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

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4 1 **Olfactory detection of trace amounts of plant volatiles is correlated with testosterone in a**
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16 **Abstract**

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

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

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4 **37 Introduction**

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6 38 Plants often emit chemical cues that protect them from damage caused by herbivores. In
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9 39 the case of herbivorous insects, volatile alarm signals released into the air attract carnivorous
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11 40 arthropods that protect the plants from damage (Turlings and Benrey, 1998; Van Poecke et al.,
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13 41 2001) by significantly reducing the number of herbivorous insects present on the plant (Kessler
14
15 42 and Baldwin, 2001). In addition, some carnivorous arthropods appear to exploit these olfactory
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17 43 cues to prepare for reproduction. For example, ladybird beetles (*Harmonia axyridis* and *Oenopia*
18
19 44 *conglobata*) experiencing a prey shortage will invest more in reproduction when presented with
20
21 45 olfactory cues from aphid-infested plants (Rondoni et al., 2017). While most work on the influence
22
23 46 of plant signals on animal behavior and physiology has focused on insects (Turlings and Benrey,
24
25 47 1998; Van Poecke et al., 2001), less is known about the abilities of vertebrates to detect and
26
27 48 exploit these plant cues (Mrazova et al., 2019).

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29
30 49 The olfactory ability of birds has become a popular area of study over the past few
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32 50 decades. Research has shown birds are capable of using smell in recognition of individuals
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34 51 (Bonadonna and Nevitt, 2004; Bonadonna and Sanz-Aguilar, 2012; Krause et al., 2012; Whittaker
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36 52 et al., 2011), selecting nest building materials (Clark and Mason, 1987; Petit et al., 2002),
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38 53 navigation (Bonadonna and Gagliardo, 2021; Gagliardo et al., 2013), and even modifying
39
40 54 behavior to avoid predators (Amo et al., 2008; Roth II et al., 2008). There is growing evidence
41
42 55 that insectivorous birds can also use their sense of smell in a foraging context, to detect infestation
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44 56 of plants by herbivorous insects (Amo et al., 2013; Mennerat et al., 2005). For example, the
45
46 57 experimental infestation of birch trees (*Betula pubescens*) increases avian predation rates
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48 58 (Mäntylä et al., 2008). Similarly, birds are attracted to branches from infested pine trees (*Pinus*
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50 59 *sylvestris*) more than branches from uninfested trees (Mäntylä et al., 2017). However, in both
51
52 60 these studies birds were able to see the infested trees and infestation can cause visual changes
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54 61 to trees (Amo et al., 2013). To our knowledge, only one published study exclusively tested the
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56 62 olfactory ability of birds to detect infested plants (Amo et al., 2013).

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

77 It has previously been shown that when young leaves are infested, monoterpene
78 emissions are produced, but in significantly smaller amounts compared to mature leaves (Brilli et
79 al., 2009). Cotton buds infested with *Helicoverpa zea* (Röse and Tumlinson, 2004) and oak buds
80 infested with first instar green oak tortrix (*Tortrix viridana*) and winter moth (*Operophtera brumata*)
81 caterpillars (about 1mm long) (Graham et al. unpublished results) already emit alarm volatile
82 compounds, but in minute amounts compared to fully developed leaves. At this early stage,
83 caterpillars live as miners in the buds, and are therefore invisible to potential predators (Du Merle
84 and Mazet, 1983). Detecting such minute amounts of volatile signals could be challenging for
85 birds, but there could nevertheless be several potential benefits to be gained from using such
86 early signals of caterpillar presence in the environment. Birds might, for example, use the timing
87 and intensity of the odorous signal to assess the quality of territories (Girling et al., 2011; Nyström,
88 1997). They could also use those signals to adjust clutch size in preparation for high or low insect

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

94 If birds are detecting trace amounts of plant HIPVs and using those cues to make breeding
95 decisions, any modification of reproductive behavior is likely to be primed by reproductive
96 hormones (Ball and Balthazart, 2009, 2004; Wingfield et al., 1987). The relationship between
97 chemosignals and their influence on the reproductive system is well documented in mammals,
98 fish, and amphibians (Kawai et al., 2009; Nikonov et al., 2017; Petrulis, 2013), but less is known
99 in birds (Caro and Balthazart, 2010), let alone for olfactory signals other than bodily odors, like
100 plant or food odors for example. Work in several avian species suggest sex-steroids, like
101 testosterone, stimulate more persistent food searching behavior (Andrew, 1972; Daisley et al.,
102 2005; Németh et al., 2015), which is likely to be beneficial in early spring for acquiring resources
103 for successful reproduction (e.g., territory, mate, food) (Lack, 1968; Wingfield, 1984). Additionally,
104 olfactory sensitivity in rats appears to be upregulated by local sex-steroid production in the
105 olfactory epithelium (Horie et al., 2017; Lupo et al., 1986), though whether this is the case in birds
106 is unknown. Alternatively, it may be the exposure to some relevant odor signals that regulates
107 sex-steroid dynamics, similarly to the stimulatory effect of pheromones on sex-steroid hormones
108 (Miller and Maner, 2010). In any case, the relationship between behavior in response to an odor
109 associated with food and pre-breeding reproductive hormones has not been studied.

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

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4 115 (*Operophtera brumata*) and green oak tortrix (*Tortrix viridana*). We hypothesized that after
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6 116 training, birds would be able to differentiate between infested and uninfested oak trees by smell
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9 117 during the early stages of bud development, and that individuals that are more reproductively
10
11 118 ready would spend more time near the infestation.
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13 119

15 120 **Methods**

17 121 *Ethics*

19
20 122 Blue tits were trapped and maintained under licenses 2015615-147 issued by the Direction
21
22 123 Régionale de l'Environnement, de l'Aménagement et du Logement Languedoc-Rousillon; and 15-
23
24 124 XIX-116 issued by the Direction Départementale de la Protection des Populations de l'Hérault.
25
26 125 Experiments were run under the license 2017-XIX-075 from the Direction Départementale de la
27
28 126 Protection des Populations de l'Hérault, and approved by the Ethical committee N°036 with
29
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31 127 reference APAFIS#8608-2017012011062214 v4.
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33 128

35 129 *Housing of blue tits (Cyanistes caeruleus)*

37
38 130 This experiment took place at the Centre National de la Recherche Scientifique (CEFE-
39
40 131 CNRS) in Montpellier, France (43°37'56"N, 3°52'E) from 15 April to 2 May 2018. We used 42
41
42 132 hand-raised blue tits (females: $n = 20$, males: $n = 22$). Birds were moved indoors between 28
43
44 133 February and 14 March 2018 and were housed individually in cages (0.8 x 0.4 x 0.35m). Males
45
46 134 and females were housed in separate rooms and females were never observed laying eggs for
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48
49 135 the duration of the study. Individuals were provided water and food *ad libitum*. Food consisted in
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51 136 a cake made of sunflower seed grease, eggs, sugar, wheat flour and high-protein pellets (Show
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53 137 1+2 crumble, Versele-Laga, Deinze, Belgium), supplemented with minerals, vitamins, amino-
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55 138 acids and carotenoids (Nutribird A21, Versele-Laga, Deinze, Belgium; Nekton-S, Günter Enderle,
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58 139 Pforzheim, Germany; Yel-Lux, Versele-Laga, Deinze, Belgium). Mealworms were provided 3
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60 140 times per week. Windows in the rooms allowed natural light to filter in while light timers were set
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141 to turn on high-frequency TL tubes (Phillips TL5 HO 90 De Luxe 49W 965, Phillips, Eindhoven,
142 The Netherlands) 15 minutes post-sunrise and turn off 15 minutes prior to sunset, providing
143 natural increases and decreases in light intensity to occur (Fleissner and Fleissner, 2002). Sunrise
144 and sunset times were determined using the United States Naval Observatory data base
145 (www.usno.navy.mil/USNO). When moved, birds' feathers were checked to confirm they were in
146 good condition, so flight capability was not inhibited.

147
148 *Oak Trees*

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

161
162 *Caterpillars and bud infestation*

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

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

168 Once caterpillars began to hatch, oak trees with buds at an appropriate stage were either
169 infested with caterpillars (using a small paintbrush to gently place caterpillars on the bud) or left
170 as a control, 72 hrs prior to being used in a Y-maze (table 1). We chose 72 hrs post-infestation
171 because data collected from experimentally infested oak buds found HIPVs significantly increase
172 48 – 72 hrs post-infestation when compared to uninfested, control buds (Graham et al.
173 unpublished data). A single oak tree was placed at the end of each active arm (4 arms total, see
174 below), so only 4 trees were used at one time. To infest a tree, 20 caterpillars were placed across
175 5 branches with stage 4 (bud elongated, swollen and green) – 6 (bud bursting, leaf shoots can be
176 distinguished but are still intricated) buds (Du Merle and Mazet, 1983). *T. viridana* were given
177 priority as they are native to the area, however, when not enough were available, *O. brumata*
178 were used to keep the number of caterpillars on infested trees consistent. We recently found there
179 is no difference in the volatile compounds emitted by oak buds when they were infested by either
180 one of these two caterpillar species (Graham et al, unpublished results). A small wire cage was
181 placed around the infested bud(s) and covered by a mesh bag to ensure caterpillars remained on
182 the bud. The cage kept the bag from coming into contact with the bud(s), to prevent alarm
183 emission due to mechanical stress (Kesselmeier and Staudt, 1999). Mean individual caterpillar
184 mass for this study was 7.799 ± 0.990 mg. Control trees remained uninfested but had cages and
185 bags on 5 branches to control for any emissions the tree may have produced in response to the
186 presence of the cages. An individual bird was never exposed to the same pair of trees more than
187 once and trees used on the day of the experiment had never been used during any of the training
188 (see below).

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190 *Y-Mazes*

191 Two large outdoor y-mazes (2.5x2m arms, figure S1) were set up facing southeast. Two
192 arms were designated as active arms (one control and one treatment arm) and a neutral arm
193 containing no stimuli where the bird entered the maze. Each active arm of the maze had five food
194 dishes and one perch, and one additional perch was placed in the neutral arm of the maze. Food
195 dishes were the same as those used to feed birds when housed in outdoor aviaries, so they were
196 already familiar to the birds. A small box was mounted on the door in the neutral arm to introduce
197 birds into the maze. Mazes were visually isolated from each other by a hedge. A wind break was
198 set up around the maze that additionally reduced visibility beyond the arms of the maze. Stands
199 placed at the far end of the two active arms of the maze (outside the maze, against the mesh)
200 contained the trees. Those stands were covered with a white fabric on three sides (those visible
201 from the inside of the maze) to block any changes in color infested trees may display compared
202 to uninfested trees (Amo et al., 2013). These enclosures also had small fans (92x92x25mm,
203 36m³/h, EbmPapst, Chelmsford, UK) to blow air from the trees toward the center of the maze
204 (placed at 80 cm, 130 cm, and 180 cm from the ground). Behavioral observations always started
205 as soon as a bird left the entrance box. Habituation, training, and testing began as early as 08:40
206 and were completed by 16:45 each day (For full experimental design, see table 1). A water bath
207 was also available in each arm of the maze, so the birds always had access to fresh water.

208
209 *Habituation*

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

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

219

220 *Training*

221 Over the course of 5 consecutive days, individual birds were released into the mazes for
222 30 min with 1 food item placed in each dish only in the side of the maze containing an infested
223 tree (Amo et al., 2013). Empty food dishes remained in the side of the maze with the control tree.
224 The type of food reward was changed each day to keep birds from learning the scent of the food
225 reward instead of the oak emissions (see table 1). The quantity of the food reward was recorded
226 so we could keep track of whether birds were consuming the reward, and therefore whether they
227 had a chance to associate the presence of oak emission with food. Additionally, the birds
228 experienced the infested tree approximately equally on each side of the maze over the successive
229 training sessions, so they would not become familiar with finding food on one side of the maze.
230 Due to an odd number of trainings, half of the birds experienced the infested tree and food reward
231 three times on the right side of the maze, while the other half of the birds experienced the infested
232 tree and food reward three times on the left side of the maze. The side the infested tree was on
233 was alternated between the left and right side daily for every individual. Order of individuals was
234 randomly assigned, but one male and one female were always being tested in parallel in the two
235 mazes with the exception of 2 males being tested at the same time during the second round of
236 the experiment.

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

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242 22.7% in the second round. In addition, inclement weather during the second round of trainings
243 resulted in 12 birds only undergoing 4 rounds of training as opposed to 5 rounds.

244

245 *Trials*

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

258

259 *Behavioral Observations*

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

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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).

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270 *Blood Sampling*

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

275

276 *Testosterone Assay*

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

290 We validated the assay in blue tits following the methods of (Caro et al., 2019). Briefly,
291 pooled plasma samples were spiked with exogenous testosterone and serially diluted. The assay

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292 was determined to work correctly as percentages of tracer bound (B/B0) from the serial dilutions
293 were parallel to the standard curve (see supplementary material, figure S2).

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295 *Estradiol Assay*

296 Plasma 17 β -estradiol (E₂) was measured using a commercially available double-antibody
297 ¹²⁵I-E₂ radioimmunoassay (DSL-4800, Ultra-sensitive Estradiol RIA, Beckman Coulter, Brea, CA,
298 USA) that was modified by dilution of the tracer and the antibody to increase the sensitivity of the
299 assay, following (Charlier et al., 2010) and (Caro et al., 2019). Like for testosterone, steroids were
300 first extracted from 35 μ L of plasma using solid phase extraction with C18 columns (100 mg C18
301 material, Sep-Pak Vac 1cc, Waters, Milford, MA, USA), dried under nitrogen gas at 40°C, and
302 reconstituted overnight with PBSg (PBS with 0.1% gelatin) containing 0.7% ethanol (Caro et al.,
303 2019). Concentration was adjusted for samples that did not have 35 μ L of plasma available ($n =$
304 11 of 20). Resuspended samples were then assayed in duplicate (300 μ l per tube) and assay
305 tubes were counted on a gamma counter (Automatic Gamma Counter, Perkin Elmer, Waltham,
306 MA, USA). Concentrations of E₂ were obtained using a linear regression with the log-transformed
307 concentrations of the standards provided in the assay kit. All samples were run in a single assay,
308 and the intra-assay coefficient of variation, as estimated by assaying one high and one low
309 concentration E₂ standard in duplicate, was 5.9%. Assay sensitivity was 0.66 pg/ml. It was defined
310 as the highest point on the standard curve whose standard deviation did not overlap that of the
311 blank standard (Wingfield and Farner, 1975). No sample was found to be below the detection
312 limit. The assay procedure has previously been validated for blue and great tits (Caro et al., 2019).

313

314 *Statistical Analyses*

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

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318 the maze (independently of which side the infested tree was on). Our results showed that birds
319 were not lateralized ($p = 0.16$) and thus we excluded this variable from future analyses. We
320 additionally ran preliminary analyses on behavior during training sessions. Briefly, we found that
321 (i) birds did not become more successful in locating the food reward as the number of trainings
322 increased ($z = -0.09$, $p = 0.93$, table S1); that (ii) training success was independent of plasma
323 testosterone ($z = -0.96$, $p = 0.34$) and estradiol ($z = 0.14$, $p = 0.89$); and (iii) the ability to
324 successfully locate the food reward was significantly repeatable within individuals at 0.49 (C/μ :
325 0.21, 0.66, $p < 0.001$, see supplementary materials for more details on these analyses).

326 After the preliminary analyses, we first ran a simple binomial test that compared the
327 number of birds that chose to visit the infested side when they entered the maze, with the number
328 of birds that first visited the control side. We then used a logistic regression to test the effect of
329 several explanatory variables on first side chosen. We tested whether the first side chosen was
330 influenced by the maze in which an individual was tested (Maze 1 or 2), the sex of the birds (M
331 or F), the period the birds were tested (group 1 = habituation, training, and testing from 15 to 21
332 April, group 2 = habituation, training, and testing from 26 April to 2 May), the order of testing within
333 a group (from 1 to 11, as a continuous variable), and the number of times no food reward was
334 consumed during training (from 0 to 5, continuous variable, listed as 'Reward Left' in tables 2 and
335 3). Statistical output for these variables is reported in table 2 and significant values are reported
336 in text. Effect sizes were calculated using package effectsize (Ben-Shachar et al., 2020). We used
337 standardized regression coefficients (β), which are reported in table 2 along with 95% confidence
338 intervals.

339 To determine if time and activity level in each side of the maze differed between infested
340 and control, paired t-tests (one for time, and one for activity) were initially used. To test additional
341 variables that could potentially affect time and activity level in each side of the maze, two linear
342 models were then run (one for each response variable, like above). The response variable for
343 time was calculated as number of seconds spent in the infested arm of the maze minus the

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4 344 number of seconds spent in the control arm of the maze. More positive values indicate more time
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6 345 spent in the infested arm, while more negative values indicate more time spent in the control arm.
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8 346 Similarly, the response variable for activity was calculated as the number of movements made in
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10 347 the infested arm minus the number of movements made in the control arm. More positive values
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12 348 indicate higher activity levels in the infested arm and more negative values indicate higher activity
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14 349 levels in the control arm. Explanatory variables included in both models were maze, sex, test
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16 350 group, testing order, and number of times the food reward was not consumed during training.
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20 351 In a final set of analyses, we tested whether plasma steroid levels played an additional
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22 352 role on the time and the activity levels of the birds tested. Because we measured a different
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24 353 hormone in each sex, we ran separate models for males and females. Like in the models above,
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26 354 maze, test group, test order, and remaining food reward were also added, together with
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28 355 testosterone (in males) and estradiol (in females) concentrations (both ln transformed to achieve
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30 356 normality). Only significant values are reported in text for variables other than hormones, but all
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32 357 values are reported in table 3. A single male had undetectable testosterone levels and was
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34 358 subsequently excluded from final hormone analyses. However, assigning the lowest detectable
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36 359 level of the kit (5.67 pg/mL) and including the male in statistical models did not change the results.
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38 360 Graphs were made using package ggplot2 (Wickham, 2016). We set $\alpha = 0.05$ and report mean \pm
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40 361 SEM.
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45 363 Results

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47 364 Individuals were more likely to visit the side of the maze with the infested tree first (29 of
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49 365 42) compared to the control tree ($p = 0.02$). On average, birds spent 250.70 s longer in the side
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51 366 of the maze with the infested tree compared to the side with the control tree ($t_{41} = 2.13$, $p = 0.04$,
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53 367 figure 1). Birds were also nearly twice as active in the infested side of the maze compared to the
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55 368 control side ($t_{41} = 3.31$, $p = 0.002$, figure 1). When additional variables are introduced into a more
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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).

371 Male-restricted analyses showed that birds with higher levels of testosterone spent more
372 time in the infested arm of the maze ($F_{1,15} = 8.01$, $p = 0.01$, $R^2 = 0.44$, figure 2A). Testosterone
373 was however not related to activity levels ($F_{1,15} = 3.35$, $p = 0.09$, $R^2 = 0.43$, table 3). Mean
374 testosterone levels were significantly higher in maze 2 males (386.49 ± 122.42 pg/mL) compared
375 to maze 1 males (259.26 ± 33.2 pg/mL; $F_{1,15} = 9.352$, $p = 0.008$), but this pattern was primarily
376 driven by two males in maze 2 with the highest testosterone levels of all 21 individuals (maze 2
377 mean with highest two males removed: 214.25 ± 32.60 pg/mL). In pre-breeding females, estradiol
378 levels showed no relationship with time ($F_{1,14} = 0.01$, $p = 0.94$, figure 2B) or activity ($F_{1,14} = 2.56$,
379 $p = 0.13$). Other variables were also all non-significant (table 3).

380

381 Discussion

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

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

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394 While our birds were originally naïve, having been in captivity from 10d of age, they were
395 trained to associate the presence of food in small dishes with the smell of one infested tree. Our
396 results clearly indicate that the detection of HIPVs from early bud infestation can be a learned
397 behavior. While one study found that naïve great tits do not discriminate between infested and
398 uninfested apple trees, suggesting use of HIPVs from fully developed leaves for locating food is
399 not innate (Amo et al., 2016), new findings in blue tits show that detection of HIPVs from
400 developing buds is innate in this species. When presented with an artificial odor bouquet
401 mimicking the HIPVs released from developing buds, naïve blue tits are more likely to choose to
402 first visit vials diffusing HIPVs as opposed to a control (Delaitre et al. unpublished data). Similarly,
403 blue petrel fledglings (*Halobaena caerulea*) appear to show an innate preference for dimethyl
404 sulfide, an olfactory cue important for locating patchily distributed prey in the ocean (Bonadonna
405 et al., 2006; Cunningham et al., 2003). Further studies are needed to determine which HIPVs are
406 innately detected, which ones need to be learned, and which ones may influence reproduction in
407 blue tits.

408

409 *Males with higher testosterone levels spent more time in the infested arm*

410 We found that spending more time in the infested arm of the maze compared to the control
411 arm was correlated with higher testosterone levels in males. Although plasma testosterone and
412 time spent in the infested arm were correlated, it is unclear which of the two variables may affect
413 the other. One potential explanation for this relationship is that male blue tits with higher levels of
414 testosterone are more sensitive to cues that could indicate territory that will have higher food
415 abundance. European starlings (*Sturnus vulgaris*) for example, only exhibit an olfactory response
416 to the odors of a preferred nesting material (De Groof et al., 2010) and a conditioned odor stimulus
417 during the breeding season (Clark and Smeraski, 1990). While artificially increasing plasma
418 testosterone levels during the non-breeding season does not seem to change olfactory sensitivity
419 to preferred nesting material in European starlings (De Groof et al., 2010) or food odor cues in

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4 420 female goldfish (*Carassius auratus*) (Ghosal and Sorensen, 2016), the lack of receptors to receive
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6 421 the increased signal might result in non-activation of seasonal changes in olfactory sensitivity.
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8 422 Olfactory processing regions in many other vertebrate species contain gonadotropin releasing
9
10 423 hormone (GnRH) and sex steroid receptors (Kawai et al., 2009). Steroid receptor density appears
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12 424 to change with reproductive status in fish (Maruska and Fernald, 2010), while olfactory sensitivity
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14 425 and preference appear to be highest during reproduction in rats (Moffatt, 2003). As the breeding
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16 426 season approaches, males may upregulate receptor density in olfactory processing regions so
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18 427 higher levels of testosterone have a greater influence on olfactory sensitivity during the breeding
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20 428 season. It should also be noted that work in mammals show that exposure to steroid hormones
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22 429 will modulate the integration of chemosensory signals, including olfactory cues. This was
23
24 430 particularly well studied in the context of hormonal-dependent onset of maternal behavior in
25
26 431 mammals. For example, females begin to show a preference for bedding soiled by pups over
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28 432 clean bedding, around the peripartum period (Kinsley and Bridges, 1990) and this is known to be
29
30 433 associated with a general rewiring of the maternal brain (Keller et al., 2019; Lévy and Keller,
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32 434 2009). Steroid-dependent changes in the neural pathways underlying chemosensory integration
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34 435 was also highlighted in copulatory context (Fiber and Swann, 1996; Paredes et al., 1998).
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36 436 Additional work should be performed to define whether sensitivity to HIPVs is modulated at the
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38 437 level of the periphery or the central nervous system, or both.
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44 438 An alternative explanation for the correlation between testosterone and time spent in the
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46 439 infested arm of the maze is the rapid response of testosterone production to certain stimuli. For
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48 440 example, males of some species can quickly upregulate hormone production in response to male-
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50 441 male competition (Archer, 2006; Mcglothlin et al., 2008; Wingfield and Wada, 1989), presence of
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52 442 a female (Coquelin and Bronson, 1979; Ronay and Hippel, 2010; Stacey, 2003), and even after
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54 443 exposure to a nest box (Gwinner et al., 2002). Male song sparrows showed a significant increase
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56 444 from baseline testosterone levels just 10 min after the start of a simulated territorial intrusion
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58 445 (Wingfield and Wada, 1989) and the introduction of green vegetation to captive-housed canaries
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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).

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

460 Another consideration in the role of testosterone in detecting HIPVs is the subsequent
461 reduction in testosterone levels during chick rearing (Hegner and Wingfield, 1987; Hunt et al.,
462 1999), which seems counterintuitive when the ability to locate prey during this period is critical for
463 reproductive success (Visser and Gienapp, 2019). However, fully developed vegetation produces
464 HIPVs in much higher quantities than the early-spring buds we studied here (Brilli et al., 2009;
465 Röse and Tumlinson, 2004), thus, increased olfactory sensitivity may not need to be maintained
466 to successfully forage once HIPVs are emitted in higher quantities in response to herbivory by
467 large caterpillars.

468
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

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

490 Testosterone on the other hand, may also be important in detecting environmental cues
491 in females. Interestingly, androgen levels in female European starlings appear to be upregulated
492 prior to rapid yolk development (Williams et al., 2004). For example, testosterone is highest during
493 territory establishment and pre-laying stages in female tree swallows (*Tachycineta bicolor*); which
494 corresponds with timing of female competition for resources and mates (George and Rosvall,
495 2018). Testosterone patterns in female blue tits could also upregulate in response to HIPVs and/or
496 influence olfactory sensitivity like in males, but future studies examining the effects of HIPV

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

499

500 **Conclusions**

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

512

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747 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.

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

Table 2. Analyses of the variables that influence the first side chosen, the time spent, and the level of activity close to one caterpillar-infested oak in blue tits ($n = 42$).

Response Variable	Explanatory Variable	B [95% CI]	Estimate	S.E.	Test Statistic	p-value
First Side Chosen	Maze	0.56 [-0.81, 2.03]	1.11	1.40	Z = 0.80	0.426
	Sex	-1.32 [-3.02, 0.15]	-1.31	0.79	Z = -1.66	0.096
	Test Group	0.09 [-0.70, 0.87]	0.18	0.77	Z = 0.23	0.820
Time	Order	-0.73 [-2.19, 0.63]	-0.12	0.12	Z = -1.03	0.302
	Reward Left	-0.45 [-1.29, 0.32]	-0.25	0.23	Z = -1.11	0.266
	Maze	-0.56 [-1.17, 0.05]	-842.70	454.72	F _{1,36} = 2.93	0.096
Activity	Sex	-0.11 [-0.75, 0.54]	-81.69	242.19	F _{1,36} = 0.17	0.682
	Test Group	-0.11 [-0.44, 0.23]	-161.72	248.89	F _{1,36} = 0.48	0.493
	Order	0.34 [-0.28, 0.95]	43.25	38.90	F _{1,36} = 1.26	0.270
Reward Left	Reward Left	0.05 [-0.29, 0.40]	22.36	74.43	F _{1,36} = 0.09	0.766
	Maze	-0.26 [-0.58, 0.07]	-30.54	19.11	F _{1,36} = 2.53	0.120
	Sex	0.10 [-0.55, 0.76]	6.10	19.56	F _{1,36} = 0.10	0.748
Test Group	Test Group	0.17 [-0.17, 0.51]	19.98	20.10	F _{1,36} = 0.96	0.333
	Order	-0.04 [-0.36, 0.29]	-0.70	3.14	F _{1,36} = 0.05	0.828
	Reward Left	0.03 [-0.32, 0.38]	0.89	6.01	F _{1,36} = 0.02	0.883

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

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Table 3: Analyses of the variables that influence the time spent and the level of activity close to one caterpillar-infested oak in male ($n = 21$) and female ($n = 20$) blue tits.

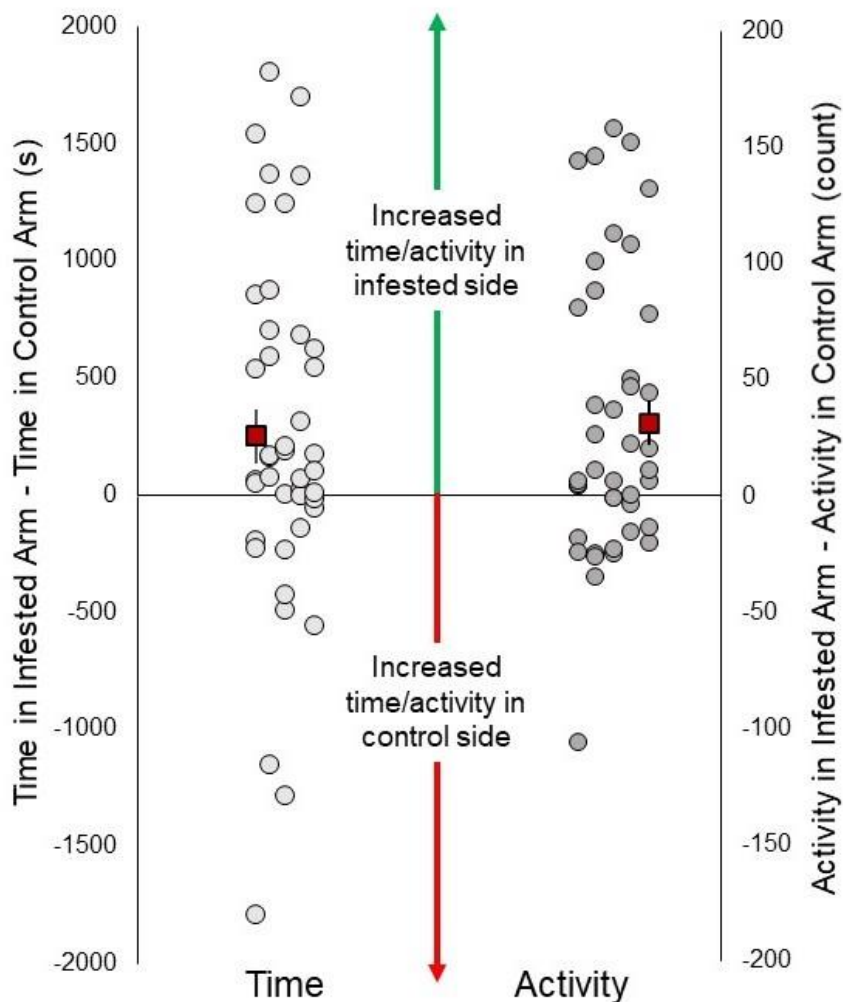
Response Variable	Explanatory Variable	B [95% CI]	Estimate	S.E.	Test Statistic	p-value	
Males	Testosterone (ln)	0.54 [0.13, 0.95]	567.19	203.46	$F_{1,15} = 8.01$	0.013	
		-0.57 [-0.94, -0.20]	-853.77	261.06	$F_{1,15} = 9.35$	0.008	
	Time	Maze	0.19 [-0.24, 0.62]	288.00	309.04	$F_{1,15} = 0.63$	0.440
		Test Group	0.28 [-0.10, 0.67]	70.41	45.16	$F_{1,15} = 2.60$	0.127
		Order	0.10 [-0.32, 0.52]	48.90	93.63	$F_{1,15} = 0.27$	0.609
	Reward Left	Testosterone (ln)	0.53 [0.12, 0.94]	39.23	14.23	$F_{1,15} = 3.35$	0.087
		Maze	-0.65 [-1.02, -0.28]	-68.43	18.26	$F_{1,15} = 14.12$	0.002
		Test Group	0.32 [-0.11, 0.76]	34.07	21.62	$F_{1,15} = 2.78$	0.116
	Activity	Order	0.11 [-0.49, 0.28]	-1.87	3.16	$F_{1,15} = 0.35$	0.563
		Reward Left	-0.02 [-0.44, 0.41]	-0.56	6.55	$F_{1,15} < 0.01$	0.932
		Estradiol (ln)	-0.07 [-0.65, 0.51]	-165.44	643.38	$F_{1,14} = 0.01$	0.940
	Females	Maze	-0.03 [-0.60, 0.53]	-47.78	394.39	$F_{1,14} = 0.01$	0.928
-0.18 [-0.78, 0.42]			-270.04	419.53	$F_{1,14} = 0.56$	0.467	
Time		Test Group	-0.04 [-0.62, 0.54]	-10.16	66.03	$F_{1,14} = 0.01$	0.930
		Order	0.08 [-0.52, 0.68]	32.89	118.07	$F_{1,14} = 0.08$	0.785
		Reward Left	-0.37 [-0.91, 0.17]	-78.11	53.53	$F_{1,14} = 2.56$	0.132
Estradiol (ln)		Maze	0.05 [-0.47, 0.58]	7.22	32.81	$F_{1,14} = 0.06$	0.807
		Test Group	0.12 [-0.43, 0.68]	16.66	34.91	$F_{1,14} = 0.21$	0.657
		Order	-0.08 [-0.62, 0.46]	-1.77	5.49	$F_{1,14} = 0.09$	0.766
Activity		Reward Left	0.03 [-0.53, 0.59]	1.14	9.82	$F_{1,14} = 0.01$	0.909

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

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770 **Figures**

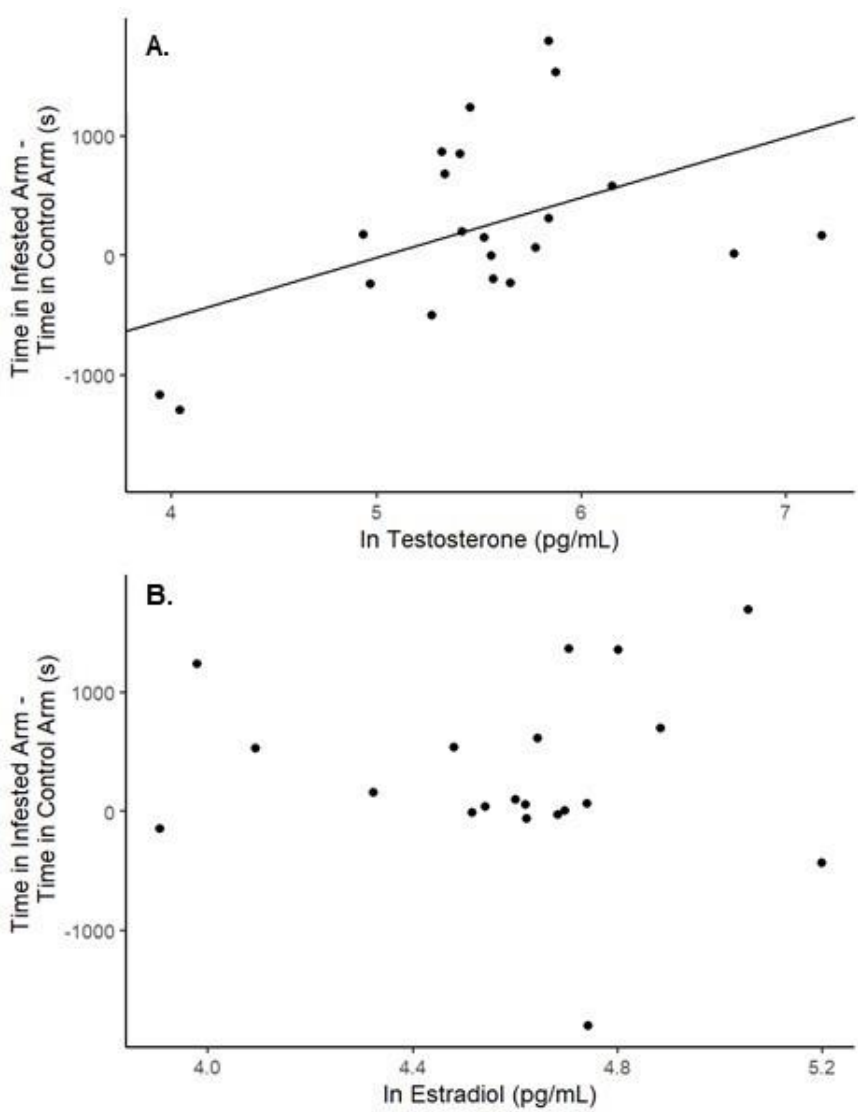
771 **Figure 1.** Blue tits detect herbivore-induced plant volatiles (HIPV) emitted by the buds of one
772 **single, small oak.** On average, individuals spent 250.7 seconds longer in the infested arm of the
773 maze than the control arm and were significantly more active in the infested arm of the maze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



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



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783 **Supplementary Material**

784 **Behavioral analyses during training**

785 We ran preliminary analyses on bird behavior related with successfully locating food
786 during training. To test whether individuals became more successful at finding the food reward
787 during trainings using a generalized linear mixed-model with binomial distribution in package lme4
788 (Bates et al., 2015). The response variable was a repeated measure coded as 1 (consumed food
789 reward) or 0 (did not consume food reward) for each of the 5 training sessions. Our fixed factor
790 of interest in the model was the training session (1 – 5). Individuals were tested in two mazes,
791 during two different time periods, thus, to determine if these variables affected the results, the
792 maze an individual was tested in (Maze 1 or 2) and the test group (group 1 = habituation, training,
793 and testing from 15 to 21 April, group 2 = habituation, training, and testing from 26 April to 2 May)
794 were both included in the model. Testing order is included as a measure of when an individual
795 was tested during the day. Those listed as 1 were the first to be tested on that day and those
796 listed as 10 or 11 were last to be tested that day. Sex (M or F) was also included. Statistical output
797 for these variables is reported in table S1 and significant values are reported in the main text.
798 Given the evidence that testosterone can influence olfactory discrimination in rats and mice
799 (Kunkhyen et al., 2018), we also decided to run 2 additional models comparing testosterone in
800 males and estradiol in females to training success. Furthermore, we calculated the repeatability
801 (logit link-scale approximation) of consuming the food reward across the five trainings using the
802 package rptR (Stoffel et al., 2017).

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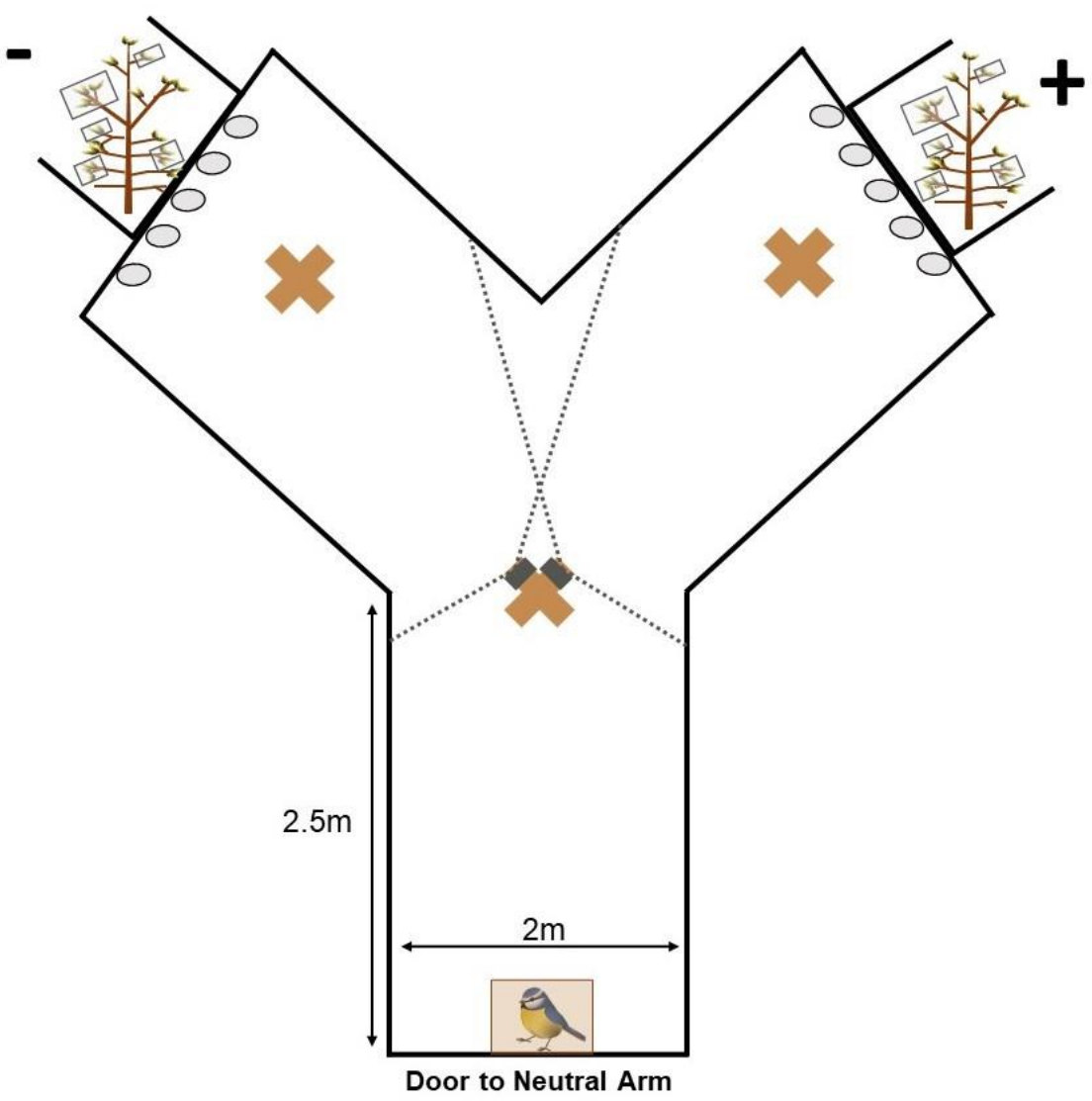
803 **Table S1.** Analyses of the variables that influence training success. All individuals were included in the initial analysis while only males and females were included in the testosterone and estradiol analyses, respectively.

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Training Success	Explanatory Variable	B [95% CI]	Estimate	S.E.	Test Statistic	p-value	
All Individuals (n = 42)	Trial Number	-0.02 [-0.40, 0.37]	-0.01	0.14	Z = -0.09	0.931	
	Maze	-0.16 [-0.88, 0.56]	-0.32	0.73	Z = -0.43	0.666	
	Sex	1.15 [-0.31, 2.61]	1.15	0.74	Z = 1.55	0.121	
	Test Group	0.68 [-0.06, 1.42]	1.35	0.75	Z = 1.80	0.072	
	Order	-0.06 [-0.76, 0.65]	-0.02	0.12	Z = -0.15	0.879	
	Males (n = 21)	Testosterone (ln)	-0.55 [-1.66, 0.57]	-0.74	0.77	Z = -0.96	0.336
	Females (n = 20)	Estradiol (ln)	0.07 [-0.92, 1.06]	0.23	1.59	Z = 0.14	0.887
	Bolded values were statistically significant ($p < 0.05$). Effect sizes (β) are standardized beta coefficients followed by the 95% confidence intervals in brackets.						
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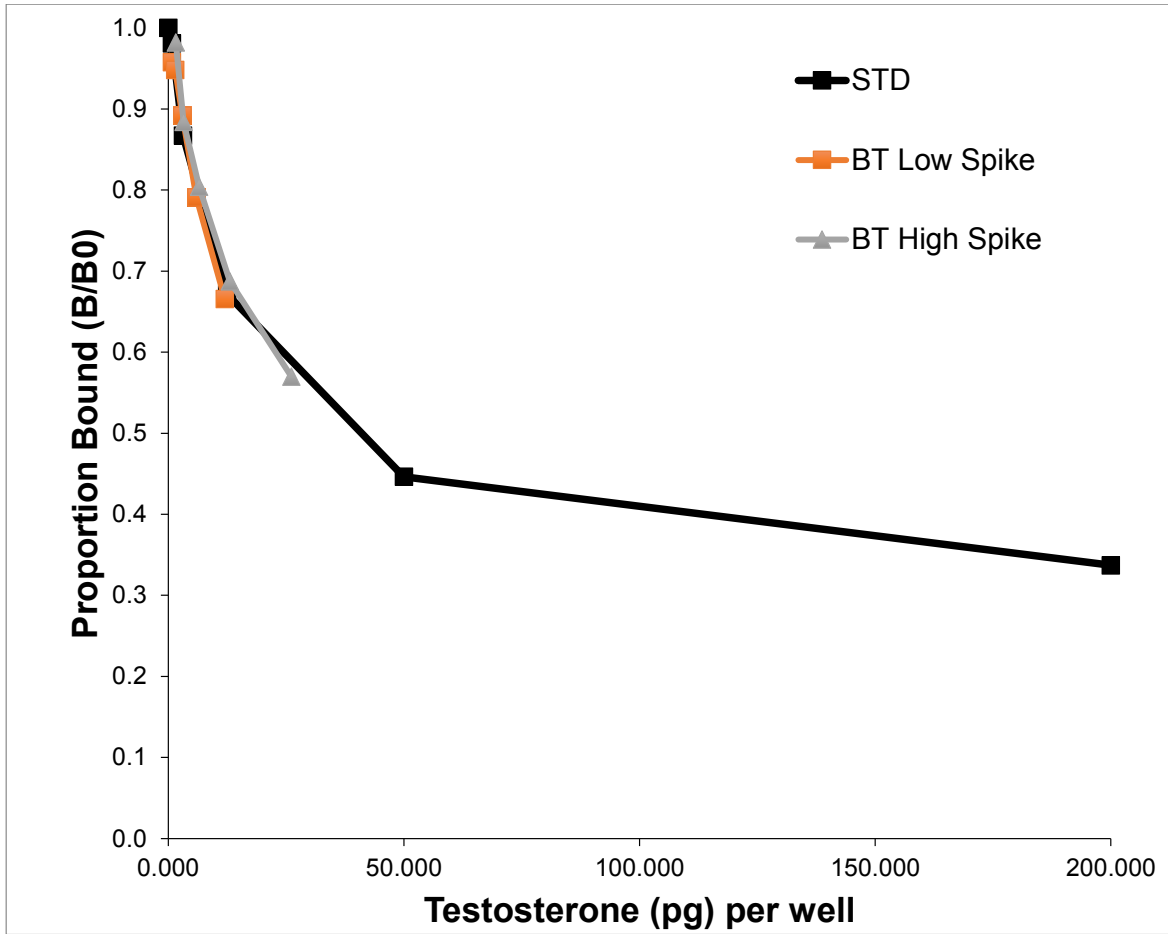
807 **Figure S1.** Diagram of the Y-mazes used in the behavioral experiment. To test the ability of
808 insectivorous songbirds to detect caterpillar infestation of oak buds by smell, a Y-maze was set
809 up so birds entered from a small box in the neutral arm. Two GoPro cameras were mounted on
810 a center perch to film activity in each of the active arms. An infested oak tree was on one side of
811 the maze and an uninfested tree on the other. Both were visually isolated from the bird to
812 obscure any visual changes resulting from infestation, but small fans were in place to help direct
813 any odors toward the center of the maze. Each trial lasted 30 minutes.



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



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