

Olfactory detection of trace amounts of plant volatiles is correlated with testosterone in a passerine bird

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16 Abstract

> In response to damage by insects, plants release herbivore-induced plant volatiles (HIPVs) into the air. Insectivorous birds exploit these cues and, consequently, reduce the damages inflicted to the plants. However, little is known about whether they solely use HIPVs as foraging cues, or if they also use them to modulate traits linked to reproduction. As caterpillars are the primary food source required for insectivorous birds to raise offspring, their ability to locate and predict future peaks in caterpillar biomass using olfaction is likely to be advantageous. Therefore, we tested whether an insectivorous songbird that naturally inhabits oak dominated forests can be trained to detect early spring infestation by hatchling caterpillars, at a time when oaks begin bursting, and birds prepare to breed. Tree buds were either infested with caterpillars or left as a control and visually obscured in a Y-Maze choice test. Additionally, we measured testosterone and 17β-estradiol as they influence olfactory perception in mammals and are linked to reproduction in vertebrates. After being trained to associate the presence of HIPVs with that of food, blue tits spent more time with, were more active around, and more frequently chose to first visit the infested trees, showing that blue tits can smell caterpillar activity. Males with higher testosterone spent more time around infested trees, suggesting that foraging behavior during the pre-breeding season is linked with a major reproductive signal. There was no relationship between foraging and estradiol in females. These results are an important foundation for further investigation of the role of hormones in avian olfaction and how smell may be useful for making breeding decisions that could improve reproductive success.

Keywords: seasonal reproduction, olfaction, alarm HIPVs, testosterone, estradiol

37 Introduction

Plants often emit chemical cues that protect them from damage caused by herbivores. In the case of herbivorous insects, volatile alarm signals released into the air attract carnivorous arthropods that protect the plants from damage (Turlings and Benrey, 1998; Van Poecke et al., 2001) by significantly reducing the number of herbivorous insects present on the plant (Kessler and Baldwin, 2001). In addition, some carnivorous arthropods appear to exploit these olfactory cues to prepare for reproduction. For example, ladybird beetles (Harmonia axyridis and Oenopia conglobata) experiencing a prey shortage will invest more in reproduction when presented with olfactory cues from aphid-infested plants (Rondoni et al., 2017). While most work on the influence of plant signals on animal behavior and physiology has focused on insects (Turlings and Benrey, 1998; Van Poecke et al., 2001), less is known about the abilities of vertebrates to detect and exploit these plant cues (Mrazova et al., 2019).

The olfactory ability of birds has become a popular area of study over the past few decades. Research has shown birds are capable of using smell in recognition of individuals (Bonadonna and Nevitt, 2004; Bonadonna and Sanz-Aguilar, 2012; Krause et al., 2012; Whittaker et al., 2011), selecting nest building materials (Clark and Mason, 1987; Petit et al., 2002), navigation (Bonadonna and Gagliardo, 2021; Gagliardo et al., 2013), and even modifying behavior to avoid predators (Amo et al., 2008; Roth II et al., 2008). There is growing evidence that insectivorous birds can also use their sense of smell in a foraging context, to detect infestation of plants by herbivorous insects (Amo et al., 2013; Mennerat et al., 2005). For example, the experimental infestation of birch trees (Betula pubescens) increases avian predation rates (Mäntylä et al., 2008). Similarly, birds are attracted to branches from infested pine trees (Pinus sylvestris) more than branches from uninfested trees (Mäntylä et al., 2017). However, in both these studies birds were able to see the infested trees and infestation can cause visual changes to trees (Amo et al., 2013). To our knowledge, only one published study exclusively tested the olfactory ability of birds to detect infested plants (Amo et al., 2013).

Among insects, caterpillars are particularly important for insectivorous birds, as they represent the primary food source required to successfully raise offspring (Naef-Daenzer and Keller, 1999). Thus, it may be expected that terrestrial birds may also use odors associated with food to detect it (Amo et al., 2013) and thereby constitute one of the most important selection factors in those bird species (Blondel et al., 1999). One such odor potentially associated with future food availability during the breeding season is herbivore-induced plant volatiles (HIPVs) released by plants in response to caterpillar herbivory. Volatile signals of caterpillar presence could modulate various behavioral and physiological processes in birds. If insectivorous birds like great tits use alarm signals from plants to forage for themselves and for feeding their chicks at a time when caterpillar biomass is large (i.e., late instar stages) and tree leaves are fully developed (Amo et al, 2013), birds may also use alarm signals from plants earlier in the season, when caterpillar biomass is still low because they just hatched and are even too small to be considered food. However, the olfactory detection of plants infested with young caterpillars has never been studied.

It has previously been shown that when young leaves are infested, monoterpene emissions are produced, but in significantly smaller amounts compared to mature leaves (Brilli et al., 2009). Cotton buds infested with Helicoverpa zea (Röse and Tumlinson, 2004) and oak buds infested with first instar green oak tortrix (Tortrix viridana) and winter moth (Operophtera brumata) caterpillars (about 1mm long) (Graham et al. unpublished results) already emit alarm volatile compounds, but in minute amounts compared to fully developed leaves. At this early stage, caterpillars live as miners in the buds, and are therefore invisible to potential predators (Du Merle and Mazet, 1983). Detecting such minute amounts of volatile signals could be challenging for birds, but there could nevertheless be several potential benefits to be gained from using such early signals of caterpillar presence in the environment. Birds might, for example, use the timing and intensity of the odorous signal to assess the quality of territories (Girling et al., 2011; Nyström, 1997). They could also use those signals to adjust clutch size in preparation for high or low insect

abundance (Gols et al., 2003; Hussell and Quinney, 1987; Marciniak et al., 2007), or potentially
even to adjust laying date in response to year-to-year variation in the date of emergence of
caterpillars. In order to determine if birds do make use of these early signals, we must first
determine whether birds are capable of detecting the trace amounts of HIPVs emitted from
developing buds, independently of visual cues. This is the main purpose of the present study.

If birds are detecting trace amounts of plant HIPVs and using those cues to make breeding decisions, any modification of reproductive behavior is likely to be primed by reproductive hormones (Ball and Balthazart, 2009, 2004; Wingfield et al., 1987). The relationship between chemosignals and their influence on the reproductive system is well documented in mammals, fish, and amphibians (Kawai et al., 2009; Nikonov et al., 2017; Petrulis, 2013), but less is known in birds (Caro and Balthazart, 2010), let alone for olfactory signals other than bodily odors, like plant or food odors for example. Work in several avian species suggest sex-steroids, like testosterone, stimulate more persistent food searching behavior (Andrew, 1972; Daisley et al., 2005; Németh et al., 2015), which is likely to be beneficial in early spring for acquiring resources for successful reproduction (e.g., territory, mate, food) (Lack, 1968; Wingfield, 1984). Additionally, olfactory sensitivity in rats appears to be upregulated by local sex-steroid production in the olfactory epithelium (Horie et al., 2017; Lupo et al., 1986), though whether this is the case in birds is unknown. Alternatively, it may be the exposure to some relevant odor signals that regulates sex-steroid dynamics, similarly to the stimulatory effect of pheromones on sex-steroid hormones (Miller and Maner, 2010). In any case, the relationship between behavior in response to an odor associated with food and pre-breeding reproductive hormones has not been studied.

The purpose of this study was to determine if insectivorous songbirds can detect the emissions of caterpillar infested trees early in the spring by smell only, and whether this is linked to their endocrine status. As blue tits (*Cyanistes caeruleus*) primarily inhabit oak dominated forests, we infested young oak trees with two common species of freshly hatched caterpillars that, later in the season, represent a major part of their natural diet in temperate zones: winter moths

(Operophtera brumata) and green oak tortrix (*Tortrix viridana*). We hypothesized that after training, birds would be able to differentiate between infested and uninfested oak trees by smell during the early stages of bud development, and that individuals that are more reproductively ready would spend more time near the infestation.

120 Methods

121 Ethics

Blue tits were trapped and maintained under licenses 2015615-147 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. Experiments were run under the license 2017-XIX-075 from the Direction Départementale de la Protection des Populations de l'Hérault, and approved by the Ethical committee N°036 with reference APAFIS#8608-2017012011062214 v4.

129 Housing of blue tits (Cyanistes caeruleus)

This experiment took place at the Centre National de la Recherche Scientifique (CEFE-CNRS) in Montpellier, France (43°37'56"N, 3°52'E) from 15 April to 2 May 2018. We used 42 hand-raised blue tits (females: n = 20, males: n = 22). Birds were moved indoors between 28 February and 14 March 2018 and were housed individually in cages (0.8 x 0.4 x 0.35m). Males and females were housed in separate rooms and females were never observed laying eggs for the duration of the study. Individuals were provided water and food ad libitum. Food consisted in a cake made of sunflower seed grease, eggs, sugar, wheat flour and high-protein pellets (Show 1+2 crumble, Versele-Laga, Deinze, Belgium), supplemented with minerals, vitamins, amino-acids and carotenoids (Nutribird A21, Versele-Laga, Deinze, Belgium; Nekton-S, Günter Enderle, Pforzheim, Germany; Yel-Lux, Versele-Laga, Deinze, Belgium). Mealworms were provided 3 times per week. Windows in the rooms allowed natural light to filter in while light timers were set

to turn on high-frequency TL tubes (Phillips TL5 HO 90 De Luxe 49W 965, Phillips, Eindhoven, The Netherlands) 15 minutes post-sunrise and turn off 15 minutes prior to sunset, providing natural increases and decreases in light intensity to occur (Fleissner and Fleissner, 2002). Sunrise and sunset times were determined using the United States Naval Observatory data base (www.usno.navy.mil/USNO). When moved, birds' feathers were checked to confirm they were in good condition, so flight capability was not inhibited.

Oak Trees

In the spring of 2018, 80 downy oak trees (Quercus pubescens Willd.; approx. 2 m high, and 0.8 m of diameter) grown in pots were maintained on the north side of a building (n = 20), south side of a building (n = 20), or at a higher elevation to delay bud burst (n = 40). All trees were treated with copper in a Bordeaux mixture (AMM 9500302) and a rapeseed oil insecticide (Naturen Eradibug, AMM 2110150) in the winter preceding each experiment to reduce the chance any fungus and wild insects infested the trees and caused other HIPV emissions than the ones induced by our caterpillar infestation. Once the oak buds began to elongate and lose their scales, they were considered ready to use in the experiment as caterpillars are unable to perforate the protective scales to enter the bud prior to this stage of development (Du Merle and Mazet, 1983). Of the 80 total trees, 24 were used in this experiment and 33 were used in a separate study specifically measuring HIPV production of oak buds experimentally infested with the same caterpillar species as here (Graham et al. unpublished results).

Caterpillars and bud infestation

We utilized 2 species of caterpillars for this project: winter moth (Operophtera brumata) and green oak tortrix (Tortrix viridana). Eggs of green oak tortrix were collected from tree branches the previous summer from Corsica, France while winter moth eggs were collected in the lab on

paper strips in The Netherlands. Eggs were kept in an unheated building overwinter and moved outdoors in spring so caterpillars and trees were experiencing the same weather conditions.

Once caterpillars began to hatch, oak trees with buds at an appropriate stage were either infested with caterpillars (using a small paintbrush to gently place caterpillars on the bud) or left as a control, 72 hrs prior to being used in a Y-maze (table 1). We chose 72 hrs post-infestation because data collected from experimentally infested oak buds found HIPVs significantly increase 48 – 72 hrs post-infestation when compared to uninfested, control buds (Graham et al. unpublished data). A single oak tree was placed at the end of each active arm (4 arms total, see below), so only 4 trees were used at one time. To infest a tree, 20 caterpillars were placed across 5 branches with stage 4 (bud elongated, swollen and green) - 6 (bud bursting, leaf shoots can be distinguished but are still intricated) buds (Du Merle and Mazet, 1983). T. viridana were given priority as they are native to the area, however, when not enough were available, O. brumata were used to keep the number of caterpillars on infested trees consistent. We recently found there is no difference in the volatile compounds emitted by oak buds when they were infested by either one of these two caterpillar species (Graham et al, unpublished results). A small wire cage was placed around the infested bud(s) and covered by a mesh bag to ensure caterpillars remained on the bud. The cage kept the bag from coming into contact with the bud(s), to prevent alarm emission due to mechanical stress (Kesselmeier and Staudt, 1999). Mean individual caterpillar mass for this study was 7.799 ± 0.990 mg. Control trees remained uninfested but had cages and bags on 5 branches to control for any emissions the tree may have produced in response to the presence of the cages. An individual bird was never exposed to the same pair of trees more than once and trees used on the day of the experiment had never been used during any of the training (see below).

190 Y-Mazes

> Two large outdoor y-mazes (2.5x2m arms, figure S1) were set up facing southeast. Two arms were designated as active arms (one control and one treatment arm) and a neutral arm containing no stimuli where the bird entered the maze. Each active arm of the maze had five food dishes and one perch, and one additional perch was placed in the neutral arm of the maze. Food dishes were the same as those used to feed birds when housed in outdoor aviaries, so they were already familiar to the birds. A small box was mounted on the door in the neutral arm to introduce birds into the maze. Mazes were visually isolated from each other by a hedge. A wind break was set up around the maze that additionally reduced visibility beyond the arms of the maze. Stands placed at the far end of the two active arms of the maze (outside the maze, against the mesh) contained the trees. Those stands were covered with a white fabric on three sides (those visible from the inside of the maze) to block any changes in color infested trees may display compared to uninfested trees (Amo et al., 2013). These enclosures also had small fans (92x92x25mm, 36m³/h, EbmPapst, Chelmsford, UK) to blow air from the trees toward the center of the maze (placed at 80 cm, 130 cm, and 180 cm from the ground). Behavioral observations always started as soon as a bird left the entrance box. Habituation, training, and testing began as early as 08:40 and were completed by 16:45 each day (For full experimental design, see table 1). A water bath was also available in each arm of the maze, so the birds always had access to fresh water.

209 Habituation

We were unable to observe 42 birds in a day, so we ran the experiment twice. Group 1 consisted of 20 birds (10 males, 10 females) sampled from 15 April to 21 April 2018. Group 2 consisted of 22 birds (12 males, 10 females) sampled from 26 April to 2 May 2018. To habituate birds to the mazes, flocks of 3 - 4 birds were released into the maze for a minimum of 2 hours. Food was removed from the birds' housing cages 60 - 90 minutes before a bird was introduced to the maze, to stimulate food searching. A mixture of food rewards (cake, mealworms) were

placed in all 10 dishes at the end of both arms of the maze to encourage exploration. No trees or
caterpillars were used during habituation. However, the empty tree enclosures and fans were in
place so birds could habituate to their visual and auditory presence.

220 Training

> Over the course of 5 consecutive days, individual birds were released into the mazes for 30 min with 1 food item placed in each dish only in the side of the maze containing an infested tree (Amo et al., 2013). Empty food dishes remained in the side of the maze with the control tree. The type of food reward was changed each day to keep birds from learning the scent of the food reward instead of the oak emissions (see table 1). The quantity of the food reward was recorded so we could keep track of whether birds were consuming the reward, and therefore whether they had a chance to associate the presence of oak emission with food. Additionally, the birds experienced the infested tree approximately equally on each side of the maze over the successive training sessions, so they would not become familiar with finding food on one side of the maze. Due to an odd number of trainings, half of the birds experienced the infested tree and food reward three times on the right side of the maze, while the other half of the birds experienced the infested tree and food reward three times on the left side of the maze. The side the infested tree was on was alternated between the left and right side daily for every individual. Order of individuals was randomly assigned, but one male and one female were always being tested in parallel in the two mazes with the exception of 2 males being tested at the same time during the second round of the experiment.

During the first training session, we observed that birds were less motivated to search for the food reward in the afternoon compared to the morning. To overcome this potential confounder, we alternated birds from the second group between morning and afternoon training sessions, so every bird experienced the maze 2 - 3 times in the morning. This reduced the number of birds that did not find the reward in at least half of the trainings from 40.0% of birds in the first round to

22.7% in the second round. In addition, inclement weather during the second round of trainings resulted in 12 birds only undergoing 4 rounds of training as opposed to 5 rounds.

Trials

Trials were performed once per individual and conducted in similar way to the training. A bird was released into the maze through the small box mounted on the door. The trial started as soon as a bird left the box and each bird was given 30 minutes to explore the maze. Food dishes were kept in the maze, but clean and empty of any food reward. Placement of the infested and control tree was randomized for each side of the maze and swapped after half of the birds had undergone a trial. Additionally, while 2 new trees were used in each maze on days 4, 7, and 9 (table 1), the trees were exchanged between mazes on days 5, 6, and 8 so that an individual bird experienced all 4 trees in up to 3 different combinations (e.g., day 1 – Trees 1 (infested) & 2 (control), day 2 - trees 3 (infested) & 4 (control), day 3 - trees 2 (control) & 3 (infested)). After trees were moved, the maze was left empty for 1 hour to allow any emissions caused by moving the tree to dissipate. Two Go Pro video cameras (GoPro, Inc., USA) were attached to the neutral perch, one facing into each active arm.

Behavioral Observations

Behavior was recorded by an observer blind to the treatment, using the free event logging software: Behavioral Observation Research Interactive Software (BORIS version 6.3.5, Friard and Gamba 2016). Videos in the left and right arm of the mazes could be watched at the same time and predetermined behavioral events were time stamped as they were observed. If a bird could not be seen in either video, they were presumed to be in the neutral arm of the maze. We measured (i) first arm of the maze visited after a bird entered the maze, (ii) time spent in each arm of the maze, and (iii) activity in each arm of the maze over the 30 min trial. Activity is the sum

267 of all movements where a bird was no longer in contact with its current perch (e.g., flying or 268 hopping to a new or the original location).

270 Blood Sampling

Immediately after being removed from the maze on the trial day, a blood sample was
collected from the wing vein. Samples were stored on ice until centrifugation to collect plasma.
Plasma was stored at -20°C until assayed for plasma 17ß-estradiol and plasma testosterone in
females and males, respectively.

276 Testosterone Assay

Plasma testosterone was measured using a commercially available enzyme immunoassay kit on a single plate (Enzo Life Sciences, ADI-900-065). Testosterone was extracted from 35 µL of plasma using solid phase extraction with C18 columns (100 mg C18 material, Sep-Pak Vac 1cc, Waters, Milford, MA, USA), dried under nitrogen gas at 40°C, and reconstituted overnight with 250 µL assay buffer (Caro et al., 2019). Concentration was adjusted for samples that did not have 35μ L of plasma available (*n* = 9 of 22). Reconstituted samples were plated in duplicate (100 µL per well) and concentrations determined using a freely available online five-parameter logistic curve-fitting program (MyAssays Ltd., 2019). Samples below detection limit (n = 1) were set at sensitivity of the assay (5.67 pg/mL). Intra-plate variation was calculated using pooled blue tit plasma from non-breeding individuals. To be certain the pool would produce results near the middle of the curve, 43.6 pg of exogenous testosterone for every 100 µL of reconstituted sample was added prior to extraction. The standard was placed in three random locations on the plate and intra-assay variation was calculated at 6.52%.

We validated the assay in blue tits following the methods of (Caro et al., 2019). Briefly, pooled plasma samples were spiked with exogenous testosterone and serially diluted. The assay was determined to work correctly as percentages of tracer bound (B/B0) from the serial dilutions
were parallel to the standard curve (see supplementary material, figure S2).

295 Estradiol Assay

Plasma 17β -estradiol (E₂) was measured using a commercially available double-antibody ¹²⁵I-E₂ radioimmunoassay (DSL-4800, Ultra-sensitive Estradiol RIA, Beckman Coulter, Brea, CA, USA) that was modified by dilution of the tracer and the antibody to increase the sensitivity of the assay, following (Charlier et al., 2010) and (Caro et al., 2019). Like for testosterone, steroids were first extracted from 35 µL of plasma using solid phase extraction with C18 columns (100 mg C18 material, Sep-Pak Vac 1cc, Waters, Milford, MA, USA), dried under nitrogen gas at 40°C, and reconstituted overnight with PBSg (PBS with 0.1% gelatin) containing 0.7% ethanol (Caro et al., 2019). Concentration was adjusted for samples that did not have 35 μ L of plasma available (n = 11 of 20). Resuspended samples were then assayed in duplicate (300 µl per tube) and assay tubes were counted on a gamma counter (Automatic Gamma Counter, Perkin Elmer, Waltham, MA, USA). Concentrations of E₂ were obtained using a linear regression with the log-transformed concentrations of the standards provided in the assay kit. All samples were run in a single assay, and the intra-assay coefficient of variation, as estimated by assaying one high and one low concentration E₂ standard in duplicate, was 5.9%. Assay sensitivity was 0.66 pg/ml. It was defined as the highest point on the standard curve whose standard deviation did not overlap that of the blank standard (Wingfield and Farner, 1975). No sample was found to be below the detection limit. The assay procedure has previously been validated for blue and great tits (Caro et al., 2019).

314 Statistical Analyses

All statistical analyses were run in R version 4.0.3 (R Core Team, 2020). As a preliminary analysis we used an exact binomial test to determine that individuals were equally as likely to go to the right side of the maze first (26 of 42) as they were to go to the left side when they entered

the maze (independently of which side the infested tree was on). Our results showed that birds were not lateralized (p = 0.16) and thus we excluded this variable from future analyses. We additionally ran preliminary analyses on behavior during training sessions. Briefly, we found that (i) birds did not become more successful in locating the food reward as the number of trainings increased (z = -0.09, p = 0.93, table S1); that (ii) training success was independent of plasma testosterone (z = -0.96, p = 0.34) and estradiol (z = 0.14, p = 0.89); and (iii) the ability to successfully locate the food reward was significantly repeatable within individuals at 0.49 (CI: 0.21, 0.66, p < 0.001, see supplementary materials for more details on these analyses). 20 325 After the preliminary analyses, we first ran a simple binomial test that compared the number of birds that chose to visit the infested side when they entered the maze, with the number of birds that first visited the control side. We then used a logistic regression to test the effect of several explanatory variables on first side chosen. We tested whether the first side chosen was influenced by the maze in which an individual was tested (Maze 1 or 2), the sex of the birds (M or F), the period the birds were tested (group 1 = habituation, training, and testing from 15 to 21 April, group 2 = habituation, training, and testing from 26 April to 2 May), the order of testing within a group (from 1 to 11, as a continuous variable), and the number of times no food reward was consumed during training (from 0 to 5, continuous variable, listed as 'Reward Left' in tables 2 and 3). Statistical output for these variables is reported in table 2 and significant values are reported in text. Effect sizes were calculated using package effectsize (Ben-Shachar et al., 2020). We used standardized regression coefficients (β), which are reported in table 2 along with 95% confidence intervals. To determine if time and activity level in each side of the maze differed between infested and control, paired t-tests (one for time, and one for activity) were initially used. To test additional variables that could potentially affect time and activity level in each side of the maze, two linear

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models were then run (one for each response variable, like above). The response variable for

time was calculated as number of seconds spent in the infested arm of the maze minus the

number of seconds spent in the control arm of the maze. More positive values indicate more time 7 spent in the infested arm, while more negative values indicate more time spent in the control arm. Similarly, the response variable for activity was calculated as the number of movements made in the infested arm minus the number of movements made in the control arm. More positive values indicate higher activity levels in the infested arm and more negative values indicate higher activity levels in the control arm. Explanatory variables included in both models were maze, sex, test group, testing order, and number of times the food reward was not consumed during training. In a final set of analyses, we tested whether plasma steroid levels played an additional 20 351 role on the time and the activity levels of the birds tested. Because we measured a different hormone in each sex, we ran separate models for males and females. Like in the models above, maze, test group, test order, and remaining food reward were also added, together with testosterone (in males) and estradiol (in females) concentrations (both In transformed to achieve normality). Only significant values are reported in text for variables other than hormones, but all values are reported in table 3. A single male had undetectable testosterone levels and was subsequently excluded from final hormone analyses. However, assigning the lowest detectable level of the kit (5.67 pg/mL) and including the male in statistical models did not change the results. Graphs were made using package ggplot2 (Wickham, 2016). We set α = 0.05 and report mean ± SEM. Results Individuals were more likely to visit the side of the maze with the infested tree first (29 of **364** 42) compared to the control tree (p = 0.02). On average, birds spent 250.70 s longer in the side of the maze with the infested tree compared to the side with the control tree (t_{41} = 2.13, p = 0.04, figure 1). Birds were also nearly twice as active in the infested side of the maze compared to the control side (t_{41} = 3.31, *p* = 0.002, figure 1). When additional variables are introduced into a more

369 complex model, they have no influence on first side chosen (all p > 0.1), time (all p > 0.09), or 370 activity (all p > 0.12, values reported in table 2).

Male-restricted analyses showed that birds with higher levels of testosterone spent more time in the infested arm of the maze ($F_{1,15}$ = 8.01, p = 0.01, R^2 = 0.44, figure 2A). Testosterone was however not related to activity levels ($F_{1,15} = 3.35$, p = 0.09, $R^2 = 0.43$, table 3). Mean testosterone levels were significantly higher in maze 2 males (386.49 ± 122.42 pg/mL) compared to maze 1 males (259.26 \pm 33.2 pg/mL; F_{1.15} = 9.352, p = 0.008), but this pattern was primarily driven by two males in maze 2 with the highest testosterone levels of all 21 individuals (maze 2 mean with highest two males removed: 214.25 ± 32.60 pg/mL). In pre-breeding females, estradiol levels showed no relationship with time ($F_{1,14} = 0.01$, p = 0.94, figure 2B) or activity ($F_{1,14} = 2.56$, p = 0.13). Other variables were also all non-significant (table 3).

381 Discussion

382 Blue tits are able to detect minute amounts of plant volatiles

We found that blue tits are capable of detecting HIPVs emitted by buds of one single small oak infested by young caterpillars. After training, individuals were not only more likely to visit the infested side of the maze first, but they also spent more time and were more active in the infested side of the maze. Our results are supported by other studies showing passerine birds select infested plants over uninfested plants (Amo et al., 2013; Mäntylä et al., 2017, 2008). In addition to the fact that visual cues were excluded in our experiment, emissions from infested buds appear to vary in both composition and quantity of HIPVs when compared to the mature foliage that older caterpillars feed on (Brilli et al., 2009; Röse and Tumlinson, 2004). This is the first study showing birds can detect the trace amounts of HIPVs from trees infested by young caterpillars during early stages of bud development, when caterpillars are not large enough to be considered a food source.

While our birds were originally naïve, having been in captivity from 10d of age, they were trained to <mark>associate the presence of food in small dishes</mark> with <mark>the smell of one</mark> infested tree. Our results clearly indicate that the detection of HIPVs from early bud infestation can be a learned behavior. While one study found that naïve great tits do not discriminate between infested and uninfested apple trees, suggesting use of HIPVs from fully developed leaves for locating food is not innate (Amo et al., 2016), new findings in blue tits show that detection of HIPVs from developing buds is innate in this species. When presented with an artificial odor bouquet mimicking the HIPVs released from developing buds, naïve blue tits are more likely to choose to 20 401 first visit vials diffusing HIPVs as opposed to a control (Delaitre et al. unpublished data). Similarly, blue petrel fledglings (Halobaena caerulea) appear to show an innate preference for dimethyl sulfide, an olfactory cue important for locating patchily distributed prey in the ocean (Bonadonna et al., 2006; Cunningham et al., 2003). Further studies are needed to determine which HIPVs are innately detected, which ones need to be learned, and which ones may influence reproduction in blue tits. Males with higher testosterone levels spent more time in the infested arm 40 410 We found that spending more time in the infested arm of the maze compared to the control arm was correlated with higher testosterone levels in males. Although plasma testosterone and time spent in the infested arm were correlated, it is unclear which of the two variables may affect the other. One potential explanation for this relationship is that male blue tits with higher levels of testosterone are more sensitive to cues that could indicate territory that will have higher food

abundance. European starlings (Sturnus vulgaris) for example, only exhibit an olfactory response to the odors of a preferred nesting material (De Groof et al., 2010) and a conditioned odor stimulus during the breeding season (Clark and Smeraski, 1990). While artificially increasing plasma testosterone levels during the non-breeding season does not seem to change olfactory sensitivity to preferred nesting material in European starlings (De Groof et al., 2010) or food odor cues in

female goldfish (Carassius auratus) (Ghosal and Sorensen, 2016), the lack of receptors to receive the increased signal might result in non-activation of seasonal changes in olfactory sensitivity. Olfactory processing regions in many other vertebrate species contain gonadotropin releasing hormone (GnRH) and sex steroid receptors (Kawai et al., 2009). Steroid receptor density appears to change with reproductive status in fish (Maruska and Fernald, 2010), while olfactory sensitivity and preference appear to be highest during reproduction in rats (Moffatt, 2003). As the breeding season approaches, males may upregulate receptor density in olfactory processing regions so higher levels of testosterone have a greater influence on olfactory sensitivity during the breeding season. It should also be noted that work in mammals show that exposure to steroid hormones will modulate the integration of chemosensory signals, including olfactory cues. This was particularly well studied in the context of hormonal-dependent onset of maternal behavior in mammals. For example, females begin to show a preference for bedding soiled by pups over clean bedding, around the peripartum period (Kinsley and Bridges, 1990) and this is known to be associated with a general rewiring of the maternal brain (Keller et al., 2019; Lévy and Keller, 2009). Steroid-dependent changes in the neural pathways underlying chemosensory integration was also highlighted in copulatory context (Fiber and Swann, 1996; Paredes et al., 1998). Additional work should be performed to define whether sensitivity to HIPVs is modulated at the level of the periphery or the central nervous system, or both.

An alternative explanation for the correlation between testosterone and time spent in the infested arm of the maze is the rapid response of testosterone production to certain stimuli. For example, males of some species can quickly upregulate hormone production in response to male-male competition (Archer, 2006; Mcglothlin et al., 2008; Wingfield and Wada, 1989), presence of a female (Coquelin and Bronson, 1979; Ronay and Hippel, 2010; Stacey, 2003), and even after exposure to a nest box (Gwinner et al., 2002). Male song sparrows showed a significant increase from baseline testosterone levels just 10 min after the start of a simulated territorial intrusion (Wingfield and Wada, 1989) and the introduction of green vegetation to captive-housed canaries

 446 (*Serinus canaria*) results in an increase in testosterone (Voigt et al., 2007). Our birds were in the
447 maze and able to interact with the infested trees for up to 30 min, providing ample time for the
448 HIPV cues experienced in the maze to produce measurable differences in testosterone based on
449 time spent near the stimulus (i.e., infested trees).

A third potential hypothesis also considers the fact that we trained individuals to associate food with HIPVs and testosterone's apparent role in olfactory sensitivity in rats and mice. For example, in gonadectomized male rats, testosterone injections can restore odor discrimination capabilities and improve task performance (Kunkhyen et al., 2018). However, our results suggest this may not be the case in blue tits. While those individuals with higher levels of testosterone may have spent more time in the infested arm of the maze, testosterone level was not correlated with increased success in locating the food reward during training. Instead, we found that the ability to locate the food reward was repeatable within an individual. Therefore, we think it is unlikely that testosterone played a role in developing a preference for HIPVs, but further work is needed to clarify this relationship.

Another consideration in the role of testosterone in detecting HIPVs is the subsequent reduction in testosterone levels during chick rearing (Hegner and Wingfield, 1987; Hunt et al., 1999), which seems counterintuitive when the ability to locate prey during this period is critical for reproductive success (Visser and Gienapp, 2019). However, fully developed vegetation produces HIPVs in much higher quantities than the early-spring buds we studied here (Brilli et al., 2009; Röse and Tumlinson, 2004), thus, increased olfactory sensitivity may not need to be maintained to successfully forage once HIPVs are emitted in higher quantities in response to herbivory by large caterpillars,

469 Estradiol is not correlated with behavior in females

470 We found no correlation between estradiol and time spent in the infested arm in female 471 blue tits. Estradiol does respond to GnRH within 30 min in female great tits (*Parus major*) and

dark-eyed juncos (Junco hyemalis) (Caro et al., 2019; Needham et al., 2019), suggesting we would see similar results to testosterone in male blue tits, if perception of a reproductively-relevant stimulus like HIPVs can indeed induce a surge in sex-steroid hormones. Yet female birds control timing of reproduction more closely than males (Caro et al., 2009). While male birds initiate reproductive activity at the beginning of the breeding season and can maintain their status for weeks (Ball and Ketterson, 2008; Caro et al., 2006), increases in plasma estradiol in females is tightly correlated with rapid yolk development (which occurs just prior to egg laying) and decreases to pre-breeding values before the final yolky follicle is ovulated (Williams et al., 2004). Our female blue tits did not lay eggs while housed indoors for this experiment. Similarly, in European starlings, social stimuli via the presence of a male is considered to be important for stimulating final maturation of ovarian follicles (Perfito et al., 2015) and we housed male and female blue tits separately during this experiment. Thus, female blue tits may not produce estradiol in response to herbivore induced HIPVs occurring early in the season because they were not ready to begin egg laying yet. Instead, females may be able to indirectly benefit from increased testosterone in males. Physiological and behavioral changes associated with reproduction are typically observed earlier in males than females (Ball and Ketterson, 2008; Caro et al., 2006). The ability to detect HIPVs may be important for males in locating a high-quality territory.

Testosterone on the other hand, may also be important in detecting environmental cues in females. Interestingly, androgen levels in female European starlings appear to be upregulated prior to rapid yolk development (Williams et al., 2004). For example, testosterone is highest during territory establishment and pre-laying stages in female tree swallows (*Tachycineta bicolor*); which corresponds with timing of female competition for resources and mates (George and Rosvall, 2018). Testosterone patterns in female blue tits could also upregulate in response to HIPVs and/or influence olfactory sensitivity like in males, but future studies examining the effects of HIPV

exposure on female reproductive physiology will be needed to unravel the pathway linking HIPV perception to behavioral decisions.

Conclusions

Our study is novel in showing that a passerine bird is able to detect trace HIPVs produced in response to caterpillar infestation of developing buds. As freshly hatched caterpillars are too small and cryptic to be used as food, individuals may use these cues when making other decisions that would improve future reproductive success. Selecting mates based on the quality of the territory they occupy (Alatalo et al., 1986), adjusting clutch size based on habitat quality (Siikamäki, 1995), and timing offspring rearing to occur during peak food availability (Post and Forchhammer, 2008; Reed et al., 2013) are a few possibilities that need to be explored. Our finding that males with higher testosterone levels spend more time with infested trees compared to controls also indicates a possible physiological interaction with HIPVs. Future studies should now focus on how insectivorous vertebrates may use these cues early in the season and how HIPVs affect vertebrate physiology.

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Tables

748 Table 1. Timeline of caterpillar infestation, habituation, training, and trials conducted in captive blue tits. Only 22 birds could be used

749 at a time; thus, the protocol was repeated twice.

Reward	Trees Used	Birds	Caterpillars	Day
			Infest Trees (1 - 4)	-
				2
Meal Worms, Cake	No Trees	Habituation		ω
Meal Worms	1 – 4	Training 1	Infest Trees (5 - 8)	4
Wax Moth Larvae	1 – 4	Training 2	Infest Trees (9 - 12)	თ
Crickets	1 – 4	Training 3		6
Beetle Larvae	5–8	Training 4		7
Wax Moth Larvae	5–8	Training 5		8
None	9 – 12	Trials		9

751 Table 2. Analyses of the variables that influence the first side chosen, the time spent, and the level of activity close to one caterpillar-

752 infested oak in blue tits (n = 42).

765	764																	
confidence interv	Bolded values we			Activity					Time						Eirct Cide		Variable	Response
als in brackets.	re statistically signi	Reward Left	Order	Test Group	Sex	Maze	Reward Left	Order	Test Group	Sex	Maze	Reward Left	Order	Test Group	Sex	Maze	Variable	Explanatory
	ficant (<i>p</i> <0.05). <mark>Effec</mark>	0.03 [-0.32, 0.38]	-0.04 [-0.36, 0.29]	0.17 [-0.17, 0.51]	0.10 [-0.55, 0.76]	-0.26 [-0.58, 0.07]	0.05 [-0.29, 0.40]	0.34 [-0.28, 0.95]	-0.11 [-0.44, 0.23]	-0.11 [-0.75, 0.54]	-0.56 [-1.17, 0.05]	-0.45 [-1.29, 0.32]	-0.73 [-2.19, 0.63]	0.09 [-0.70, 0.87]	-1.32 [-3.02, 0.15]	0.56 [-0.81, 2.03]	B [95% CI]	
	<mark>tt sizes (β) are s</mark>	0.89	-0.70	19.98	6.10	-30.54	22.36	43.25	-161.72	-81.69	-842.70	-0.25	-0.12	<mark>0.18</mark>	-1.31	<mark>1.11</mark>	Estimate	
	tandardize	6.01	3.14	20.10	19.56	19.11	74.43	38.90	248.89	242.19	454.72	0.23	0.12	0.77	0.79	<mark>1.40</mark>	S.E	
	d beta coefficient	$F_{1,36} = 0.02$	$F_{1,36} = 0.05$	$F_{1,36} = 0.96$	$F_{1,36} = 0.10$	$F_{1,36} = 2.53$	$F_{1,36} = 0.09$	$F_{1,36} = 1.26$	$F_{1,36} = 0.48$	$F_{1,36} = 0.17$	$F_{1,36} = 2.93$	<mark>z = -1.11</mark>	z = -1.03	z = 0.23	<mark>z = -1.66</mark>	<mark>z = 0.80</mark>	Test Statistic	
	s followed by	0.883	0.828	0. <u>33</u> 3	0.748	0.120	0.766	0.270	0.493	0.682	0.096	0.266	0.302	0.820	0.096	<mark>0.426</mark>	p-value₃4	
	the 9																	

Bolded values were statistically significant (*p*<0.05). Effect sizes (β) are standardized beta coefficients followed by the 95% confidence intervals in brackets.

<u>0</u>	$F_{1,14} = 0.01$	9.82	1.14	.03 [-0.53, 0.59]	Reward Left 0	_		
0	$F_{1,14} = 0.09$	5.49	-1.77	.08 [-0.62, 0.46]	Order -0			
0	$F_{1,14} = 0.21$	34.91	16.66	. <mark>12 [-0.43, 0.68]</mark>	Test Group 0		Activity	
0	$F_{1,14} = 0.06$	32.81	7.22	.05 [-0.47, 0.58]	Maze 0			
0	$F_{1,14} = 2.56$	53.53	-78.11	. <mark>37 [-0.91, 0.17]</mark>	Estradiol (In) -0	m		
0.	$F_{1,14} = 0.08$	118.07	32.89	.08 [-0.52, 0.68]	Reward Left 0			
.0	$F_{1,14} = 0.01$	66.03	-10.16	.04 [-0.62, 0.54]	Order -0			
0.	$F_{1,14} = 0.56$	419.53	-270.04	.18 [-0.78, 0.42]	Test Group -0		Time	
0.	$F_{1,14} = 0.01$	394.39	-47.78	.03 [-0.60, 0.53]	Maze -0			
0.	$F_{1,14} = 0.01$	643.38	-165.44	.07 [-0.65, 0.51]	Estradiol (In) -0	п	S	Female
0.	F _{1,15} < 0.01	6.55	-0.56	.02 [-0.44, 0.41]	Reward Left -0			
0.	$F_{1,15} = 0.35$	3.16	-1.87	.11 [-0.49, 0.28]	Order -0			
0.	$F_{1,15} = 2.78$	21.62	34.07	. <mark>32 [-0.11, 0.76]</mark>	Test Group 0		Activity	
<u>0</u>	$F_{1,15} = 14.12$	18.26	-68.43	65 [-1.02, -0.28]	Maze -0.			
0	$F_{1,15} = 3.35$	14.23	39.23	0.53 [0.12, 0.94]	sterone (In)	Testc		
0.	$F_{1,15} = 0.27$	93.63	48.90	.10 [-0.32, 0.52]	Reward Left 0			
0.	$F_{1,15} = 2.60$	45.16	70.41	.28 [-0.10, 0.67]	Order 0			
0	$F_{1,15} = 0.63$	309.04	288.00	. <mark>19 [-0.24, 0.62]</mark>	Test Group 0		Time	
<u>.</u>	$F_{1,15} = 9.35$	261.06	-853.77	57 [-0.94, -0.20]	Maze -0.			
<u>.</u>	$F_{1,15} = 8.01$	203.46	567.19	1.54 [0.13, 0.95]	sterone (In)	Testos	-,	Males
va	Statistic			B [95% CI]	riable	Va	Variable	
~	Test	S.E	Estimate		lanatory	e Exp	Response	

confidence intervals in brackets.

Table 3: Analyses of the variables that influence the time spent and the level of activity close to one caterpillar-infested oak in male

Figure 1. Blue tits detect herbivore-induced plant volatiles (HIPV) emitted by the buds of one single, small oak. On average, individuals spent 250.7 seconds longer in the infested arm of the maze than the control arm and were significantly more active in the infested arm of the maze compared to the control arm. Activity is the sum of all movements where a bird was no longer in contact with its current perch (e.g., flying or hopping to a new or the original location). Circles represent individual data points while squares represent mean of all individuals ± SEM.



Figure 2. Testosterone correlates with HIPV detection. (A) Males with higher levels of testosterone spent significantly more time in the infested arm of the maze compared to the control arm of the maze, (B) but this was not the case for the relationship with estradiol in females.



783 Supplementary Material 784 <u>Behavioral analyses during training</u>

We ran preliminary analyses on bird behavior related with successfully locating food during training. To test whether individuals became more successful at finding the food reward during trainings using a generalized linear mixed-model with binomial distribution in package Ime4 (Bates et al., 2015). The response variable was a repeated measure coded as 1 (consumed food reward) or 0 (did not consume food reward) for each of the 5 training sessions. Our fixed factor of interest in the model was the training session (1 - 5). Individuals were tested in two mazes, during two different time periods, thus, to determine if these variables affected the results, the maze an individual was tested in (Maze 1 or 2) and the test group (group 1 = habituation, training, and testing from 15 to 21 April, group 2 = habituation, training, and testing from 26 April to 2 May) were both included in the model. Testing order is included as a measure of when an individual was tested during the day. Those listed as 1 were the first to be tested on that day and those listed as 10 or 11 were last to be tested that day. Sex (M or F) was also included. Statistical output for these variables is reported in table S1 and significant values are reported in the main text. Given the evidence that testosterone can influence olfactory discrimination in rats and mice (Kunkhyen et al., 2018), we also decided to run 2 additional models comparing testosterone in males and estradiol in females to training success. Furthermore, we calculated the repeatability (logit link-scale approximation) of consuming the food reward across the five trainings using the package rptR (Stoffel et al., 2017).

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	Bolded values were	Males (<i>n</i> = 21) Females (<i>n</i> = 20)	1 1 1	All Individuals $(n = 42)$	Training Success	males and females v	Table S1. Analyses
	statistically significan	Testosterone (In) Estradiol (In)	Test Group Order	Maze Sex	Explanatory Variable Trial Number	vere included in the t	of the variables that i
	nt (p<0.05). Effect sizes	-0.55 [-1.66, 0.57] 0.07 [-0.92, 1.06]	0.68 [-0.06, 1.42] -0.06 [-0.76, 0.65]	-0.16 [-0.88, 0.56] 1.15 [-0.31, 2.61]	B [95% CI]	estosterone and estra	influence training succ
34	<mark>s (β) are standardize</mark>	-0.74 0.23	1.35 -0.02	-0.32	Estimate	diol analyses, respec	<mark>ess. All individuals w</mark>
	d beta coefi	0.77 1.59	0.75 0.12	0.73 0.74	<mark>S.E.</mark> 0 14	<mark>xtively.</mark>	<mark>/ere include</mark>
	ficients followed b	z = -0.96 z = 0.14	z = 1.80 z = -0.15	z = -0.43 z = 1.55	Test Statistic		d in the initial ana
	y the 95%	0.336 0.887	0.072 0.879	0.666 0.121	p-value 0.931		lysis while only

Figure S1. Diagram of the Y-mazes used in the behavioral experiment. To test the ability of insectivorous songbirds to detect caterpillar infestation of oak buds by smell, a Y-maze was set up so birds entered from a small box in the neutral arm. Two GoPro cameras were mounted on a center perch to film activity in each of the active arms. An infested oak tree was on one side of the maze and an uninfested tree on the other. Both were visually isolated from the bird to obscure any visual changes resulting from infestation, but small fans were in place to help direct any odors toward the center of the maze. Each trial lasted 30 minutes.



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Figure S2. Testosterone assay validation for blue tit plasma. To validate the assay in blue tits, 2 plasma pools were created from samples collected from blue tits in January 2018. The first pool was spiked with 19.7 pg of exogenous testosterone per well (low spike) and the second pool was spiked with 39.5 pg of exogenous testosterone per well (high spike) to ensure the entire serial dilution of the pooled samples would fall on the curve. Each plasma pool was then serially diluted to create a 5-point curve. During validation, testosterone was extracted from 35 µL of plasma using solid phase extraction with C18 columns (100 mg C18 material, Sep-Pak Vac 1cc, Waters, Milford, MA, USA), dried under nitrogen gas at 40°C, and reconstituted overnight with 250 µL assay buffer (Caro et al., 2019). Recovery after extraction was 57.3% and 64.4% for low and high spiked curves, respectively.

