.001** ***
Treatment Exposed	0.28	0.46	60.16	0.62	0.540
Sex M	- 0.49	0.51	60.16	-0.96	0.343
Time 2	**- 1.39**	**0.10**	**157.00**	**- 13.43**	**<0.001** ***
Temperature 25 x Sex M	**1.01**	**0.31**	**157.00**	**3.29**	**0.001** **
Treatment Exposed x Sex M	0.47	0.70	60.16	0.67	0.507
Temperature 25 x Treatment Exposed	**1.11**	**0.28**	**157.00**	**4.03**	**<0.001** ***
Temperature 25 x Treatment Exposed x Sex M	**-1.07**	**0.42**	**157.00**	**- 2.54**	**0.012** *

Table G : Effects of temperature of metabolic measurements (12°C or 25°C), treatment (control or exposed to tebuconazole), and their interaction on the delta mass lost overnight according to linear mixed effects modelling. The identity of the individuals was added as a random factor. Significant terms are indicated with the following codes: '***' < 0.001 '**' < 0.01 '*' < 0.05 '.' < 0.1.

Fixed effects :	Estimate	Std. Error	df	t value	p-value
Intercept	1.38	0.06	95.17	23.98	<0.001 ***
Temperature 25	- 0.03	0.07	52.00	- 0.41	0.683
Treatment Exposed	- 0.01	0.08	95.17	- 0.15	0.883
Temperature 25 : Treatment Exposed	0.11	0.09	52.00	1.23	0.224