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## The influence of plant odours on sexual readiness in an insectivorous songbird

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## **Abstract**

Many organisms rely on environmental cues to predict and anticipate the annual optimal timing of reproduction. In insectivorous birds, preparation for breeding often coincides with the time vegetation starts to develop in spring. Whether there is a direct relationship between the two, and through which mechanisms this link could come about, has rarely been investigated. Plants release Herbivore-Induced Plant Volatiles (HIPVs) when they are attacked by insects, and recent studies have shown that birds can detect and orient to those odours when searching for food. If those volatiles also stimulate sexual reproductive development and timing of reproduction remains to be discovered. We tested this hypothesis by monitoring gonadal growth in pairs of blue tits (*Cyanistes caeruleus*) exposed to air from caterpillar infested oak trees or from a control, in spring. We found that while males and females grew their gonads over time, gonads grew at the same rate in both odor treatments. More exploratory (i.e. a proxy of personality) females did however have larger ovarian follicle sizes when exposed to the HIPVs than to the control air, which is consistent with earlier results showing that fast explorers have larger gonads in spring and are more sensitive to HIPVs. If HIPVs constitute powerful attractants in foraging birds, their influence on gonadal development prior to breeding appears to be relatively subtle and only enhance reproductive readiness in some individuals. These results are nevertheless important as they set olfaction as a new player in seasonal timing of reproduction in birds.

Keywords: olfaction; herbivore induced plant volatiles; seasonal timing; reproduction; gonadal cycles; birds

## Introduction

Photoperiod (i.e., day length) is the predominant cue used by temperate breeding organisms to determine the appropriate time of year to undergo the physiological and behavioral changes to prepare for seasonal breeding (Dawson, 2003). However, photoperiod does not vary annually, which means that it is of no use for predicting year-to-year variation in environmental conditions. This is what supplementary, or non-photic cues do (reviewed by Chmura et al., 2019). Temperature has received vast attention as a supplementary cue (Caro et al., 2013a) since rapidly occurring changes to the environment are shifting timing of life history traits across a broad array of taxa (Parmesan, 2006). However, results from studies manipulating temperature and observing effects on timing of breeding have been mixed. If a causal relationship between ambient temperature and timing of reproduction has been shown in some songbirds (Meijer et al., 1999; Visser et al., 2009), the amplitude of this effect has proven variable and, in all cases, smaller than expected, particularly on the physiological mechanisms of reproduction (Caro et al., 2013b; Dawson, 2005; Perfito et al., 2005; Schaper et al., 2011a; Verhagen et al., 2020; Visser et al., 2009; 2011; Watts et al., 2018). This suggests that temperature is not the only player regulating annual variation in seasonal timing of reproduction in temperate-zone species and it calls for investigations on other non-photic signals.

Vegetation development, and more specifically timing of bud burst in trees, has also received attention as a possible reproductive cue, particularly in insectivorous birds. Relying on bud development to decide when to start breeding could be a good proxy for predicting the optimal timing for breeding in several bird species because buds and young leaf shoots constitute the exclusive food of the caterpillars that these birds provide to their chicks (Du Merle and Mazet, 1983; Naef-Daenzer and Keller, 1999; van Asch et al., 2013). Many studies have indeed found significant correlations between tree phenology and laying date in wild populations of insectivorous birds (Bison et al., 2020; Bourgault et al., 2010; Cole et al., 2015; Hinks et al., 2015; Nilsson and Källander, 2006; Shutt et al., 2019; Slagsvold, 1976). However, bud burst is tightly linked with temperature and only a handful of studies have experimentally

tested the effect of vegetation cues independently from temperature cues on bird reproduction (Schaper et al., 2011b; Visser et al., 2002; Voigt et al., 2007; 2011). To complicate things further, the effect of plants on seasonal timing could occur through various sensory channels, which include visual, gustatory, tactile and olfactory pathways (Ettinger and King, 1981; Voigt et al., 2011).

Plants have been shown to defend against herbivores by releasing volatile alarm signals (Herbivore-Induced Plant Volatiles: HIPV) that attract predators. This ability to attract predators has been well studied in insects (Turlings and Benrey, 1998), where alarm signals emitted by plants in response to caterpillar grazing attract parasitoid wasps (Van Poecke et al., 2001), but also in seabirds that use degradation products of phytoplankton to identify fish-rich zones (Dell'Araccia et al., 2014; Savoca and Nevitt, 2014). It was shown plants are capable of reducing the number of herbivores by greater than 90% through release of such volatiles (Kessler and Baldwin, 2001). Recent evidence also reveals that songbirds are capable of detecting caterpillar infestation of apple trees completely by smell (Amo et al., 2013). However, there is very little knowledge concerning the effects of plant volatile emissions in contexts other than foraging. Carnivorous arthropods will invest more in reproduction when presented with olfactory cues from infested plants while undergoing a food shortage (Rondoni et al., 2017). We have also recently shown that birds with higher levels of testosterone, the main reproductive hormone in males, were more attracted to areas diffusing HIPVs characteristics of a caterpillar infestation (Graham et al., 2021), and that more eggs and fledglings were produced in forest zones diffusing artificial HIPVs (Delaitre et al., unpublished results). Whether and how these volatile emissions from infested plants directly influence the physiological mechanisms underlying reproduction is however still unknown.

The goal of this study was to determine if insectivorous songbirds use HIPVs emitted by tree buds in response to initial caterpillar infestation in the spring as a cue to prepare for breeding. We exposed breeding pairs of blue tits (*Cyanistes caeruleus*) in captivity to the odors of caterpillar infested trees or a control odor, over two consecutive years using a complex experimental setting in which we controlled the air birds breathed. While photoperiodic and

temperature cues were identical to all breeding pairs, we only exposed birds to the tree odors so any potential visual, gustatory or tactile cues provided by vegetation would not be present (Amo et al., 2013). We monitored breeding pairs for gonadal development and hypothesized that pairs exposed to odors from caterpillar infested trees would develop their gonads more quickly than pairs in the control group.

Gonadal development might not only differ between the experimental and the control groups, but also among birds exposed to the odor treatment. Exploring variance among individuals is central to understand ecological and evolutionary processes, and it has long been neglected by endocrinologists (Williams, 2008). One way individuals differ reliably is in their temperament or personality, which in songbirds is often measured through exploratory behaviors (Réale et al., 2007). Several of these behaviors have indeed been shown to be repeatable, heritable, and to covary with one another, like exploration of new environments, new objects and aggression (Dingemanse et al., 2002). Interestingly these temperaments also covary with physiological traits and environmental perception (van Oers et al., 2011). In great tits for example, ovarian follicle volumes differ between selection lines for personality, with fast exploring females having larger ovarian follicles (Caro et al., 2019). We also recently showed that more exploratory male blue tits are more attracted to HIPVs than less exploratory males (Delaitre et al., unpubl. results). Exploration behavior thus seems to be linked to both HIPV sensitivity and reproductive mechanisms in tits, and therefore constitutes a potential source of variation in how individuals integrate and respond to HIPV signals.

## **Methods**

### *Ethical note*

Blue tits were trapped and maintained under licenses 2018-s-11 issued by the Direction Régionale de l'Environnement, de l'Aménagement et du Logement Languedoc-Rousillon; and 15-XIX-116 issued by the Direction Départementale de la Protection des Populations de l'Hérault. These experiments were carried out under license D34-172-11 from the Direction Départementale de la Protection des Populations de l'Hérault, and approved by the animal

experimentation ethical committee of the French Ministry of Higher Education and Research (APAFIS#8608-2017012011062214 v4).

### *Animal Housing*

The experiment took place over two years at the CNRS in Montpellier, France (43°37'56"N, 3°52'E). Twenty-four hand raised blue tits (born in 2015 in our long-term studied population of La Rouvière, near Montpellier; Caro et al., 2021) were housed in 12 terrariums (120 x 50 x 50 cm, one opposite-sex pair per terrarium) on 20 February 2019 and 21 February 2020 to allow approximately one month to adjust to new housing before starting the experiment ( $n = 48$  birds total). We randomly assigned 12 male-female pairs each year to either a HIPV exposure treatment ( $n = 6$  per year) or control ( $n = 6$  per year). Within each terrarium, a wire mesh was used to separate males and females (see figure 1), because males are usually ready to reproduce before females (Caro et al., 2006; 2009) and can therefore become overly aggressive toward females in confined environments (Caro et al., 2007). If physical contacts were prevented, males and females could still interact visually and acoustically, which has been shown to be sufficient for birds to develop their gonads and often for laying eggs (Kroodsma, 1976). Every individual had access to a nest box (not shown on figure 1) that was built around the wire mesh, with one entrance hole on each side, in a way that males and females could also see each other inside the nest box. *Ad libitum* food and water were replaced three times per week.

Terrariums were housed indoors to remove any potential visual cues provided by vegetation. We housed the birds in three different rooms with four breeding pairs per room. Pairs were visually isolated from other pairs and the terrariums also strongly reduced the ability of the pairs in the same room to hear each other. One terrarium in each room received air flow from one of four outdoor enclosures, so that each room had two treatment and two control terrariums. A window allowed natural light into the room. We additionally supplemented artificial light starting 15 min post-sunrise, until 15 min pre-sunset, so that birds were exposed to the natural increase and decrease in light intensity from the relatively small window (55 x 42

cm) (Fleissner and Fleissner, 2002). Extra light aimed at increasing and homogenizing light intensity in the room during the day. Sunrise and sunset times were determined using the United States Naval Observatory data base ([www.usno.navy.mil/USNO](http://www.usno.navy.mil/USNO)).

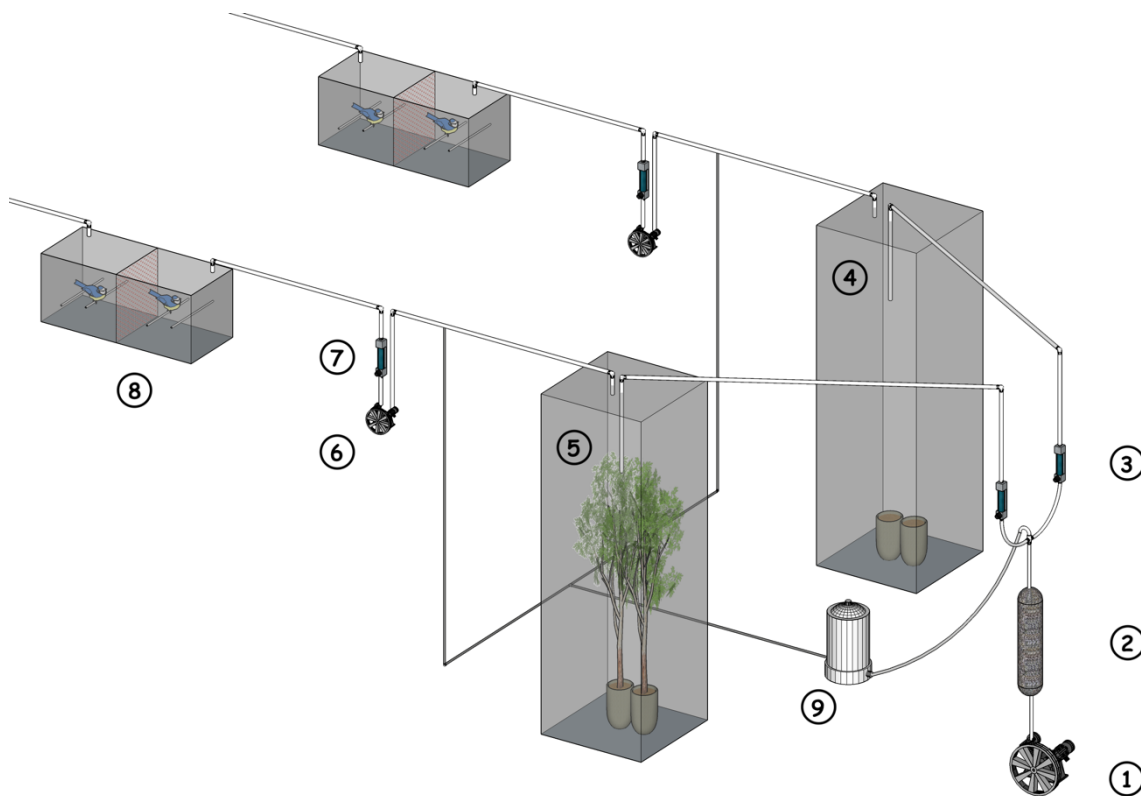
### *Equipment design*

To control the odor cues individuals received, two pumps (KNF Neuberger, Cat #N026.1.2AN.18) pushed 40 L/min of air through a respective charcoal filter to purify the air (Doughty et al., 1998) (figure 1). Flow from each pump was then split to push air into one control and one experimental enclosure. The four enclosures were constructed by stainless steel structure (250 x 80 x 80 cm) covered with thermowelded, chemically resistant Flonfilm 300 ETFE film (100 µm thick, PolyFlon Technology, LTD, Cat #ETFE1000/1550). Acrylic flow meters (Dakota Instruments Inc, Cat #6A0111BV-AB) kept the flow to ~19 L/min in each enclosure. Three smaller pumps (KNF Neuberger, Cat #N86KN.18) then each pulled air from one enclosure and pumped through an acrylic flow meter at 5 L/min (Dakota Instruments Inc, Cat #6A0107BV-AB) and into the terrarium (figure 1). An overflow of ~4 L/min in the enclosures ensured outward pressure of the system in case any leaks were present, so unfiltered air was less likely to enter the system. Air passively exited the terrariums through two exhaust tubes that led outside.

Air could also be diverted through a center enclosure (made of glass in 2019, aluminum container in 2020) shared by all 12 terrariums (see 9 on figure 1). When trees needed to be watered or changed out, birds continued to receive filtered, uncontaminated air while the treatment and control enclosures were open. A third pump (not shown on figure 1) was used to flush contaminated air out of the enclosures for several hours before resuming airflow from the enclosures. Due to high humidity levels inside the terrariums in 2019, particularly later in the season, several changes were made to the system in 2020 (figure S1). First, the tubing leading from the small pumps to the terrariums was run through a cooling system to remove water. The condensed water vapor was collected in glass jars below the cooling system before



the air entered the terrarium. These jars were emptied at least once per week. Second, the center enclosure described above was filled with silica gel. Two times per week at the time trees in the other enclosures were manipulated, air was diverted through this silica gel for approximately 24 hours to dry air in the terrariums (Yu et al., 2001).



**Figure 1.** Diagram of the experimental setup. Air was pumped (1) and pushed through a charcoal filter (2). Purified air was then split and flow regulated at 19L/min (3) before reaching one control (4) and one experimental (5) enclosure. Smaller pumps (6) pulled air from the enclosures at 5 L/min (7) and to the terrariums (8). Silica gel (9) was used to remove moisture from the air when flow was not through the enclosures. For simplicity, diagram represents only a fraction of the experimental setup. In reality, there were 4 enclosures, each leading to 3 terrariums (12 in total). See figure S1 for a more detailed view.

## *Infestation of Trees*

Green oak tortrix moths (*Tortrix viridana*) were collected from Corsica in June 2018 and 2019 and placed in large nets on oak branches to lay eggs. Branches were returned to Montpellier and kept outside at ambient temperature to synchronize hatching of eggs with oak bud burst. Green oak tortrix caterpillars are oak specialists and hatching needs to be closely timed with elongation and development of oak buds so caterpillars can perforate the bud and eat young developing leaves inside the bud (Du Merle, 1999; Ivashov et al., 2002). Thus, we utilized downy oak trees (*Quercus pubescens* Willd.) of approximately 2.5 meters high, grown in pots. To spread out timing of bud burst over the season, we housed 20 trees on the south side of a building, 20 trees on the north side, and 40 trees at higher elevation to delay bud burst. We began to expose birds to HIPV air as soon as bud burst and caterpillar hatching occurred simultaneously.

When bud development began to reach an appropriate stage for caterpillar infestation, four trees were placed into each of the two treatment enclosures and infested with freshly hatched green oak tortrix caterpillars (approx. 20 caterpillars per tree, depending on caterpillar availability). Control enclosures contained four soil-filled pots identical to those the trees were planted in. All pots (including those with trees) were wrapped in ETFE film bags to reduce any odors emitted from the soil and pots from entering the terrariums. When buds on the tree began turning into mature leaves, they were replaced with newly infested trees (generally 1 – 2x per week). We used freshly hatched caterpillars on new trees until ~29 April 2019, when nearly all caterpillar eggs had hatched. We then began to transfer caterpillars from trees being removed to trees being added. This likely kept the HIPV production at a very early season stage for most of the experiment. We stopped changing trees and let them progress in the enclosures after the third laparotomy (details below). However, in 2020, the French lockdown caused by the COVID-19 pandemic made it impossible to transport the high elevation trees to the campus. Thus, when we ran out of trees (21 April 2020), trees and caterpillars were left to continue development. HIPVs emitted by buds are to our knowledge similar to those emitted

by developed leaves, only the emission rates seem lower in buds than in leaves (unpublished results).

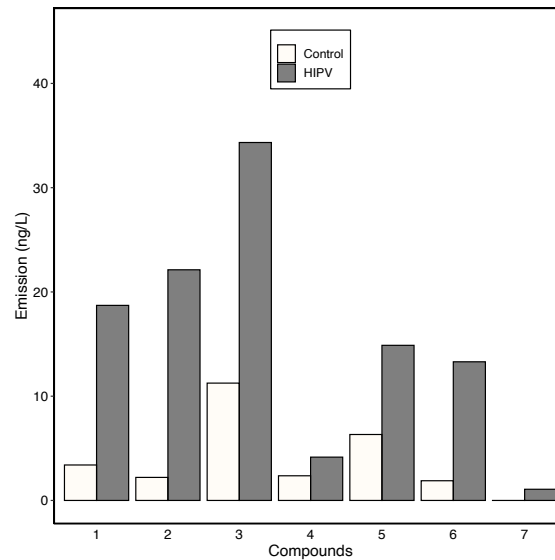
Control enclosures were treated exactly as treatment enclosures. When treatment enclosures were opened to water the trees, control enclosures were also opened and water added to the soil-filled pots. Similarly, when two old trees were exchanged for new ones, two soil filled pots would be replaced with two new soil filled pots.

Even if this was beyond the scope of the present study, a small number of air samples reaching the terrariums were pulled into cartridges containing an adsorbent to collect volatile compounds. These compounds were subsequently separated over a Gas Chromatograph - Mass Spectrometer coupled system (GC-MS) using a semi-standard non-polar column. Mass spectrum and linear retention index comparison enabled us to identify the compounds present. Those samples revealed that (*E*)- $\beta$ -ocimene, (*E*)-4,8-dimethyl nona-1,3,7-triene (DMNT), methyl salicylate, (*E*)-caryophyllene, aromadendrene,  $\alpha$ -humulene and (*E,E*)- $\alpha$ -Farnesene, all known HIPVs (Röse and Tumlinson, 2004, Graham et al. unpublished data), were found in the terrariums of the HIPV treatment group (figure 2).

### *Reproductive measures*

Three laparotomies were conducted each year to measure gonadal growth in all birds. Initial laparotomies were conducted when photoperiod was 11 hours of light (11L:13D) to obtain a pre-breeding measure of the gonads and confirm the two treatment groups did not differ. Two subsequent laparotomies were performed at four-week intervals (photoperiods 12.5L:11.5D and 14L:10D). Individuals were unilaterally laparotomized under isoflurane anesthesia. Left testis length and width were recorded in males and diameter of the largest developing ovarian follicle was measured in females. Measures were collected using the scale engraved in the ocular of a binocular microscope (Optika SZM-4) to the nearest 0.1mm. Testis volume was calculated as:  $V = \frac{4}{3}\pi\alpha^2\beta$  where  $\alpha$  is half the testis width and  $\beta$  is half the testis length. Follicle volume was calculated as:  $V = \frac{4}{3}\pi\alpha^3$  where  $\alpha$  is half the follicle width (Visser

et al., 2011). The observer was blind to the treatments. Throughout the experiment, birds were provided with moss to build nests and monitored for possible nest building and egg laying.



**Figure 2.** Concentrations of the compounds detected in the terrariums. Concentrations depicted here are the means of the 6 aquariums either exposed to air from caterpillar infested trees (HIPV group), or to control air. List of the compounds: (1) (*E*)- $\beta$ -ocimene, (2) DMNT, (3) methyl salicylate, (4) (*E*)-caryophyllene, (5) aromadendrene, (6)  $\alpha$ -humulene, (7) (*E,E*)- $\alpha$ -Farnesene.

### Exploration scores

We scored exploration by releasing each bird separately in a novel environment, which here consisted in an artificially lid room (3.5 x 2 m) equipped with five two-storey perches. Once the bird entered the room, its movements were observed for two minutes. The total number of movements between the five perches was counted, as well as hops up, and down, and based on these measures, birds were given an exploration score on a continuous scale with higher scores indicating faster exploration, and lower scores indicating slower exploration (Dingemanse et al., 2002; Reparaz et al., 2014).

### *Statistical Analysis*

All analyses were performed with R version 4.1.0. Linear mixed-effects models were completed using the lme4 and lmerTest packages (Bates et al., 2015) (Kuznetsova et al., 2017). We set  $\alpha = 0.05$ .

We started by running some preliminary analyses on the possible influence of variables that were not of primary interest to our study. Using two mixed-effect models (one for each sex), we tested whether the tree enclosures (four in total), the rooms housing the terrariums (three in total), the spatial position of the terrariums within a room (up or down the shelves, near or far from the window providing sunlight), the year, and body mass of the birds had any influence on gonadal size. We only found a significant influence of body mass on gonadal size in females, none of the other variables had any significant effect on gonadal size (all p-values >0.1, data not shown). We therefore decided to keep body mass in subsequent analyses, and ignored the other variables.

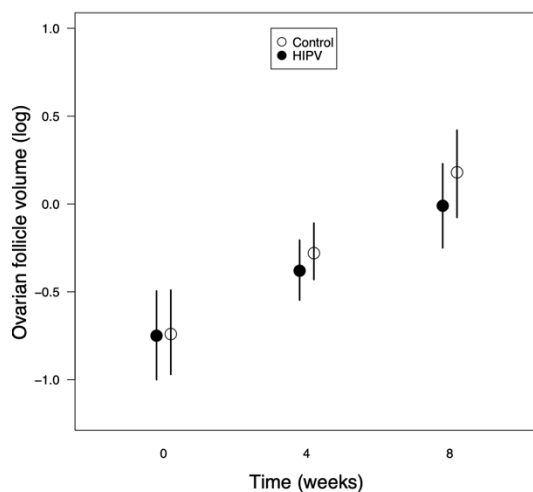
Male and female gonad size was analyzed separately as volume is not comparable between the sexes. In 2019, 24 individuals were measured during the first ( $T_0$ ) and second ( $T_1$ ) time point. There were only 22 measures for the third time point ( $T_2$ ) as we could not locate the ovary in one control female and one treatment male died during the experiment. Additionally, one female in 2019 laid an egg before the third laparotomy. Since the female was laying, we did not operate it and rather assigned the mean yolk diameter calculated from 23 unfertilized blue tit eggs collected from our aviaries ( $\bar{x} = 8.24 \pm 0.12$  mm) as a proxy for its largest ovarian follicle diameter. In 2020, 24 individuals were measured at the first and second time points. Twenty-three individuals were measured at the third time point because one treatment male died during the experiment.

Gonadal volume was log transformed before analysis to account for exponential growth. Linear mixed effects models included treatment (factorial), time point (continuous), exploration score (continuous), body mass (continuous), treatment by exploration score interaction, and treatment by time point interaction, as fixed factors. Individual ID was included as a random intercept to account for repeated measures. Non-significant interactions were

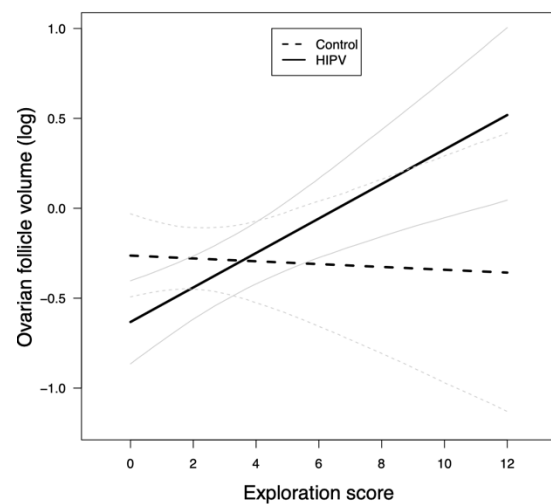
removed from the models. Since the HIPV treatment was only provided after  $T_0$ , we also ran the analyses described above, restricting data to  $T_1$  and  $T_2$ . Results were very similar to those obtained with all three time points (data not shown). Not enough individuals built nests (7 out of 24; 3 in the HIPV group, 4 in the control group) or laid eggs (6 out of 24; 3 in each group), thus no statistical analyses were performed on breeding data.

## Results

Ovarian follicle size increased over the course of the experiment ( $p < 0.001$ , table 1, figure 3), but follicles grew at the same rate in HIPV and control treatments (interaction treatment x time:  $p = 0.505$ , table 1, figure 3). There was a significant interaction between odor treatment and exploration score, with fast exploring females having larger ovarian follicles when exposed to HIPVs than to the control odor treatment (interaction treatment x exploration score:  $p = 0.037$ , table 1, figure 4). Finally, heavier females had larger ovarian follicles ( $p < 0.001$ , table 1).

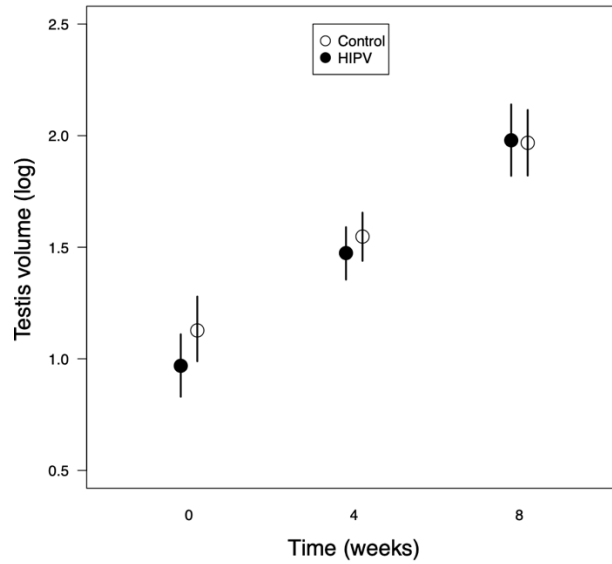


**Figure 3.** Ovarian follicle growth in blue tits. While there was a significant increase in follicle size over time (LMM,  $F=36.83$ ,  $p<0.001$ ), they grew at the same rate in both treatments (LMM,  $F=0.45$ ,  $p=0.505$ ). Points indicate mean  $\pm$  SEM;  $n=24$ , 12 birds per treatment.



**Figure 4.** Effect of odour treatment and personality on ovarian follicle size in female blue tits. Fast explorers had larger ovarian follicles when exposed to air containing HIPVs than to control air (LMM,  $F=5.04$ ,  $p=0.037$ ). Lines depict model estimates and 95% CI,  $n=24$ , 12 birds per treatment.

Male testes also increased in volume over time ( $p < 0.001$ , table 1, figure 5). Like in females, male gonads grew at the same rate in HIPV and control treatments (interaction treatment x time:  $p = 0.236$ , table 1, figure 5). Body mass or exploration score did not influence testis volume (all  $p \geq 0.1$ , see table 1).



**Figure 5.** Testes growth in blue tits. While there was a significant increase in testes volume over time (LMM,  $F=165.31$ ,  $p<0.001$ ), they grew at the same rate in both treatments (LMM,  $F=1.45$ ,  $p=0.236$ ). Points indicate mean  $\pm$  SEM;  $n=24$ , 12 birds per treatment.

**Table 1.** Analysis of the variables that potentially influence the volume of the gonads of female (n=24) and male (n=24) blue tits. Eliminated interactions (in grey) are presented in the reverse order in which they were removed from the model. Intercept includes Treatment = control. Volumes are log-transformed.

	<b>Variable</b>	<b>Estimate</b>	<b>S.E.</b>	<b>F</b>	<b>p-value</b>
<i>Females</i>					
	(Intercept)	-0.26	0.12		
	Treatment	-0.37	0.17	4.98	0.038*
	Time	0.41	0.07	36.83	<0.001***
	Exploration score	-0.01	0.04	3.68	0.070
	Body mass	0.23	0.05	18.73	<0.001***
	Treatment x Expl. score	0.10	0.05	5.04	0.037*
	Treatment x Time	-0.09	0.13	0.45	0.505
<i>Males</i>					
	(Intercept)	1.07	0.07		
	Treatment	-0.08	0.08	0.88	0.362
	Time	0.46	0.04	165.31	<0.001***
	Exploration score	-5e-04	0.01	0.01	0.914
	Body mass	0.06	0.04	2.68	0.113
	Treatment x Time	0.09	0.07	1.45	0.236
	Treatment x Expl. score	0.01	0.01	0.35	0.562



## Discussion

Our results suggest that HIPVs produced in response to caterpillar infestation do not exert a strong influence on pre-breeding sexual development in blue tits. While gonad volume increased over time in both males and females, there were no striking differences in size between the control and the treatment pairs. A significant response to the odor treatment was nevertheless visible in more exploratory females, which had larger ovarian follicles in the HIPV than in the control group.

Experiments manipulating non-photoc cues related to the initiation of reproduction in strictly seasonal breeding birds rarely result in striking differences between groups, owing to the fact that those cues are fine-tuning cues that only modulate the powerful effect of photoperiod (Wingfield and Kenagy, 1991). The case study of the influence of temperature cues on reproduction in great tit, highlighted in introduction, is probably the best example (Schaper et al., 2011a; Visser et al., 2009). Experimental evidence for an effect of temperature on great tit timing of reproduction in an ecologically relevant context requested nearly 10 years of experiments in captivity. Furthermore, temperature was never found to influence some of the main physiological mechanisms underlying egg-laying, which includes gonadal size, luteinizing hormone, and prolactin concentrations (reviewed in Caro et al., 2013b). Only patterns of gene expression related to seasonal timing were found to differ between temperature treatments (Laine et al., 2019), suggesting that the substrate for temperature-induced changes in reproductive physiology is there, but hard to detect. It is possible that the same complexity applies to environmental cues linking vegetation to timing of breeding, as suggested here by the absence of an effect of HIPVs on gonadal development, except for a subset of females (see below). Only a few previous studies have manipulated vegetation cues in captivity in tits (mainly through the addition of cut branches to the aviaries where the birds were), and they found no effect on timing of reproduction, or at least not in the predicted direction (Schaper et al., 2011b; Visser et al., 2002). In both cases however there was evidence for a positive effect of the leafing branches on luteinizing hormone, a central reproductive hormone, which called for more investigation on the topic. Other studies on other

bird and mammal species sometimes found stronger evidence. In meadow voles (*Microtus montanus*), adding sprouted wheatgrass, or one of its constituent (6-methoxybenzoazolinone: 6-MBOA), triggered reproduction and modulated sex-ratio (Berger et al., 1987; Negus and Berger, 1977), while in white-crowned sparrows (*Zonotrichia leucophrys*), adding wheat sprout leaves to the diet accelerated ovarian development (Ettinger and King, 1981). In canaries (*Serinus canaria*) the presence of vegetation in the aviaries advanced egg-laying by several weeks (Voigt et al., 2007; Voigt et al., 2011). While the number of experimental studies on the possible influence of vegetation development on bird reproduction is already very limited, the sensory pathways through which the plant stimuli were provided varied tremendously and mixed in various proportions tactile, gustatory, olfactory and visual signals. The drawback is that in several of those earlier studies, animals from the experimental groups could physically interact with the plants, while the control animals received none, which introduces environmental enrichment as a confounded factor. Disentangling pathways by carefully controlling sensory modes of perception of plants, like Voigt et al. (2011) did for example, will be key for clarifying whether and how vegetation cues potentially affect reproduction in vertebrates. In our case, the only possible sensory mode of perception of infested oak trees was olfaction, and we show that more exploratory females responded to these HIPVs. Future studies will need to determine whether combining olfactory with other carefully selected cues (either visual or gustatory cues from plants, or other environmental cues like photoperiod or temperature) could enhance the results obtained here.

More exploratory females grew their ovarian follicles more rapidly when exposed to HIPVs. This is not the first time that a link between a behavior related to personality and HIPVs is discovered. In a recent study testing whether blue tits can innately detect and orient to a blend of artificial HIPVs mimicking an infestation of oak buds by caterpillars, we found that the preference for the side of a Y-maze containing the HIPVs was driven by fast exploratory males (Delaitre et al., unpub. results). The results of both experiments suggest that fast explorers might be more sensitive to HIPVs. Through which mechanisms this link operates is still unclear at present, but we know that high sensitivity to HIPVs in male blue tits is associated with

testosterone (Graham et al., 2021). We also know that testosterone is locally produced in the olfactory epithelium in rats (Horie et al., 2017; Lupo et al., 1986), providing a possible mechanistic link between sex steroids and olfactory sensitivity for HIPVs. On the other hand, in female great tits we have recently shown relationships between exploration behavior, ovarian follicle sizes and plasma concentrations of 17 $\beta$ -estradiol, the main sexual steroid in female. Females from a line artificially selected for fast exploration more rapidly increased 17 $\beta$ -estradiol after a GnRH challenge, and had larger ovarian follicles than females from the line selected for slow exploration (Caro et al., 2019). This interplay between sex steroid hormones, HIPV sensitivity and personality clearly needs further investigation, in particular to understand if the enhanced olfactory sensitivity of fast explorers is driven through the action of steroid hormones.

There may also be technical limitations that could explain why we did not find stronger differences between the treatment and controls. First, birds were housed in relatively small compartments and blue tits are known to necessitate large enclosures to reproduce (Caro et al., 2007). Not enough birds may have approached reproductive readiness to detect an effect of HIPVs given the small sample sizes. Some females however laid eggs (6 out of 24, see methods), but we will need to experiment in larger settings to test this properly. A second potential issue is that HIPVs might not have reached the terrariums in sufficient concentrations. That early in the season, the HIPV production in response to caterpillar herbivory is very low (Graham et al. unpublished data). With overflow in the enclosures used to keep contaminated air from entering through any leaks in the system, we may have further lowered the concentration of HIPVs. Additionally, water condensation in the system due to high humidity levels can lead to adsorption on solid surfaces and oxidation of some VOCs, particularly polar compounds, before they reach the bird enclosures (Niinemets et al., 2011). Despite this however, we still detected several HIPVs compounds in the air arriving to the terrariums (figure 2), showing that air to which birds were exposed in the treatment group included oak bud HIPVs.

In conclusion, our results do not provide strong evidence that HIPVs released by developing tree buds in response to caterpillar herbivory, trigger gonadal recrudescence in captive blue tits. This might however not be surprising given that vegetation cues, in the same way as temperature or rainfall, are considered supplementary cues that refine the preponderant influence of photoperiod in temperate zones. Our finding that more exploratory females had slightly larger gonads when exposed to HIPVs should be considered as promising insight and it calls for more investigation on the possibility that olfaction could be a new player in seasonal timing of reproduction in birds

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