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Environmental and social cues used by tits for reproductive timing and investment

Ségolène Delaitre

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THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

En Sciences de l'évolution et de la Biodiversité

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Filière EERGP – Ecologie, Evolution, Ressources Génétiques et Paléobiologie

Unité de recherche CEFE - Centre d'Ecologie Fonctionnelle et Evolutive – UMR5175

**Environmental and social cues used by tits for
reproductive timing and investment**

**Indices environnementaux et sociaux utilisés par les
mésanges pour décider de leur période et leur
investissement dans la reproduction**

Présentée par Ségolène DELAITRE
Le 5 Décembre 2023

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UNIVERSITÉ
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List of publications and communications

Articles

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- Caro S. P., Delaitre S., Buatois B., Bonadonna F., Graham J. L. (2023). The influence of plant odours on sexual readiness in an insectivorous songbird. *Journal of Experimental Biology*, 226(6), jeb245313.
- Delaitre S., van Oers K., Visser M. E., Caro S. P. (2023). Female great tits (*Parus major*) reproduce earlier when paired with a male they prefer. *Ethology*.
- Delaitre S., Doutrelant C., Caro S. P. (2023). Whoever their partner, female blue tits breed the same. *Behavioral Ecology*, arad082.
- Delaitre S., Visser M. E., van Oers K., Caro S. P. (2024) Odours of caterpillar-infested trees increase testosterone concentrations in male great tits. *Hormones and Behavior*, 160, 105491.

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Communications

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Introduction



I. Adapting to a changing environment

1. Natural selection ready for action

As stated by Darwin in *The Origin of Species* (1859), “Natural Selection [...] is a power incessantly ready for action, and is immeasurably superior to man's feeble efforts, as the works of Nature are to those of Art”. When he wrote these lines, Darwin was describing the process experienced by every organism on the planet that leads to the formation and change of biological systems in response to their environment: evolution. A species' inability to adapt to changes in the environment is often synonymous with a loss of competitive advantage and, ultimately, extinction. Natural selection can lead to micro-evolution, i.e. the tendency of beneficial heritable traits to increase in frequency in populations to make individuals better able to survive and reproduce ¹. The average phenotype of the population is therefore the result of the greatest contribution in each generation of individuals with the traits best adapted to their environment ^{2,3}. The success of these individuals lies in their ability to regulate their physiological state and adapt their behaviour in response to variations in the environment to be able to grow and reproduce. This mechanism, known as phenotypic plasticity, is a key process in evolutionary biology ⁴.

2. Plasticity in the seasonality of life cycles

Naturalists have long been interested in recording the annual occurrence of the first flowers, migrating birds, and leafing trees in spring, either for agricultural purposes or simply to determine the transition from one season to the next ^{5,6}. Phenology, the study of the timing of recurring seasonal biological events ⁷ is a scientific field intensely studied ⁸. As a result of the recent and rapid environmental changes, many organisms have shifted the timing of some of their life history traits ⁹⁻¹¹. In a recent study, the phenological advancement of spring events was estimated at four days per decade across the northern hemisphere ¹². In terrestrial ecosystems, primary consumers are often more able to bring forward their phenological events than species at higher trophic levels ¹³⁻¹⁵. For example, terrestrial vertebrates lag behind terrestrial invertebrates in shifting their phenology (4.1 days of advancement per decade for invertebrates, 2.6 days per decade for vertebrates) ¹⁵.

To maximize their fitness (their ability to survive and reproduce), organisms must adapt to seasonal changes in light, temperature, rainfall or humidity. Seasonality thus represents an important selective factor that results in adaptations of life-history traits of individuals. They have to modify their biological processes at the physiological and behavioural levels to adapt to annual variations in the environment. Migration in birds, hibernation in mammals, dormancy

in plants and diapause in insects are all examples of biological processes that organisms have developed to adapt to the seasons. Seasonal adaptations are not only crucial for surviving a certain period of the year, but they are usually required for the general synchronisation of the whole life cycle to the seasonal availability of resources. In the case of reproduction, which is the stage of the life cycle that I have focused on in this thesis, the optimal timing of breeding of seasonal breeders is generally synchronised with a period of high food availability. This way, individuals ensure that the period of maximum energy requirement for their offspring coincides with the time when food is most abundant ¹⁶.

As environmental conditions vary from one year to the next, the timing of the food peak also varies from year to year. The plasticity of a trait like timing of breeding enables organisms to adapt rapidly to changes in their environment, and thus maintain the synchrony of their life cycle with seasonality. A lack of adaptation to the environment could result in a phenological mismatch between trophic levels, i.e. a desynchronisation between the energy demand of the consumers and food availability, which can have negative consequences both at the individual and at the population levels ^{17–20} (**figure 1**). In a French population of roe deer (*Capreolus capreolus*) for example, females failed to track environmental changes over a 27-year study period ¹⁸, which resulted in a mismatch between vegetation development and the birthdate of fawns. Individual survival consequently decreased, and so did the mean population fitness ¹⁸.

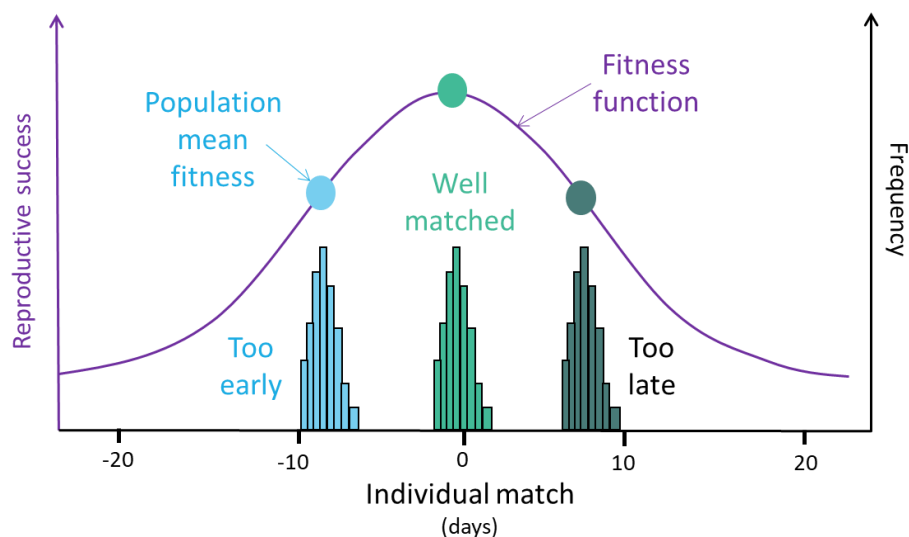


Figure 1: Phenological synchrony between trophic levels and its effect on population fitness. The purple line depicts a theoretical fitness curve in relation to individual matching between offspring energy demand and food availability. The frequency distributions of individual phenology in a population under three different scenarios of matching are depicted (blue, too early reproduction compared to the food peak; green, well matched; grey, too late). The dots on the fitness curve indicate the population mean fitness for each scenario. On average, populations too early or too late have a reduced mean reproductive success. Adapted from (Visser and Gienapp, 2019) ²¹.

To ensure a match across trophic levels during reproduction, i.e. between the consumer needs and the food availability, the future breeding individuals must undertake considerable physiological and behavioural preparation to develop their gonads, find a partner and, in some cases, build a nest, lay eggs and incubate them well before the actual food peak occurs. In some bird species, these events can take up to 6 to 8 weeks to be completed and must therefore be initiated well in advance of any seasonal increase in food availability²². In mammals with gestation periods of several months, the timing of parturition is often a function of conception date and adjustment of gestation length to environmental conditions^{23–25}. Organisms consequently have to adapt their reproductive physiology and engage in reproductive behaviours at the right time to be able to synchronise the period of maximal food requirement of offspring with the timing of the food peak. To accomplish this, they need to use proximate factors, also called cues, to predict and anticipate their prey phenology, and thus time their reproductive events accordingly^{22,26–31}. The factors used can be numerous and, in this thesis, I focus on two of them, of different kinds: a social factor linked to preference for the partner, and an environmental cue linked to vegetation phenology.

II. Factors influencing reproductive phenology and investment

1. The interplay between social and seasonal behaviours

a) The importance of coordination between partners in a breeding pair

Although the seasonal behaviours of organisms are ultimately linked to environmental factors that I will develop further below, all reproductive behaviours first take place in a social context, and interactions with conspecifics are intimately linked to seasonal activities³². In monogamous pairs, particularly common in birds, but also found in some species of fish and primates, a male and a female pair for the breeding season^{33–35}. This mating system occurs in particular when the survival of offspring depends on a concerted effort by both parents^{34,35}. For example, in the case of emperor penguins (*Aptenodytes forsteri*), which breed in the middle of the Antarctic winter, the chick has no chance of survival without the cooperation of both parents. While one parent is away feeding at sea, the other incubates the egg alone for many weeks. When the first one returns, the roles are reversed^{36,37}. Such examples of coordination among pair members in feeding the offspring, or incubating the eggs are numerous in birds^{38,39}, and it has been shown that a good coordination may increase reproductive success^{40–42}. In zebra finches (*Taeniopygia guttata*) for example, nest visits to feed the chicks are highly synchronised, with parents visiting the nest together on 78% of the visits, and nest visit synchrony is positively correlated with the number of offspring in the nest a few days before

fledging⁴¹. This synchronised behaviour could consequently decrease nest predation rate, or improve food partitioning among nestlings⁴¹.

Coordination between partners is not only important at an advanced stage of reproduction, to raise the offspring for example, but also at the start of the breeding season. Individuals forming a monogamous couple express many behaviours that are exclusively directed to their partner. These behaviours help to strengthen the bonds between individuals in the pair⁴³ and are particularly emphasised during courtship. In some species, these displays require great coordination between the two partners⁴⁴. For example, blue-capped cordon-bleus (*Uraeginthus cyanocephalus*) are characterised by courtship displays shared by both sexes. Males and females sing songs and sometimes they add a unique tap dance^{45,46}. Such displays may be used to synchronise and coordinate reproductive physiology and behaviour, for example by communicating the reproductive status of each individual^{44,47}. Mutual stimulation between partners may then accelerate breeding preparation and allow temporal flexibility in timing of breeding⁴⁸.

Although both males and females can stimulate the reproductive system of their partner reviewed in 27,49,50, the importance of social factors on reproduction can differ between sexes. Males usually achieve gonadal development and are ready to reproduce even when kept individually, whereas females often require male stimulation to complete their gonadal activation^{51–53}. A majority of studies find that the presence of a male partner stimulates follicle maturation in females^{54–58}. Male sexual traits and social behaviours not only modulate female gonadal development, but also their hormone secretions, reproductive decisions and investment^{54,59–63}. For example, male canary songs stimulate both female ovarian development, nest-building activity and egg laying (**figure 2**)^{61,64,65}.

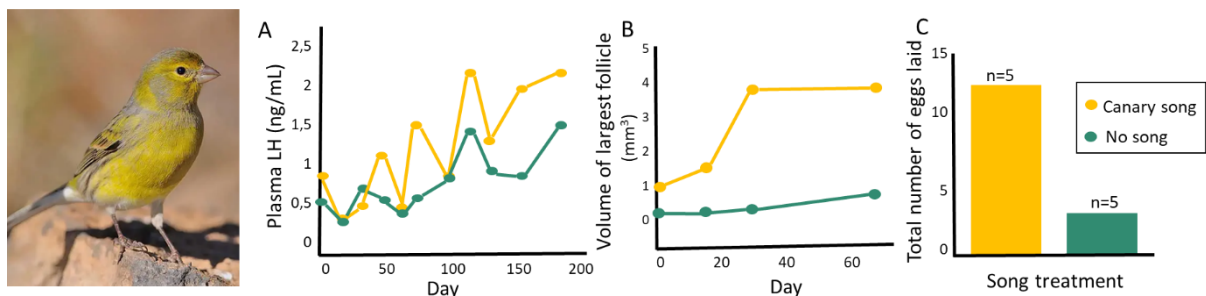


Figure 2: Reproductive responses of female canaries exposed to male song playbacks.

(A) Plasma LH concentrations (see **box 1**) were more elevated in female canaries exposed to male song playbacks, (B) they developed larger follicles and (C) laid more eggs. Dots correspond to mean. © Martin Pelanek. Adapted from (Bentley et al., 2000)⁶⁴.

b) Modulation of reproduction according to the partner preference

The choice of a mate and the amount of time the partners spend together affect the capacity of the pair to coordinate their behaviours and improve reproductive success^{66,67}. Consequently, not only the presence of the male, but also the degree of female preference for its partner can influence her reproduction^{68–70}. Several theories have been developed around this effect of male quality on reproduction. The differential allocation theory^{71,72} suggests that perceived male quality and attractiveness modulate female reproductive decisions and investment^{73–76}, as well as the viability of the offspring^{77,78}. For example, female mallards (*Anas platyrhynchos*) lay larger eggs and produce better quality offspring after copulating with a preferred male^{76,79} and female zebra finches incorporate higher levels of testosterone in their eggs when mated with a preferred male⁷⁵. The sex-allocation theory on the other hand, predicts that, when the fitness of sons and daughters differ, parents should adjust the sex ratio of their brood accordingly^{80–83}. Consequently, when sons inherit from their fathers some traits that determine their attractiveness, females are expected to adjust their brood sex ratio depending on the male quality they are paired with^{82,84–87}. For example, great tits (*Parus major*) have been suggested to adjust the sex ratio of their offspring in response to their mate's tarsus length⁸⁸.

Being mated to a non-preferred partner may consequently reduce an individual's motivation to reproduce, or cause physiological stress altering reproductive performances^{70,89–92}. In Gouldian finches (*Erythrura gouldiae*), experiments have shown that females paired with non-preferred mates had levels of circulating corticosterone, the main avian stress hormone, that were three to four times higher than those of females paired with their preferred mates. Moreover, females paired with non-preferred males delayed their onset of reproduction (**figure 3**)⁸⁹. Females that are not able to acquire the male they want may also adopt a compensatory strategy, which consists in increasing their reproductive investment to compensate for the lesser quality of, or the lower preference for, their male partner^{77,90,93}.

We have seen in this section that seasonal reproduction takes place in a social context and that interactions between partners significantly modulate the reproductive cycle. However, the members of a breeding pair must coordinate not only with each other, but also with the environment in which they live. Therefore, organisms need to rely on environmental cues in addition to social factors to predict the environmental conditions at the time of offspring rearing.

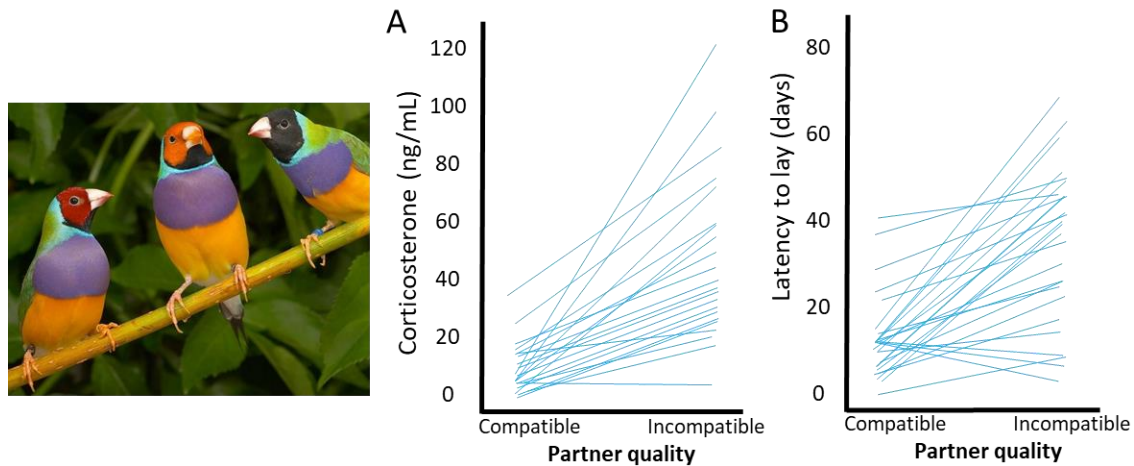


Figure 3: Compatibility between partners and its effects on stress and on the date of egg laying in Gouldian finches (*Erythrura gouldiae*).

In Gouldian finches (*Erythrura gouldiae*), birds preferentially mate with individuals of the same head colour morph as themselves. Females (A) have a lower level of circulating corticosterone, and (B) lay earlier, when paired with a compatible male. Blue lines connect the data points for the two conditions of the same individual females. © Gerhard Hofmann and Claudia Mettke-Hofmann. Adapted from (Griffith et al., 2011) ⁸⁹.

2. Environmental cues used to match offspring needs with food abundance

a) Photoperiod as the primary seasonal cue for reproduction

In most vertebrates, photoperiod is the main predictive cue used to initiate and regulate the physiological and behavioural cascades of most seasonal events ^{94–96}. In mammals, the eyes are the only photoreceptors ⁹⁷ but in birds, photoperception occurs through the eyes, the pineal gland and deep brain photoreceptors ^{94,96,98,99}. The seasonal response to photoperiod then takes place through the Hypothalamus-Pituitary-Gonadal (HPG) axis, where a complex neuro-endocrine cascade is initiated (**box 1**) ⁹⁴. In most species living at mid and high latitudes, gonadal maturation occurs when photoperiod increases, corresponding to spring ^{94,100}. The magnitude of the gonadal response to the increasing day length is equivalent to a five-fold increase in testicular weight in hamsters ¹⁰¹ and up to a 30-fold increase in a tropical finch (*Zonotrichia capensis costaricensis*), for example ¹⁰². At the end of the breeding period, many birds, contrary to mammals, enter a photorefractory state, in which they no longer respond to long days and start regressing their reproductive system and start moulting ⁹⁸.

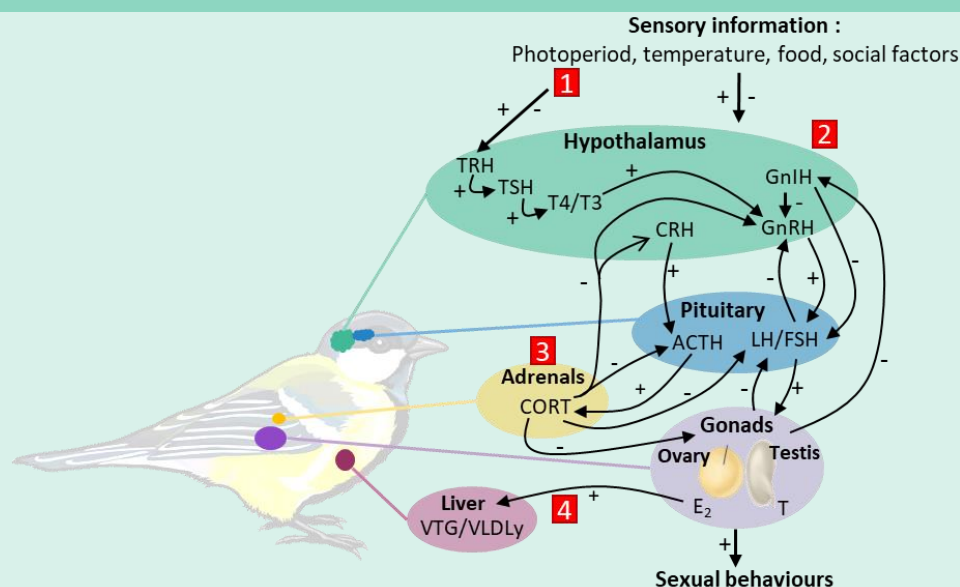
In male birds, the annual change in day length constitutes the main trigger of gonadotropin release, gonadal growth, initiation of spermatogenesis and steroidogenesis in the testes ^{22,98}. However, it appears that variations in the timing and rate of development of the male reproductive system do not always translate into variations in female breeding time. For example, in two populations of Mediterranean blue tits living in two different habitats, the onset

of egg-laying occurs with a one-month difference, but the initiation of the seasonal HPG axis (**box 1**) development in males occurs at the same time in both populations, in late winter. The subsequent phases of the seasonal testicular growth and singing activity remain only partly differentiated between the populations despite the one-month difference in egg-laying^{103,104}. These results show that strong variation in the timing of breeding can occur even without clear differences in photoperiodic responses. Experiments in captivity also showed that long photoperiods lead to complete sexual activation of the male testes, but are insufficient to trigger the final stages of ovarian development in females^{105–108}, suggesting that photoperiod alone is insufficient for females to develop a fully functional reproductive system.

One reason why seasonal changes in photoperiod are insufficient to trigger reproduction is that the seasonal changes in day length are invariable across years. Predicting inter-annual variations in the optimum reproductive period is therefore impossible using photoperiod as the only cue^{109,110}. Photoperiod rather opens a window of time in which reproduction can occur, and supplementary cues help males and females to refine when reproduction actually takes place.

b) Adapting reproduction to local environmental conditions using supplementary cues

There are many non-photic environmental cues that interact with the HPG axis (**box 1**) to speed up or slow down reproduction, and act through various sensory pathways, such as vision, audition, and olfaction¹¹¹. In this section, I will first describe a supplementary cue that has been extensively studied for its involvement in reproductive cycles, particularly in avian species: temperature. It represents a cue of main interest in ecology because it influences all trophic levels and it is currently the major factor of change in our warming world. I will then focus on the cue I investigated most in this thesis, which is vegetation phenology. Vegetation is the first level of most food chains, including those of the insectivorous birds that I study (**box 2**). I will detail why this cue might represent a reliable predictor of future environmental conditions, and why birds could use it for anticipating their reproductive decisions.

Box 1|Main hormonal networks involved in bird reproductive control.

Key components of the Hypothalamic-Pituitary-Gonadal (HPG) and Adrenals (HPA) axes, controlling bird gonadal development and sexual behaviours. Adapted from (Chmura et al., 2019; Williams 2012)^{22,29}.

1 Under increasing day length, light information activates the secretion of Thyrotropin Releasing Hormone (TRH) in the mediobasal hypothalamus, which transmits the information to the pituitary gland where it induces Thyroid-Stimulating Hormone (TSH) secretion. This increases the expression of the DIO2 enzyme that converts Thyroxine (T₄) into its active form, the thyroid hormone Triiodothyronine (T₃). This conversion promotes morphological changes in the brain region secreting Gonadotropin-Releasing Hormone (GnRH) and regulates its release into the portal vein system located at the interface of the hypothalamus and pituitary^{94,112,113}.

2 GnRH then promotes the secretion of gonadotropins (Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH)) from the anterior pituitary, which stimulate gonadal growth, production of gametes and sex steroids (mainly testosterone (T) and oestrogens (E₂)) by the gonads^{114–116}. In addition, the hypothalamic neuropeptide Gonadotropin Inhibitory Hormone (GnIH) can oppose gonadotropin synthesis and may also regulate the GnRH system directly¹¹⁴.

3 The Hypothalamic-Pituitary-Adrenals (HPA) axis influences the hormonal network in the HPG axis mostly under unfavourable conditions (CORT-flexibility hypothesis) when birds are under stress¹¹⁷. In the hypothalamus, Corticotropin Releasing Hormone (CRH) induces the release of Adrenocorticotrophic Hormone (ACTH) from the pituitary. Corticosterone (CORT), the main stress hormone in birds, is then released from the adrenal glands and contributes to the decrease of gonadal steroid levels (T, E₂), consequently altering sexual behaviours¹¹⁷.

4 In females, E₂ stimulates follicle development and production of vitellogenin (VTG) and yolk-targeted Very Low-Density Lipoprotein (VLDLy) by the liver^{118–121}. Females have been suggested to use supplementary cues to regulate vitellogenesis, follicle development and the timing of egg-laying downstream of the brain, in the ovary and/or liver, and could thus fine-tune the timing of reproduction^{118,120,122}.

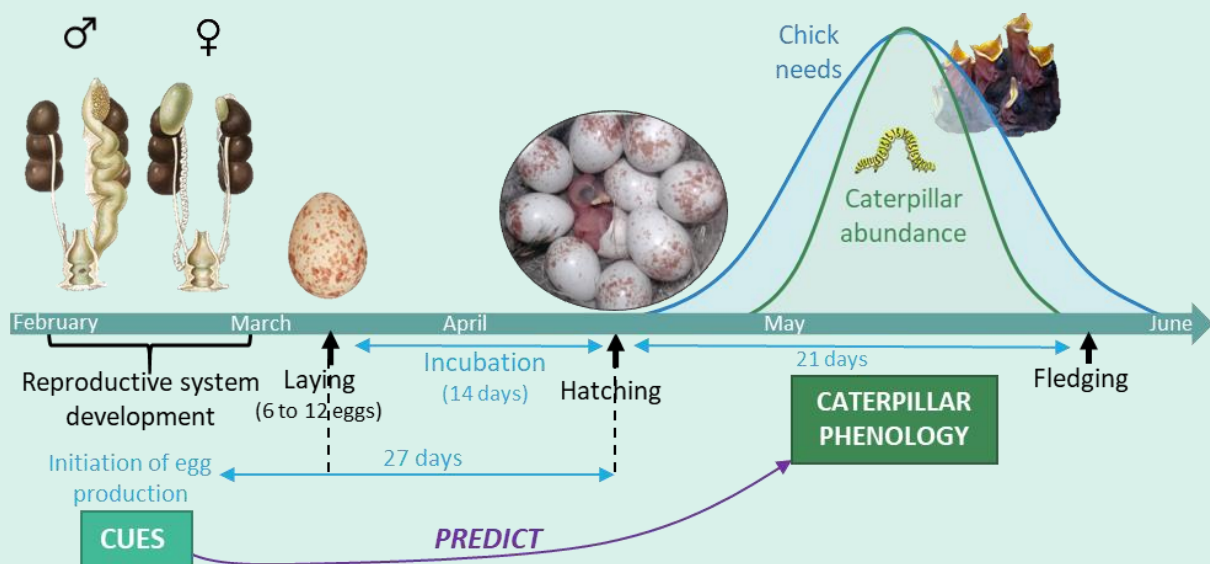
i. Temperature as a key signal for reproduction

Global warming has important ecological consequences and evolutionary impacts on wild populations^{123,124}. A growing number of studies are now reporting alterations of plant and animal cycles from a wide range of ecosystems in response to climate change¹²⁴. For example, insects are expected to undergo faster development during their larval stages and become adults sooner in response to rising temperatures¹²⁵ and frog calling has been reported to occur 10 days earlier in New York state in the 1990s than at the beginning of the twentieth century¹²⁶. The effect of temperature has been extensively studied in the field of animal reproduction in all main taxa^{127–131}. Generally speaking, temperature affects many components of the reproductive cycle, from endocrine control of reproduction, puberty, duration of oestrus, and gonadal development, to spermatogenesis and ovulation. For example, studies on molluscs *Aplysia* have shown that individuals maintained under 15 °C laid eggs significantly less frequently than those maintained at 20 °C¹³², and in Atlantic salmon (*Salmo salar*), elevated temperatures inhibit the ovarian synthesis of E₂ (**box 1**), leading in turn to reductions in VTG synthesis (**box 1**) and ultimately decreasing egg size and fertility¹³³.

In birds, the model system for this thesis (**box 2**), the effect of temperature on laying dates has been intensely studied both in wild populations^{134–142} and in controlled conditions^{143–146}. Studies find that warmer spring temperatures significantly advance laying dates^{143,144,147}, but see^{148,149}. At the same time, the main food sources of birds have also advanced their phenology in response to the increasing temperatures^{125,150,151}. Advancing laying dates thus enables birds to track the advancement of the period of food availability for offspring, and consequently avoid, or at least reduce, desynchronisation between food availability and offspring needs (**box 2**)^{134,152,153}. However, numerous experimental studies have explored the mechanisms underlying the advancement of laying dates in response to increasing temperatures, but these studies have produced mixed results^{reviewed in 147,154}. For example, Visser and colleagues conducted an experiment in which they exposed birds to temperature patterns from two specific years in which the wild population laid either early (the “warm” treatment) or late (the “cold” treatment). Although birds in the warm treatment laid earlier than those in the cold treatment in five out of the six years of the study, they did not advance as much as what was observed in the wild with the same temperature patterns¹⁴⁴. Although there is a link between temperature and timing of breeding, no such relationship has been found between temperatures and reproductive investment. For example, temperatures and clutch sizes are not correlated in field studies on tree swallows (*Tachycineta bicolor*), nor are they in field^{155–157} or laboratory¹⁴⁸ studies on

great tits. A great deal of research has also been carried out into the physiological effects of temperature on bird reproductive development, particularly in model avian systems such as the white-crowned sparrow (*Zonotrichia leucophrys gambelii*)^{158,159}, great tit^{143,160}, and European starling^{161,162}. Some studies highlighted an acceleration of gonadal development with increasing temperatures^{159,163–165}, while others found no effect^{143,146,148,166–168}. The difference noted between predictions made from the field observations and results obtained in the laboratory may be attributed to the need for birds to have other supplementary cues, potentially more integrative than temperatures, to predict the phenology of their food source. Vegetation phenology has been suggested to be one of these cues.

Box 2 | Reproductive cycle of the model system of this thesis



*Reproductive phenology of blue tits (*Cyanistes caeruleus*) and great tits (*Parus major*).*

In this thesis, I focus on two small passerine bird species, the blue and the great tits. In these species, synchronising the timing when chicks require the greatest quantity of food with the timing of the maximal biomass of caterpillars is crucial to ensure a successful reproduction^{169–171}. For example, in a blue tit population of Poland, the nestling diet is composed of more than 70% of caterpillars¹⁷² and they even constitute more than 80% of their diet in a Corsican population¹⁷³. However, this surge in caterpillar abundance occurs only at a specific moment in late spring. Birds consequently have to anticipate their physiological and behavioural preparations for reproduction, including reproductive system development, nest site selection, and partner acquisition, to be able to synchronise the period of maximal food requirement of offspring with the timing of the caterpillar peak. To accomplish this, they need to use proximate factors, also called cues, to predict and anticipate the peak of caterpillar biomass¹³, and thus time their reproductive events accordingly^{13,22,26,27}.

ii. Birds and buds: vegetation phenology as an integrative cue for bird reproduction?

Buds constitute an ideal food source for many insect species^{174–176}, with a maximum of protein and a minimum of tannins that inhibit insect growth^{176,177}. Many studies have shown that the emergence of herbivorous insects, including but not restricted to caterpillars, therefore coincides with local bud development^{175,178–181}. For example, some caterpillar species, like the green oak leafroller (*Tortrix viridana*), used by several bird species to feed their nestlings, enter the buds as soon as they hatch and spend their first instars hidden inside the buds¹⁷⁸. Synchrony between the hatching of caterpillars and the opening of the host tree buds is consequently a major component of the caterpillar's capacity to survive^{125,174,179,181–185}.

Vegetation phenology, and particularly bud development, has thus been suggested to be a predictive cue that birds could use to predict the future peak of caterpillar biomass, and thus fine-tune their egg production to local annual conditions^{186,187}. While other supplementary cues, such as temperatures or precipitations, provide information about current environmental conditions, vegetation phenology may be a more integrative cue. Indeed, bud development integrates the temperatures over a long period and provides information on the environmental conditions of the preceding days and weeks, so it may be a more integrative cue than temperature *per se*, which fluctuates on a short-term basis. Birds could therefore use a certain stage of bud development as a cue to time their reproduction.

The hypothesis that vegetation phenology could be a supplementary cue used by birds to decide when to breed seems to be supported by observations both in the field and in captivity. Voigt and colleagues showed that island canaries (*Serinus canaria*) are capable of adjusting their reproduction to vegetation, with birds advancing their laying dates when offered green plants¹⁸⁸. Another striking example concerns one of my model species, the blue tit (**box 2**). Two populations of blue tits on the island of Corsica breed in two very different types of habitats, only 25 km apart. One of the habitats is an evergreen oak forest, which phenology is characterised by late bud burst, while the other is a deciduous oak forest, in which bud burst occurs one month earlier^{189,190}. Although photoperiod and temperatures are similar at both sites due to their geographic proximity, birds lay about one month later in the evergreen habitat than in the deciduous one (**figure 4**)^{186,191–194}. This difference in timing of breeding, linked to the vegetation composition of the forests, appears adaptive because it closely matches the respective caterpillar peaks at the two sites¹⁹⁰. In other populations of blue and great tits in the United Kingdom and Sweden, studies have shown that the onset of egg laying was positively

correlated with local vegetation phenology^{187,195,196} but see²⁰³. All these studies consequently support the idea of a role for vegetation phenology as a cue for the reproductive decisions of insectivorous birds. However, those studies remain descriptive. Demonstrating a causal link between bird reproduction and vegetation development requests experimental work manipulating the cues to which birds are exposed before they start reproducing. I will review some of that work in the next section.

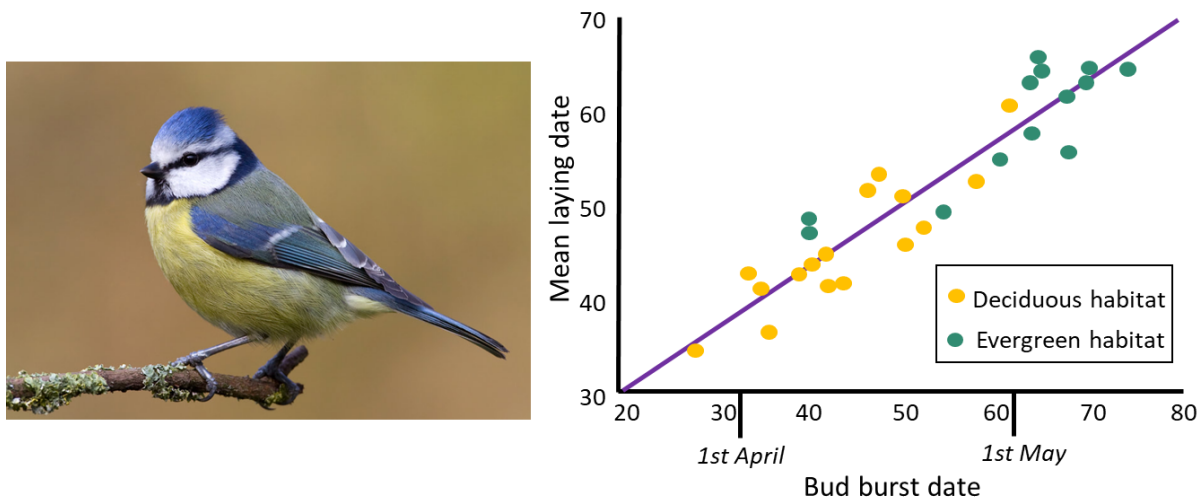


Figure 4: Relationship between annual mean laying dates of Corsican blue tits and bud burst dates of oak trees in two different habitats.

The timing of breeding in Corsican blue tits is strongly correlated with the phenology of the dominant vegetation, with birds in deciduous forests laying one month earlier compared to birds living in evergreen forests. Points depict annual mean laying dates for different sites, characterised by either evergreen (green points) or deciduous (yellow points) vegetation. For dates: 1 = 1st March. © Liz Cutting. Adapted from (Bourgault et al., 2010)¹⁸⁶.

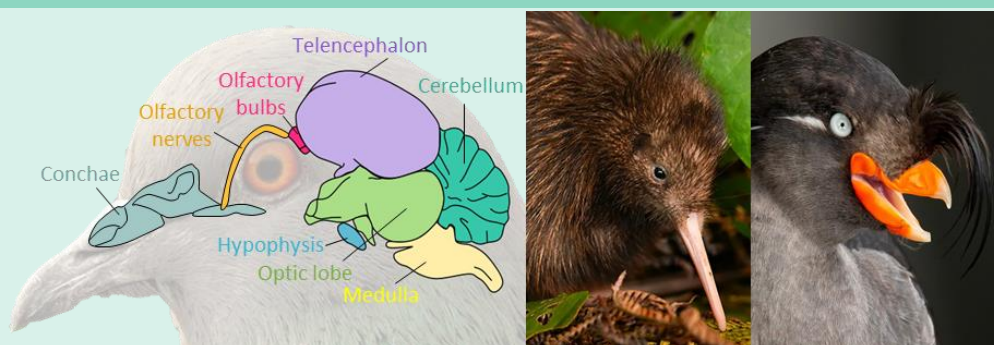
- Visual and olfactory assessment of vegetation development by birds

The effect of vegetation phenology on bird breeding phenology could occur through various sensory channels. We cannot know whether birds see the world in “colour” the same way we do, as colour is a property of the nervous system and not of the environment¹⁹⁸. However, it is clear that birds detect light over a wider range of wavelengths than humans¹⁹⁹. They consequently have a broader visible spectrum. It also seems that birds are able to discern more colours within this spectrum and some birds, including passerines, are also able to detect light in the ultraviolet part of the spectrum^{199–201}. Leaves damaged by insects have been shown to reflect less light in the birds’ visual range than uninfested leaves²⁰², suggesting that birds may be able to discriminate between the two using a visual mechanism. However, a recent study used a discrimination threshold model, which uses information about the visual system such as the sensitivity and relative abundance of different photoreceptor types, to assess whether birds are able to discriminate the visual difference between insect-infested and uninfested leaves.

Although the birds could distinguish branches with and without infestations, the model suggested that they could not perceive the colour difference between them²⁰³. Consequently, the birds' discrimination between infested and uninfested branches was probably based on a sensory system other than vision. Vegetation can be perceived not only by its colours but also by its smells, so olfaction is a sense that birds might use to assess vegetation development.

Only in the last 20 years has smell been acknowledged as a potential source of information for birds. For many years, birds were considered anosmic, or at best to have a rudimentary sense of smell compared to mammals. Recent research has however revealed that birds have an olfactory system that allows them to detect odours at low concentrations and in very different contexts²⁰⁴ (**box 3**). As a result, it is possible that they rely on their olfactory abilities to extract information from the odours generated by trees. Indeed, plants produce a wide array of Volatile Organic Compounds (VOCs) involved in plant communication and ecophysiological functions^{205,206}. When subjected to different types of stress (e.g. temperature, light intensity, pathogens, drought), plants release new compounds and alter their constitutive emissions^{reviewed in 207}. Insect herbivores feeding on plant leaves represent one of these stressors and they trigger specific emissions of VOCs, called Herbivore-Induced Plant Volatiles (HIPVs) (**figure 5**)²⁰⁸⁻²¹⁰. The "cry for help hypothesis" suggests that plants emit HIPVs to defend themselves against herbivores by attracting their predators²⁰⁸. These chemical compounds are emitted by plants in different quantities and ratios depending on the plant species²⁰⁵, the level of infestation that the plant undergoes²¹¹⁻²¹³, and sometimes even the herbivore species attacking them^{214,215}. HIPVs may thus serve as a straightforward indicator for birds, reflecting both caterpillar phenology and abundance.

Box 3|Olfaction in birds



Location of olfactory bulbs in the avian brain and examples of two good smellers.

Left: Schematic morphology of the brain, showing the conchae, the olfactory nerves and the olfactory bulbs at the front of the brain. Adapted from (Ruiz i Altaba et al., 2002) ²¹⁶. Middle: the brown kiwi (*Apteryx mantelli*) uses olfaction to find prey buried in the ground ²¹⁷. © Neil Robert Hotton. Right: the crested auklet (*Aethia cristatella*) exhibits a tangerine-like scent closely associated with courtship ²¹⁸. © Christopher Dodds.

For a long time, birds were considered anosmic, or at best microsmatic ^{219–221}, but many avian species are equipped with a functional olfactory system. Birds have a similar olfactory system to mammals ²²². Inhaled air passes from the nostrils and then successively goes through three chambers (conchae), which filter, warm and chemically sample the air ²²³. The third conchae is innervated by olfactory receptors that detect the presence of specific chemical compounds before the air passes into the respiratory system. From these receptors, neurons reach the brain at the level of the olfactory bulbs ²²⁴. Olfactory bulbs are usually paired structured and situated at the front of the brain, although their shape and size vary markedly across bird species. Their size relative to the rest of the brain varies from a ratio of 25% in Procellariiforms to less than 5% in Passeriformes ²²⁵. The olfactory bulbs then project to different brain areas homologous to those processing olfactory information in mammals ^{226–228}.

Nowadays, a wide number of studies have shown that birds use olfaction in a large variety of contexts, from foraging ^{229–231}, to predator recognition ^{232,233}, selection of nest materials ^{234,235}, nest location ²³⁶, navigation ^{237,238}, conspecifics recognition ²¹⁸ or even mate recognition ^{239,240}. One of the most impressive examples of how birds use scent to locate their prey comes from pelagic seabirds. They use one particular chemical compound, dimethyl sulphide (DMS) that does not directly indicate the presence of food to birds but that is produced by marine phytoplankton when grazed by zooplankton. Areas with high concentrations of DMS are thus associated with areas of high primary productivity in the ocean, attracting larger animals that exploit this food chain and on which seabirds feed ^{230,241}.

Birds not only use the scents of their environment but also those of individuals, particularly in a reproductive context ^{242,243}, reviewed in ²⁴⁴. Body odours in birds are mostly emitted by preen oil from the uropygial gland, located at the dorsal base of the tail ²⁴⁵. These odours have been suggested to be implied in species and sex recognition ^{246–249}, mate choice and synchronisation ^{250,251} and parental care ^{252,253}. For example, in two sympatric sibling species of waxwings, *Bombycilla garrulus* and *B. japonica*, it has been shown that females *B. garrulus* prefer the body odour of a conspecific while they avoid the scents of the sibling species *B. japonica*, suggesting that divergence of body odours between sympatric species could be a good mechanism for avoiding hybridization ²⁴⁸.

- *Avian responses to Herbivore-Induced Plant Volatiles (HIPVs): from a foraging to a reproductive cue?*

Carnivorous arthropods and parasitoids are known to use HIPVs to locate herbivorous insects^{254–257}. For example, *Cotesia marginiventris*, a general parasitoid of noctuid moths, uses the terpenoids (**figure 5**) emitted by noctuid caterpillar-infested corn seedlings to locate their host²⁵⁵. The first evidence that birds are also attracted to damaged trees attacked by herbivorous insects, without the need to see the larvae, dates back less than twenty years²⁵⁸. This first study showed that willow warblers (*Phylloscopus trochilus*) preferentially orient towards branches of mountain birches previously damaged by sawfly larvae, than branches from intact trees²⁵⁸. Mäntylä and colleagues repeated this experiment with blue and great tits²⁰², and also in the field²⁵⁹, and in both studies, birds were attracted to infested trees. While these studies showed the ability of birds to differentiate between infested and non-infested trees, they did not investigate the sensory pathway used to make this distinction. A few years later, Amo and colleagues brought convincing responses to some of the questions that Mäntylä and colleagues' studies left unanswered²⁶⁰. Amo et al. notably tested the exact mechanisms responsible for the birds' attraction to trees infested with lepidopteran larvae. To this aim, they exposed great tits to apple trees (*Malus silvestris*) in a Y-shaped aviary. They placed two apple trees in each arm of an aviary. In both arms, one tree was visible behind a glass, while the other was hidden behind a permeable fabric, in such a way that birds could see one tree without being able to smell it, and smell the other without seeing it. One arm contained two uninfested apple trees (control pair), while the other arm contained trees that were treated in different ways (experimental pair). To disentangle whether birds were attracted to caterpillar-infested trees based on visual or olfactory cues, or a combination of both, the experimental pair of trees could have the (i) “olfactory” (tree seen uninfested and tree smelled infested), (ii) “visual” (tree seen infested and tree smelled uninfested) or (iii) “olfactory and visual” treatment (seen and smelled trees infested). With this complex set-up, Amo and colleagues found that bird preference for infested trees was still exhibited when the only cues available were the plant odours, but not when there were only visual cues. They, therefore, showed that bird attraction to infested trees was mainly mediated by olfactory cues from trees²⁶⁰.

As we have just seen, to understand to what extent birds use olfactory cues in complex tritrophic systems involving plants, herbivorous insects, and birds, experimental approaches are essential. Besides the seminal studies by Mäntylä et al. in 2004 and Amo et al. in 2013, detailed above, numerous studies have investigated these interactions in the field or in captivity (summarised in **Table 1**). In field experiments, bird attraction to HIPVs is often estimated by

the signs of pecking on fake plasticine caterpillars^{259,261–268}. Unfortunately, variables like the bird species, the number of individuals attracted and their identity, or the distinction between chemical and visual cues are difficult to disentangle with this method. Experiments performed in captivity can therefore overcome some of the limitations of experiments performed in the wild, with a better control of environmental conditions. Moreover, experimental set-ups can be developed to better isolate visual from olfactory cues. In captivity, assessing birds' attraction to HIPVs is typically done by testing their response to the olfactory cue in an aviary with two possible directions. One direction contains the olfactory cue, either from an infested tree or from artificial compounds (see details below), while the other direction has no HIPV emissions. Experiments conducted using this methodology usually investigate the initial direction the bird chooses upon release inside the aviary, as well as the frequency of visits, and the proportion of time spent on each side of the aviary^{260,269–271}. Such analyses allow for an estimation of the bird's attraction to the olfactory cue through quantifiable measurements, which remains challenging to accomplish in the field.

Studying the attraction of birds to HIPVs requires developing methods for inducing these odours. One method is to infest a tree, or a part of the tree (branch, leaves), with a herbivorous insect^{202,203,258–261,263,269,270,272}. This method can be used both in the field, directly on trees in a forest for example, or in captivity on potted plants. In both cases, if insects are kept on the tree at the time of testing the bird's attraction to HIPVs, insects are enclosed inside an opaque mesh bag to prevent birds from seeing them^{259,261,263}. In captivity, it is even the entire infested tree that can be hidden in a large opaque bag so that the birds have no access to any visual cues^{260,263,269,270,272}. Another approach involves applying Methyl Jasmonate (MeJA) (**figure 5**) to trees to induce the production of HIPVs^{261,262,264–266,273}. Indeed, MeJA is a phytohormone involved in the jasmonic acid signalling pathway that ultimately leads to the release of HIPVs^{209,274,275}. Other studies have implemented a technique wherein a part of the plant is intentionally injured and insect saliva is subsequently applied to the wound, thereby replicating the mechanical and chemical action that occurs during a herbivorous insect attack on leaves^{267,271}. Finally, only two studies have investigated how birds respond to odour mixtures artificially created to imitate natural HIPVs^{263,276}. Artificially recreating HIPVs is an interesting method for studying the behavioural response of birds to these odours, as it allows the compounds emitted and their concentrations to be controlled. Some studies have also focused on individual compounds, such as MeJA, or Methyl Salicylate (MeSA) (**figure 5**)^{268,277–279}, rather than mixtures of compounds. While MeJA triggers HIPV emissions after

damage caused by chewing herbivores such as caterpillars, MeSA triggers them after an attack by sap-sucking herbivore such as aphids ^{280–282}.

Although methods differed between studies, most have found that birds are attracted to HIPVs without the need to use any visual cue of insect presence (sight of the insects or of the damages caused by the insects on the leaves) ^{202,203,267,269,271,272,276,277,258–261,263–266}. Attraction to these compounds, however, appears to be a trait learned by birds over the course of their lives. Indeed, Amo et al. in 2016 tested the attractiveness of caterpillar-infested apple trees on hand-reared great tits that had never foraged on trees, nor smelled HIPVs. They found that great tits were equally attracted to infested than to control trees ²⁷⁰. A few years later, Sam et al. in 2021 found similar results when they tested the attraction of hand-reared great tits to scotch elms that had been mechanically damaged and wounds covered with insect saliva ²⁷¹. Birds were only attracted to the trees after having been trained to associate the olfactory cue emitted by wounded trees with food presence ²⁷¹. The attraction of insectivorous birds to infested trees does therefore not appear to be innate, but learned ^{270,271}. Great tits have also been observed to take longer to associate MeSA with the presence of food than MeJA ²⁷⁸, suggesting that the ability to learn olfactory cues could depend on the nature of the compounds.

Among the studies finding no preference of birds for HIPVs, two of them used MeJA to induce the production of HIPVs by the trees ^{261,273}. However, the authors found that trees treated with MeJA emitted a mixture of HIPVs that significantly differed from the HIPVs that trees emit when they are attacked by herbivorous insects ^{203,264,273}. Similarly, Koski et al. in 2015 investigated the attraction of birds to an artificial mixture mimicking the HIPVs emitted by defoliated mountain birch trees (*Betula pubescens*) and found not attraction of bird to the artificial blend, although they found that some of the compounds tested, such as DMNT (**figure 5**), were positively associated with higher bird predation rates ²⁶³. Consequently, the olfactory signal emitted by trees infested with herbivorous insects might constitute a much more complex mixture than the one artificially recreated in the study of Koski et al., or triggered by MeJA.

Table 1: List of studies that investigated the response of birds to HIPVs.

It indicates whether the study was conducted in the wild or in captivity ("Study"), which method was used to emit the HIPVs ("HIPV emission"), whether birds came from the wild or was raised by hand ("Bird origin"), whether birds had already smelled HIPVs ("Bird experience of HIPVs"), whether different visual and olfactory cues were available in the experiment ("Type of cues available"), how the authors assessed the birds' attraction to HIPVs ("Assessment of bird attraction to the OC") and whether birds were attracted to the odour cue ("Bird response to the OC").
OC: olfactory cue.

Ref.	Author	Year	Bird species	Tree species	Insect species	Study		HIPV emission				Bird origin		Bird experience of HIPVs		Type of cues available			Assessment of bird attraction to the OC			Bird response to the OC	
						Field	Lab	Insects	MeJA	Mechanical damage	Artificial blend	MeJA	MeSA	Nature	Hand reared	Native	Trained	Visual	Olfactory	If both, tested separately?	Predation on artificial caterpillar	First choice	Proportion of visits and/or time to the OC
258	Mäntylä et al.	2004	Willow warbler	Mountain birch	Sawfly larvae	x						x				x					x		
202	Mäntylä et al.	2008a	Blue and great tit	Silver birch	Autumnal moth	x						x									x		
259	Mäntylä et al.	2008b		Mountain birch	Autumnal moth	x						x											
261	Mäntylä et al.	2014		Mountain birch	Autumnal moth	x						x											
203	Mäntylä et al.	2017	Blue and great tit	Scot pine	Sawfly larvae	x						x											
272	Mäntylä et al.	2020	Blue and great tit	Scot pine	Sawfly larvae	x						x											
262	Mäntylä et al.	2022		3 species of Ficus		x						x											
260	Amo et al.	2013	Great tit	Apple tree	Winter moth	x							x										
270	Amo et al.	2016	Great tit	Apple tree	Winter moth	x							x										
263	Koski et al.	2015	Pied flycatcher	3 species of birch		x																	
			Blue and great tit	Silver birch	Autumnal moth	x																	
264	Mrazova and Sam	2017		Grey willow		x																	
265	Mrazova and Sam	2019		Ficus hatiana		x																	
266	Mrazova et al.	2023		English and Sessile oak		x																	

Ref.	Author	Year	Bird species	Tree species	Insect species	Study		HIPV emission				Bird origin		Bird experience of HIPI's		Type of cues available			Assessment of bird attraction to the OC			Bird response to the OC	
						Field	Lab	Insects	MeJA	Mechanical damage	Artificial blend	MeJA	MeSA	Nature	Hand reared	Naive	Trained	Visual	Olfactory	If both, tested separately?	Predation on artificial caterpillar	First choice	Proportion of visits and/or time to the OC
	Mrazova	unpub (thesis)	Blue and great tit	European privet	Allanthus silkmoth	x	x					x	x						x	x			
	Mrazova	unpub (thesis)		Pedunculatae and sessile oaks		x						x								x			
276	Hiltbold et al.	2018		Maize field		x		x				x								x			
				Pyrenean oak				x				x									x		
273	Saavedra and Amo	2018	Blue tit	Pyrenean oak		x		x				x									x (number of nesting begging and duration of first begging)	x	
267	Sam et al.	2015		12 tree species		x						x										x	
				Scotch elm, Cattle guava								x (insect saliva)										x (only time)	x
271	Sam et al.	2021	Great tit	Scotch elm, Cattle guava		x						x (insect saliva)											x
				Scotch elm, Cattle guava		x						x (insect saliva)											x
277	Rubene et al.	2019		Agricultural landscapes		x																	x
																							x
278	Rubene et al.	2022	Great tit			x																	x
						x																	x
279	Rubene et al.	2023	Great tit			x																	x
						x																	x
268	Nguyen et al.	2022		2 tree species, 2 herbaceous species		x																	x
						x																	x
269	Graham et al.	2021	Blue tit	Downy oak	Winter moth, green oak tortrix	x																	x

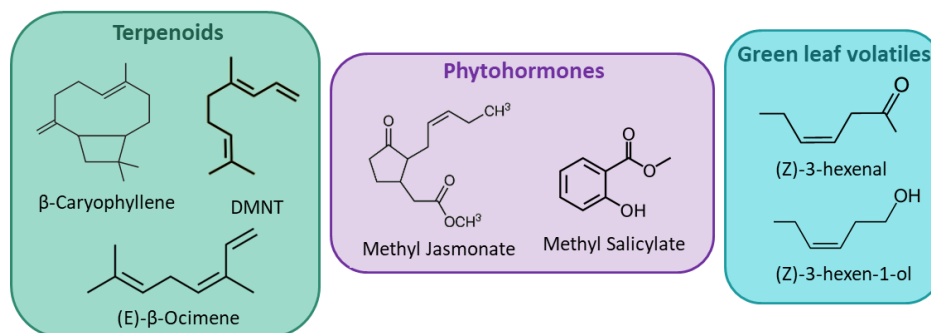


Figure 5: Main Herbivore-Induced Plant Volatiles (HIPVs) emitted by plants after an attack by herbivorous insects.

The terpenoids group includes monoterpenes (C₁₀, e.g. (E)- β -Ocimene), sesquiterpenes (C₁₅, e.g. β -Caryophyllene) and homoterpenes (C₁₁ or C₁₆, e.g. (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT)). They are synthesised rapidly and locally by trees infested with herbivorous insects. Phytohormones MeJA and MeSA, derived from jasmonic acid and salicylic acid, respectively, are considered two of the most important molecules released by plants in response to feeding herbivores and are implied in the signalling pathways leading to the production of HIPVs. Green leaf volatiles (C₆), including aldehydes, alcohols and esters, are other compounds immediately released by leaves after wounding. Adapted from (Arimura et al., 2005, Dicke et al., 2009, Holopainen and Blande, 2012)^{205,209,283}.

All studies cited above examined whether birds use the HIPVs emitted by mature leaves in a foraging context, and during the period when caterpillars are fully developed, i.e. when they constitute a suitable food source. Recent results by Graham and colleagues (Graham, Buatois, Staudt, Caro; unpublished data) have however shown that oak trees already start emitting HIPVs in early spring, while they are still in buds, and caterpillars just hatched from their eggs. Those first instars only measure a few millimetres and are therefore unsuitable preys for birds. Most are even invisible to the birds, as they live in miners inside buds^{174,284}. But it is those early mining activities that trigger the buds to start producing HIPVs. Graham and colleagues also showed that these early HIPVs are relatively similar to those emitted by mature leaves (Graham et al., unpublished data). Several field studies on insectivorous bird species have shown that, on average, females start laying around the time tree buds start opening^{186,187,195}, thus at about the time the tiny caterpillars are mining inside buds, chewing young shoots, and triggering the first seasonal HIPV emissions. Because bud HIPVs are relatively similar to those emitted by mature leaves, and because earlier studies have shown that HIPV emissions are proportional to the level of insect infestation^{211–213}, it is therefore tempting to postulate that birds might not only use HIPVs in late spring, to find mature caterpillars for their chicks, but also much earlier in the season, this time to take informed reproductive decisions about when to initiate laying, and how many eggs to produce. The time it then takes to caterpillars to grow and reach their maximal biomass, approximately corresponds to the time it takes to female birds to lay their eggs, incubate them and start raising their chicks, up to an age

when chick needs are maximal (around day 10 in blue and great tits). Laying when they start perceiving the first HIPV emissions might therefore be adaptive and guarantee female birds to find suitable caterpillars for their chicks about a month later. Adjusting their clutch size to the amount of HIPVs emitted in their breeding territory in early spring would also seem adaptive, as it should be a guarantee to find enough food for the number of chicks they decided to produce. I am only aware of one study that has tried to link early HIPVs with a reproductive trait in birds. This was also done by Graham and colleagues²⁶⁹. In this study, they demonstrated that blue tits were able to detect those early bud HIPVs triggered by young caterpillars, but most interestingly, they also showed that males with higher levels of testosterone spent more time around the infested trees. As testosterone levels are closely linked to reproductive behaviour in males, it was the first evidence that bud HIPVs could be linked to reproduction in insectivorous birds. However, whether and how these volatile emissions from infested plants influence physiological mechanisms underlying the reproductive decisions in females and reproductive success of birds is currently unknown.

III. Thesis aims and hypotheses

As we have seen in this introduction, breeding decisions are made in a social context, which is itself exposed to variation in the local environmental conditions; conditions that organisms must consider to maximise their reproductive success. This thesis aimed to shed light on how social and environmental cues influence the reproductive decisions of two insectivorous birds, the blue and the great tits. More specifically, I studied **the effect of mate preference on female breeding decisions, and the importance of vegetation development and Herbivore-Induced Plant Volatiles (HIPVs) on those same reproductive decisions.**

1. Social factors shaping avian reproductive timing and investment: a focus on mate preferences

In **Chapter 1**, I investigated **the effect of female mate preferences on reproductive timing and investment**, in both tit species. I determined how females modify their reproductive investment when they are paired with a male that matches their preferences. Although some studies have investigated the effect of male ornamentation on reproductive success in these species^{88,285,294,286–293}, none of them have examined the effect of the female preferences on their reproductive decisions. Moreover, many studies investigating female preference for a mate have been conducted in the wild^{295–299}, where it is often impossible to disentangle the respective roles of the different components involved in mate choice, such as competition with other females for males and territories, or male availability^{300–303}, on reproduction. Captive studies

on the other hand, have often involved experimental manipulations of individuals' characteristics and have offered females a limited choice^{304,305,314–320,306–313}. In this chapter, I adopted an intermediate approach, combining the advantages of wild and captive studies.

I conducted two distinct experiments with blue (**article 1**) and great tits (**article 2**) in which I asked females to express their mate preferences in panels of six males. I first analysed which male phenotypic traits drive female choices. I then tested how being paired with a preferred, or a non-preferred male impacted their reproductive parameters, by measuring female reproductive timing and investment. In line with the existing hypotheses that I presented in this introduction, I hypothesised that females showing a strong preference for a given male would show a faster reproductive physiological development when mated with this male (differential allocation hypothesis), compared to when they are mated to a less-desired male. I thus expected them to show higher levels of sex steroids in the blood circulation, to lay earlier, more and larger eggs, and to produce more fertilised eggs. I also hypothesised that females paired with a more preferred male would bias the sex ratio of their brood in favour of sons, in order to confer the perceived quality of their mate on their offspring (sex allocation theory).

2. Hidden signals of nature: Investigating avian reproductive strategies based on vegetation phenology and smell.

A central topic in the study of the impact of environmental changes on biodiversity is to try to understand how organisms perceive their environment, in order to be able to predict how they are likely to respond to these changes. A second research avenue of my thesis project was therefore to investigate **whether** two widely distributed and extensively studied bird species, the **blue and great tits, use vegetation phenology as a source of information to orchestrate their reproduction, and whether the influence of vegetation on bird reproduction operates through plant chemical compounds signalling future insect availability**. To investigate these questions, I combined descriptive (**Chapter 2**) and experimental approaches, both in the laboratory (**Chapters 3 and 4**) and in the field (**Chapter 4**).

I started by investigating **the link between the leafing of oak trees, and the reproductive timing and investment of blue and great tits, in the wild (Chapter 2)**. This relationship has been described in several populations^{186,187,195,321}, but never at the level of a breeding pair territory. Establishing this correlation requires assessing the phenological stages of the trees surrounding each nest-box, and on multiple years; meaning a labour-intensive and time-consuming activity. To overcome this constraint, I used images from two high-spatial-resolution satellites, on which I quantified the leafing of trees via an index of plant

photosynthetic activity: the Normalised Difference Vegetation Index (NDVI). In parallel, I monitored birds' laying dates, and tested whether they can be predicted from the phenology of the trees located in each bird territory. Finally, I tested whether the reproductive success of blue and great tits varies as a function of their adjustment to the local tree phenology (**article 3**). I hypothesised that earlier vegetation development would be associated with earlier bird laying dates, and conversely. Additionally, I predicted that individuals whose breeding phenology better synchronised with that of the local trees would have a greater reproductive success.

Chapter 2 outlines the relationship between bird reproduction and plant phenology. However, this chapter solely offers a descriptive approach to this interplay and thus does not provide a clear comprehension of the causal links between these two parameters, neither by which sensory channel birds could use plant phenology as a reproductive cue. The second objective of this section was therefore to determine whether bird reproductive phenology and investment are influenced by the olfactory perception of the chemical compounds emitted by growing buds attacked by herbivorous insects. I achieved this aim by adopting an approach mixing experiments conducted under controlled conditions (**Chapter 3 and 4**) and in natural populations (**Chapter 4**).

In **Chapter 3**, I investigated **the effect of the volatile emissions released by caterpillar-infested tree buds on the reproductive behaviours and physiology of captive birds**. HIPV emissions indicate the timing of emergence and the abundance of caterpillars in the environment^{211–213,322}. Consequently, if birds are able to detect this alarm signal in early spring, they could adjust the start of their reproduction and their clutch size to match the predicted food availability in their territory at the time of chick rearing. In a first experiment with blue tits (**article 4**), we exposed pairs of blue tits housed in airtight compartments, to a continuous air flow that either came from enclosures containing oak trees infested with caterpillars, or from empty enclosures. We then quantified gonadal growth in the spring, with the hypothesis that the perception of alarm odours emitted by infested buds would induce a more rapid growth of the female ovaries and male testes. In a second experiment (**article 5**), I have set up a larger-scale version of the previous experiment, housing pairs of great tits in 36 climate-controlled aviaries, an experimental infrastructure unique in Europe. Pre-breeding great tits were exposed to caterpillar-infested oak trees hidden behind an opaque screen, so that birds could only smell, and not see the trees. Every other week, blood was sampled from the birds to monitor their reproductive hormone levels, and birds' reproduction (laying date, clutch size) was followed over the duration of the experiment. I hypothesised that females and males

exposed to olfactory emission of caterpillar-infested trees would show an earlier and greater increase of their sex steroid concentrations, and would lay earlier and more eggs.

Studying organisms in captivity allows us to control some parameters of their environment, thereby helping us to understand the factors that influence their physiological mechanisms and their behaviours. Nevertheless, keeping animals in captivity subjects them to a relatively artificial environment, which can cause stress due to the various disturbances to which they are regularly exposed (feeding, artificial light, noise). Furthermore, replicating the complexity of the natural environment is challenging, if not impossible. Such disruptions may alter the birds' response to the factor being tested. Therefore, captive studies must be completed with field studies. In **Chapter 4**, I examined **the influences of an artificial bouquet of olfactory compounds, mimicking the emissions of caterpillar-infested oak buds, on blue and great tit reproductive decisions, directly in the wild**. Before I began my thesis, the team in which I worked identified the chemical compounds emitted by oak buds infested with caterpillars. We subsequently created an artificial blend of HIPVs, replicating the alarm signals emitted by trees when attacked by herbivorous insects. I first tested the behavioural attractiveness of this blend on captive blue tits (**article 6**). The aim of this experiment was twofold: to validate the artificial bouquet of odours for the experiments conducted in the field (see below), and to test whether the perception and recognition of HIPVs by birds were innate or learned. I then used the artificial bouquet in natural populations, to test whether the presence of these odours in early spring would lure the birds into an earlier reproduction and a higher investment. Since the olfactory cue mimicked a signal of earlier presence of caterpillars, and increased the overall concentration of HIPVs in the field, I hypothesised that birds would lay earlier and more eggs in areas where the odour was supplemented. However, as I only modified the signal of caterpillar presence but not the abundance of food itself, I also expected that birds would experience reduced fledging success.

General method



Species studied

General ecology

The great tit (*Parus major*) and blue tit (*Cyanistes Caeruleus*) are two small passerine birds belonging to the family of Paridae, the great tit being slightly larger than the blue tit (9-12 g for the blue tit, 16-21 g for the great tit, **figure 6**). They are usually resident and non-migratory birds and are widespread throughout Europe and the western Palearctic. They are common in deciduous and mixed woodlands with a high proportion of oak trees, but also occupy urban areas such as gardens and parks. In spring, they feed on insects, especially lepidopteran caterpillars at the time of nestling rearing. Outside of the breeding season, they become granivorous, continue to feed on insect-resistant forms such as eggs and chrysalises, and occasionally consume tree buds and fruits ^{323,324}.

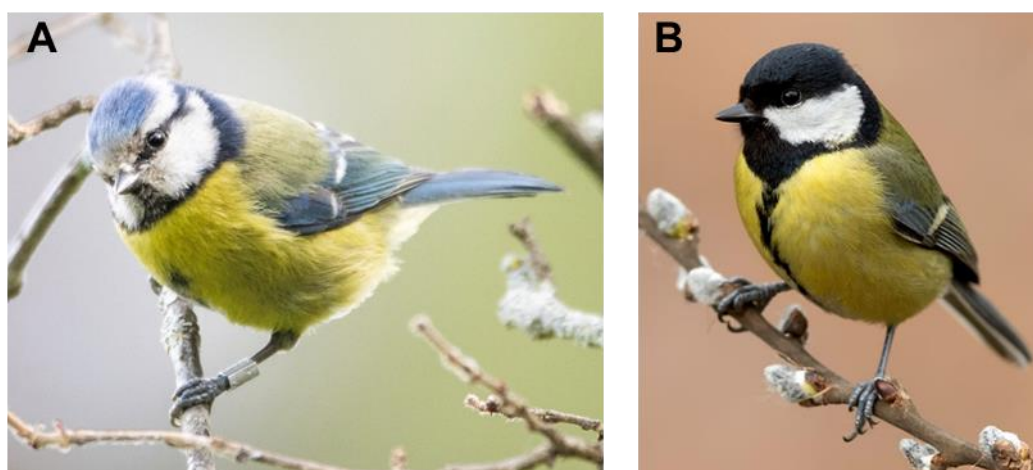


Figure 6: Studied species.

(A) Blue tit (*Cyanistes caeruleus*) © David López Idiáquez. (B) Great tit (*Parus major*) © Piotr Krzeslak.

Reproduction and its monitoring

Both species are socially monogamous breeders and establish breeding territories as early as late January ^{323,325}. They nest in cavities and readily occupy artificial nest-boxes. Nest-building is a female-restricted activity. The nest is made of moss, feathers, hair, and wool. The female lays one egg per day, and clutches range 6 to 13 eggs for the blue tit and 5 to 12 eggs for the great tit, usually in April. The female then incubates the eggs alone for 14 days. After hatching, the nestlings stay in the nest for approximately 21 days and are fed by both parents. Once they have left the nest, parents continue to feed them for approximately three weeks, before they finally become independent. Second clutches are common in great tits, but less common in blue tits ^{323,324}.

Birds' reproduction was monitored every breeding season, from nest-building until clutch completion in captivity, or until chick fledging in the field. The laying date of the first egg, egg size, the number of eggs, hatchlings, and fledglings were monitored. In the field, adults were captured while feeding at the nest. Each bird was identified with a metal ring and morphological measurements were taken (**figure 7**). All chicks were measured and ringed before fledging (around 15 days old). In captivity, I also took blood samples from adults to analyse steroid hormone levels (**Chapter 1 article 2, Chapter 3 article 5**), and gonads were measured (**Chapter 3 article 4**).



Figure 7: *Reproduction monitoring and biometric measurements.*

(A) Different stages of reproductive monitoring, from nest-building to measuring a 15-day-old chick. (B-C) Ringing and measurement of the wing length of an adult blue tit © Anne Charmantier, Christophe de Franceschi.

Study locations

Although descriptive field studies provide a good assessment of the natural behaviours of living organisms, understanding the mechanisms controlling behaviours and physiology often requires complementary experimental methods in captivity. I have therefore used both approaches in my thesis. I describe in this section the housing conditions of the birds in captivity (**Chapters 1, 3 and 4**), as well as my field study sites (**Chapters 2 and 4**).

Captive studies

In **Chapter 1 (article 1)**, **3 (article 4)** and **4 (article 6)**, I studied blue tits, originating from the long-term studied population of La Rouvière (see below) at the Centre d'Ecologie Fonctionnelle et Evolutive (CEFE) in Montpellier, France. They were born in the wild in 2019,

collected as chicks (10 days old) in nest-boxes, and hand-raised in captivity until independence (35 days old), following the procedure described in Reparaz et al., (2014)³¹³ and Caro et al., (2021)³¹². Adult birds were kept in large outdoor aviaries (2.5 x 3 x 3 m) containing deciduous (*Quercus humilis*, *Ficus carica*) and/or evergreen (*Quercus ilex*, *Ligustrum ovalifolium*) vegetation as standard housing. They were fed with cake made of sunflower seed grease, eggs, sugar, wheat flour and high-protein pellets, supplemented with minerals, vitamins, amino-acids and carotenoids and mealworms. Food and water were provided *ad libitum*. Two nest-boxes were available in each aviary.

In **Chapter 1 (article 2) and 3 (article 5)**, I studied great tits at the Netherlands Institute of Ecology (NIOO-KNAW) in Wageningen, the Netherlands. Birds were the second generation of a population originated from Boslust near Arnhem, the Netherlands, a 70 ha field site consisting of mixed pine-deciduous forest. Birds were hand-reared until independence as described in van de Heuvel et al., (2022)³²⁶ and Drent et al., (2003)³²⁷. Fully-grown birds were fed daily with a mixture of minced beef heart, canary egg-food, proteins, vitamins, minerals and trace elements, *ad libitum* dry food, a piece of apple three times a week, and water for drinking and bathing. Birds were housed in pairs in indoor climate-controlled aviaries (2 x 2 x 2.25 m) containing a perch and an artificial tree so that birds could perch and hide. Birds were kept under an artificial light regime mimicking a natural daylight pattern. The main source of light consisted of three high frequency fluorescent light tubes and an additional 7W incandescent light bulb mimicked dawn and dusk. Temperatures were set to mimic the temperatures in the wild. Three nest-boxes were available in each aviary.

Field studies

In **Chapter 2 (article 3) and 4 (article 6)**, I studied tit populations on two different sites in France.

Mainland site

The Rouvière forest, located 20 km north-west of Montpellier, near the town of Montarnaud (**figure 8**) is mainly composed of evergreen oaks (*Quercus ilex*) and downy oaks (*Quercus pubescens*). The populations of great and blue tits have been monitored since 1991 using artificial nest-boxes. 228 nest-boxes are placed at a distance of about 50 m from each other's, with 61% of the nest-boxes having a hole of 28 mm diameter in which only blue tits can breed, and 39% having a hole of 32 mm diameter in which both blue and great tits can breed.

Corsican site

I studied the blue and great tit Corsican populations in the north-west of the island in the Muro valley. This area is dominated by deciduous downy oaks. The monitoring of the population is spread over three different sites (20 nest-boxes in Avapessa since 1993, 20 nest-boxes in Feliceto since 2000; 60 nest-boxes in Muro since 1994 (**figure 8**)), located less than five kilometres apart. These populations are mainly composed of blue tits. 90% of the nest-boxes therefore have a hole of 28 mm diameter.

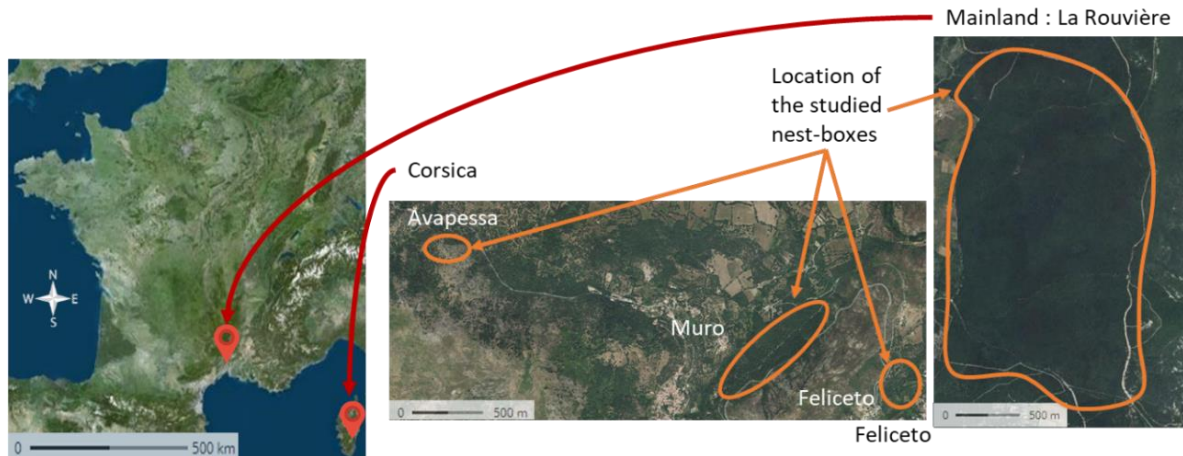


Figure 8: Map of the location of the two study sites.

Blue and great tit reproduction is monitored every year in the forest of La Rouvière, located 20 km north-west of Montpellier, in mainland France, and in the Corsican population around Muro. Photos modified from géoportail.fr.

Why these species and sites for this thesis?

Why study the effect of female preference on the reproduction of blue and great tits?

Both blue and great tits are socially monogamous species with biparental care, and previous studies indicate that females rely on male traits to decide with whom to mate, suggesting that mate choice might play a key role in reproductive success in these species ^{294,295,328–332}. Some studies have investigated the influence of mate choice on their reproductive investment and showed, for example, that blue tit females modify the sex ratio of their embryos ^{285–291,333}, their parental investment ^{292,334} or the amount of testosterone included in their eggs ²⁹³ in response to proxies of male quality, and that sex ratio and weight of great tit chicks are related to the male tarsus length ^{88,335}. All these studies were either conducted after experimental manipulation of the male phenotype, or female choice was investigated in the wild ^{295–299}. However, pairing is the result of a complex series of events mixing mate preferences, competition with other females for males or for territories, and male availability, leading finally to mate choice ^{300–303}. Many steps are therefore involved between the observed pairings in the wild and the initial mate preferences of females. In this thesis, I wanted to be able to control

some of these steps, and thus understand the direct effect of the female's initial preference for her mate on her reproductive investment.

Why study the effect of vegetation phenology on the reproduction of blue and great tits in the Mediterranean region?

Blue and great tits are common breeders throughout Europe and western Asia, where they readily breed in nest-boxes, which facilitates the monitoring of their reproduction. Several studies have already linked blue and great tit reproduction to vegetation phenology in different populations, but most of them suggest that, if a relationship exists between both, this correlation occurs at the population level ^{187,195–197}. Tits in Corsica and on the mainland have been monitored since the 1990s, providing ample data for studying this correlation ¹⁹³. Bourgault et al. in 2010 ¹⁸⁶ found a strong correlation between oak phenology and annual means of laying dates of blue tits in Corsica. This link is however largely driven by the fact that birds breed in very contrasting habitats, with populations breeding in evergreen forests having late phenologies, and populations breeding in deciduous habitats having early phenologies. Szulkin et al. in 2015 ³²⁹ also found in La Rouvière forest that the number of evergreen oaks was positively associated with later breeding dates in blue tits. As selection at the population level may operate differently than at the individual level ³³⁶, in this thesis, I wanted to investigate whether the relationship described between oak phenology and bird reproduction was still maintained at a level relevant for a single breeding pair.

In addition, as blue and great tits reproduce in captivity, they are suitable models to study the physiological mechanisms that regulate their reproduction throughout the breeding season. Some studies have explored the link between vegetation phenology and bird reproductive timing and physiology. Visser et al. in 2002 ³³⁷ and Schaper et al. in 2011 ³³⁸ tested whether birds use the visual detection of tree phenology to time their breeding, but found no effect on either breeding timing or gonadal growth. However, birds were only exposed to tree branches and not whole trees, which may not provide a relevant cue for the birds to make reproductive decisions. Finally, the growing body of literature suggesting that birds can use their olfactory capacities to detect herbivorous insects include blue and great tits in most cases ^{202,203,278,279,260,263,267,269–273} (see **Table 1** in introduction). The only study carried out so far investigating the link between volatile emissions of caterpillar-infested trees and blue tit reproduction has produced encouraging results, namely that males with higher levels of testosterone spent more time close to the olfactory cue ²⁶⁹. This study suggested a potential link between the perception of HIPVs and bird reproduction, and calls for further investigations.

Methods to study female mate preference and their effects on reproduction

In **Chapter 1**, I investigated the role of mate preference of blue (**article 1**) and great tit (**article 2**) females on their reproductive investment. On the one hand, studies investigating the traits used in mate choice, and their consequences for reproductive investment, have usually been conducted in the wild^{295–299}, where it is often impossible to control, and even know, all the factors that influence mate choice. On the other hand, in captive studies, females have only been offered a limited number of potential mates^{312–320} and male phenotypic traits are often manipulated^{304,306,307,310,312}. I have adopted an intermediate approach, combining the advantages of wild and captive studies, to test the effect of female mate preference on reproductive investment.

Testing female mate preference

I tested female preferences for a mate in a carousel-shaped six-choice chamber inspired by Zandberg et al., (2017)³²⁸ (**figure 9**). In this set-up, each female could see all stimulus males from a central platform, but when in the choice zone of a stimulus male, she could not see the other males. Each female was tested once with a group of six stimulus males, for 90 min. The experiment was video-recorded using a central camera affixed above the test chamber. Each group of six males was randomly selected from the available males, with the constraint that the males could not belong to the family of the tested female. The time spent by the females in the choice zone of each stimulus male bird was extracted from each video and used as a proxy for mate choice^{339,340}.

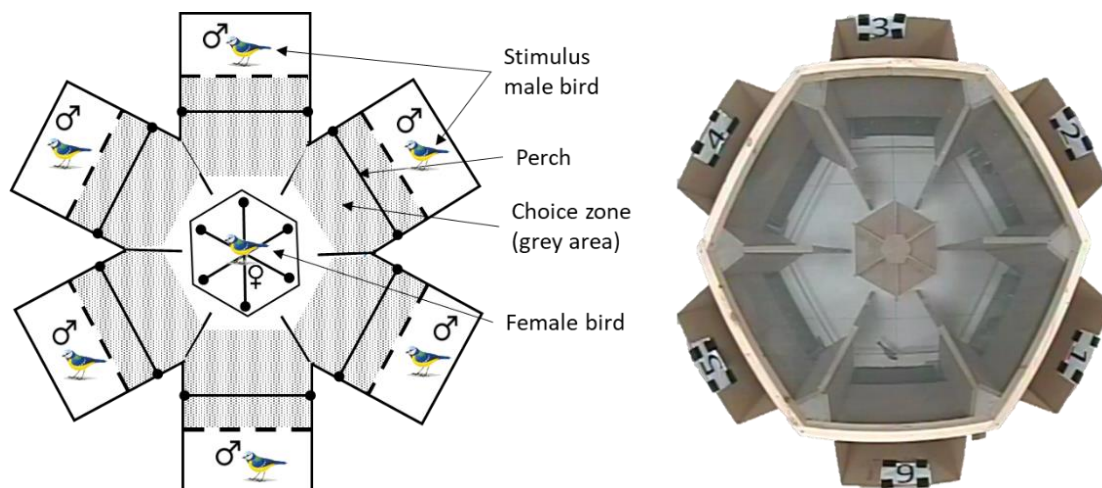


Figure 9: *Experimental set-up for the mate preference test.*

The preferences of blue and great tit females were tested in a carousel-shaped six-choice chamber for 90 min. From the hexagonal platform in the centre, the focal female could observe all males, whereas, in a choice zone, only one male was visible. On the picture on the right, the female is in the choice zone of male 6.

Testing the effect of mate preference on reproductive timing and investment

In order to assess whether female mate preferences influenced female reproductive timing and investment, I conducted a first experiment with blue tits (**article 1**) in which I randomly distributed females across two groups: one group of females paired with the male with which they spent the most time in the preference test (preferred male) and one group of females paired with the male they avoided most (avoided male). I conducted this experiment over two years. On the second year, I reversed the groups, i.e. a female that had been paired with its preferred male the first year was paired with its most avoided male the second year. That way each female was used as its own control. Pairs were kept in outdoor aviaries (see above) during the breeding season.

With great tits (**article 2**), the idea was to proceed the same as for blue tits (**article 1**) (i.e. one group of females with their preferred male and one group of females with their avoided male). However, the preference test conducted with great tits was originally designed as a preliminary test to ensure successful breeding in climate-controlled aviaries, for the purpose of another experiment investigating the effect of HIPVs on bird reproduction (see **Chapter 3, article 5**). I consequently paired all females with their preferred male. Despite this, females differed in the total amount of time they spent with their preferred male during the mate preference test, thus I recorded variability in the strength of female preferences. After pairing, birds were housed in opposite-sex pairs in climate-controlled aviaries (see above) for the breeding season and I monitored their reproduction (see below).

Methods to study the effects of vegetation phenology on reproduction

Two methods can be adopted when studying the influence of environmental factors on organism reproduction: a descriptive approach to establish correlations between the factors of interest, or an experimental approach to investigate the causal links and gain a deeper understanding of the mechanisms involved in the relationship between the parameters investigated. This latter approach can be conducted in captivity, to control environmental conditions more precisely, or in the wild to get as close as possible to the real conditions experienced by the organisms. In my thesis, I decided to combine these approaches to benefit from their respective advantages. First, I conducted a descriptive analysis of the link between vegetation phenology and avian reproduction (**Chapter 2**). Second, I used an experimental approach, both in captivity (**Chapter 3**) and in the field (**Chapter 4**), to explore the sensory pathway by which birds are likely to use vegetation as a cue for their reproductive decisions.

A descriptive approach: on the use of satellite imaging to assess vegetation phenology

In **Chapter 2**, I describe the correlation that exists in nature between the budburst of oak trees and tit reproductive timing and investment. Classical methods to measure vegetation phenology are based on visual assessment of the phenological stages of buds on several trees around a point of interest, making it a relatively time-consuming activity that can hardly be applied to an entire forest^{186,187,197,341}. Remote sensing has proven an effective alternative to data collection in the field in research on phenology^{342–346}. Furthermore, the potential to study phenological timing across large surfaces, and at a spatial resolution relevant for a single individual, can now be achieved through the use of satellite-derived vegetation indices^{321,347–351}. I calculated the Normalised Difference Vegetation Index (NDVI), a satellite-derived indicator of vegetation primary productivity used to determine the length and the peak of the growing season³⁵², from high-resolution satellite images, to estimate the date of leaf emergence (“date of vegetation green-up”) around each nest-box in La Rouvière forest (see above). I then compared the vegetation green-up dates to the bird laying dates and measured the reproductive success of breeding pairs as a function of their adjustment to the local tree phenology (details in **article 3**). While similar methods have already been used to link vegetation phenology with animal distributions, or breeding phenology and performance, at low spatial resolution, i.e. between 240 m and 8 km^{195,353–360}, my study is the first to use a vegetation index derived from satellite images to investigate the effect of vegetation development on bird reproductive traits at a level relevant to one single breeding pair.

Investigating the use of Herbivore-Induced Plant Volatiles in avian reproduction: complementary experiments in captivity and in the field

I adopted a complementary approach to study the underlying mechanisms linking HIPVs and avian reproduction. I conducted experiments in captivity (**Chapters 3 and 4**), as well as in natural populations (**Chapter 4**).

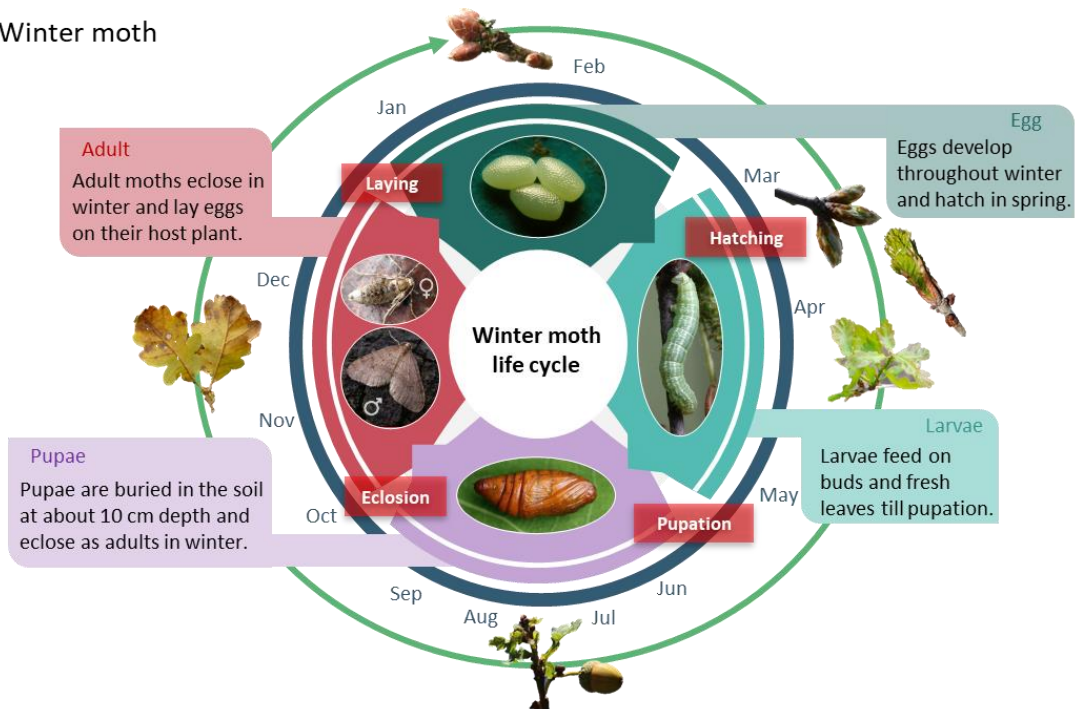
Experiments in captivity: bird reproductive decisions in response to natural HIPVs

In previous studies investigating the effect of HIPVs on bird behaviours, the volatile compounds tested were HIPVs emitted by mature leaves infested with large caterpillars, and the response of the birds were examined in a foraging context^{260,263,270}. Since HIPVs start to be emitted as soon as freshly hatched caterpillars (~1 mm) enter tree buds early in spring (Graham et al., unpublished data), and because studies have shown that the amount of HIPVs emitted is proportional to the level of infestation^{211–213}, HIPV emissions could also reveal the timing of emergence and the abundance of caterpillars in the environment. Whether insectivorous birds

use this early spring information for deciding when to start reproducing, and how much to invest in clutch size and chick feeding, has never been considered. I therefore investigated whether blue and great tits perceive and use HIPVs emitted by oak buds, rather than leaves, when they start to be attacked by freshly hatched caterpillars, to adjust their reproductive behaviour (laying date, clutch size) and try to match the predicted food availability in their breeding territory.

In **Chapter 3**, I studied reproductive behaviours and physiology of captive birds in response to the perception of HIPVs released by oak tree buds infested with very young caterpillars. As I used Mediterranean blue tits (**article 3**) and Dutch great tits (**article 5**) as model species, I needed to select caterpillar species that these birds use as a food source for their chicks. In the Netherlands, the main caterpillar species infesting the common oak is the winter moth (*Operophtera brumata*)³⁶¹, while in the Mediterranean region, the green oak leafroller (*Tortrix viridana*) is one of the main prey species of tits³⁶². I consequently worked with green oak leafrollers for the blue tit experiment (**article 4**) and winter moths for the great tit experiment (**article 5**). These two caterpillar species have slightly different life cycles. Adult winter moths emerge in winter from their pupae that were buried in the soil, mate, and wingless females lay eggs on branches of host trees in late December/early January (**figure 10A**). The eggs hatch in early spring and the emerging caterpillars feed on a wide range of broadleaved trees, of which oak is the primary host¹⁸¹. The green oak leafroller is a more specialised species than the winter moth, as its host range is restricted to the genus *Quercus*^{178,363}. Adults emerge in late spring and early summer (**figure 10B**). Females lay eggs in pairs on the bark of twigs. The eggs experience diapause during fall and winter and hatch the following spring. Both species are highly selected to synchronise their hatching with oak budburst^{174,184,185}, making them good models for studying the release of HIPVs following herbivore attack on tree buds. If for each experiment, I used the caterpillar species that best corresponds to the tit diet in the wild, the HIPV emissions they trigger from oaks do not significantly differ between the two species, neither do caterpillars emit significant volatile compounds themselves (Graham et al., unpublished results).

A: Winter moth



B: Green oak leafroller

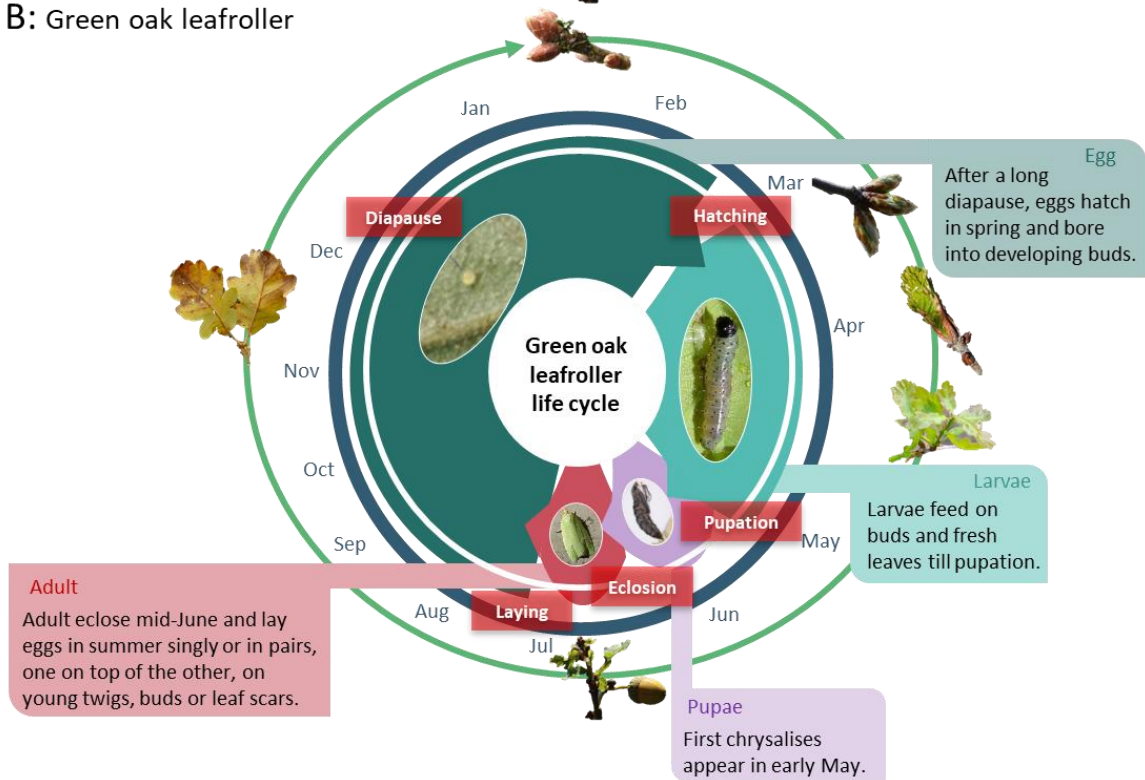


Figure 10: (A) Winter moth (*Operophtera brumata*) and (B) green oak leafroller (*Tortrix viridana*) life-cycles in parallel with the phenological stages of oaks.

These two caterpillar species have slightly different life cycles, but both are highly selected to emerge at the same time as oak bud burst. Adapted from (van Asch et al., 2007, Du Merle 1983) 174,178

In a first experiment, opposite-sex pairs of blue tits were housed indoor in terrariums (120 x 50 x 50 cm) (**article 4, figure 11A**), away from the stimulus trees, to remove any potential visual cues provided by vegetation. Within each terrarium, a wire mesh was used to separate males and females, because males are usually ready to reproduce before females^{103,118} and can become aggressive toward females in confined environments³⁶⁴. However, males and females could still interact visually and acoustically, which has been shown to be sufficient for birds to develop their gonads and lay eggs³⁶⁵. Every individual had access to a nest-box, and *ad libitum* food and water. Terrariums received air flow from one, out of four, outdoor enclosure. When oak buds of potted trees began to burst, four trees were placed into each of two treatment enclosure and were infested with freshly hatched green oak tortrix caterpillars. When buds on the trees began turning into mature leaves, trees in the enclosure were replaced with newly infested trees with buds at an earlier stage of development. Putting the trees into an enclosure enabled us to control the infestation level of the trees and to concentrate the HIPVs within the enclosure. The control enclosure contained four pots filled with soil, instead of four trees. We used pots filled with soil rather than uninfested trees in the control enclosure because this experiment was the first of a series of experiments. The idea was that if we obtained significant results regarding the effect of HIPVs on bird reproduction in this experiment, a subsequent step was to repeat the experiment with one group of birds exposed to HIPVs and another group exposed to uninfested trees, thus emitting constitutive volatile compounds from developing buds. This second experiment would allow to determine whether it is the alarm signal emitted by caterpillar-infested buds or the odour emitted by developing buds themselves that triggers the birds' reproductive responses. This second experiment could not be realised with the time available for this thesis. Bird reproduction was monitored during the breeding season, and three laparotomies were performed to measure gonadal growth in all birds.

This first experiment had a number of technical limitations. First, the sample size was relatively limited, with only six pairs of birds per treatment and year. Second, high humidity levels were observed in the terrariums. Even if some changes were made to the system in the second year of the experiment, this did not completely prevent humidity from forming in the terrariums, and water droplets in suspension have been shown to sequester some volatile molecules³⁶⁶. This may thus have reduced the odour signal reaching the birds. Finally, very few birds laid eggs during this experiment, presumably because of the limited space available for the birds³⁶⁴. As a result, birds may not have responded to HIPVs as strongly as they would have in a more natural environment. Therefore, in the second experiment with great tits in the

Netherlands (**article 5**), I have set up a larger version of the previous experiment. Opposite-sex pairs of great tits were housed in 36 climate-controlled aviaries (200 x 200 x 225 cm) (**figure 11B**), a unique system in Europe that allows to precisely control photoperiod and temperatures of the bird environment. In this system, a bigger housing volume prevents humidity from building-up in the aviaries, and birds can express more natural reproductive behaviours. Males and females were not separated and could interact acoustically, visually and physically, while they could only interact visually and acoustically in the terrariums. As in the previous experiment, when tree buds began to burst, two trees infested with freshly-hatched caterpillars were placed in the treatment aviaries, while two pots filled with soil were placed in the control aviaries. Trees and pots were hidden behind an opaque screen, so that the birds could only smell the trees, but not see them. In contrast to the enclosures/terrariums system, where the concentration of HIPVs might have decreased during the transfer from enclosures to terrariums due to ambient humidity, trees were here placed directly in the birds' aviary, allowing HIPVs to be emitted directly into the birds' environment. I monitored bird reproduction and every two weeks, blood samples were collected from the birds to measure their reproductive hormone levels. The improvements in the birds' living conditions with this device enabled 35 out of 36 females to lay eggs. As carrying out blood samples and laparotomies during the same breeding season is too invasive for such small birds, conducting two experiments enabled me to access complementary parameters of the birds' reproductive physiology.

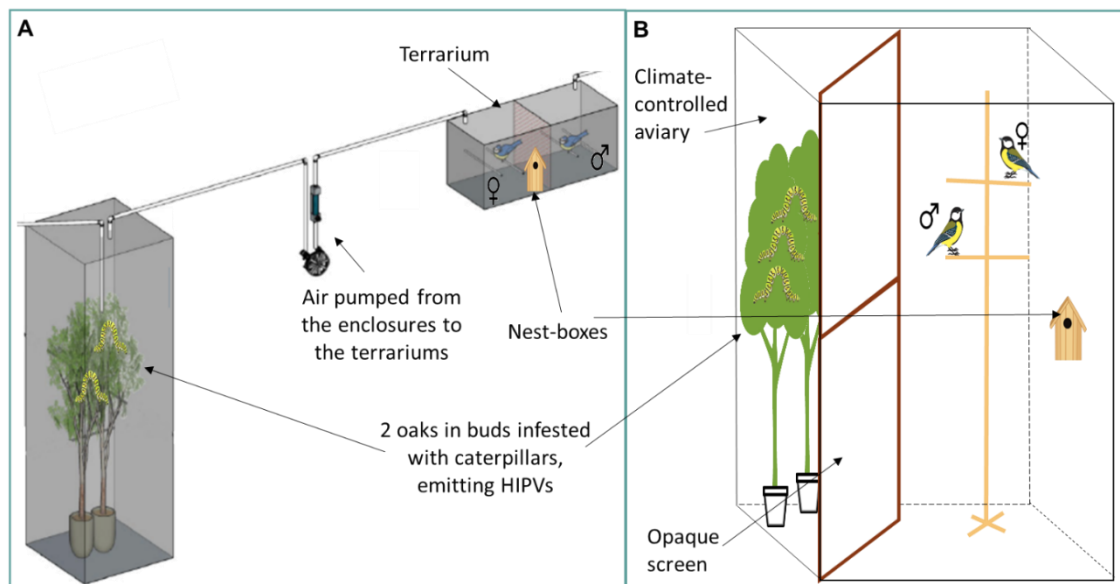


Figure 11: Experimental set-up used to expose captive birds to the odours of caterpillar-infested oak buds.

In both experiments described in **Chapter 3**, captive birds were exposed to the odours of caterpillar-infested tree buds. Birds were housed either (A) in terrariums (**article 4**) or (B) in climate-controlled aviaries (**article 5**). In both experiments, the birds could not see the trees or the caterpillars. (A) Adapted from (Caro et al., 2023)³⁶⁷.

Experiment in the field: bird reproductive decisions in response to artificial HIPVs

Experiments in captivity allow the fine control of some environmental factors and the focus on the effect of a variable of interest. However, the environment to which organisms are exposed in captivity is inevitably simplified. For instance, in my experiments, I have studied pairs of birds that were isolated from the other pairs, preventing any form of interactions, be it for a territory, food, mates, or breeding decisions. Returning to the field is therefore necessary to answer some questions about how natural populations adapt to, and interact with, the multiple factors constituting their environment.

In **Chapter 4**, I investigated the effects of artificial HIPVs mimicking the emissions of insect-infested oak buds, on blue and great tit reproductive decisions in the Mediterranean region. To set up this experiment, I relied on the work previously conducted by the team in which I worked at the CEFE. They identified the chemical compounds emitted in higher concentrations by downy oak buds infested with green oak leafroller and winter moth caterpillars compared to uninfested buds (Graham et al., in prep). Eight compounds were identified to be emitted in higher concentrations in infested buds (**figure 12A**) and we recreated an artificial blend with those same HIPVs (described below, **figure 12B**). I first tested the attractiveness of this blend on captive blue tits (**article 6**), mainly to validate its use for the experiment conducted in the field (see below). However, since the captive blue tits were collected as nestlings, hand-raised in captivity indoors and were not released into outdoor aviaries until late June (i.e. after the period when caterpillars forage on oak buds), I assumed that they were naïve to HIPVs emitted by oak buds in early spring. As a consequence, I also tested whether the perception and use of these alarm signals by birds were innate, or needed to be learnt, as it has so far been described in the literature ^{270,271,273,278} (see **Table 1** in introduction). Birds were tested for 30 min in a Y-shaped aviary (**figure 13**), in which one side of the aviary contained two flasks of artificial HIPVs and the other side contained two empty control flasks. The first side visited, the number of visits to each side and the time spent in each side were measured, to evaluate the bird attraction to the artificial blend of HIPVs.

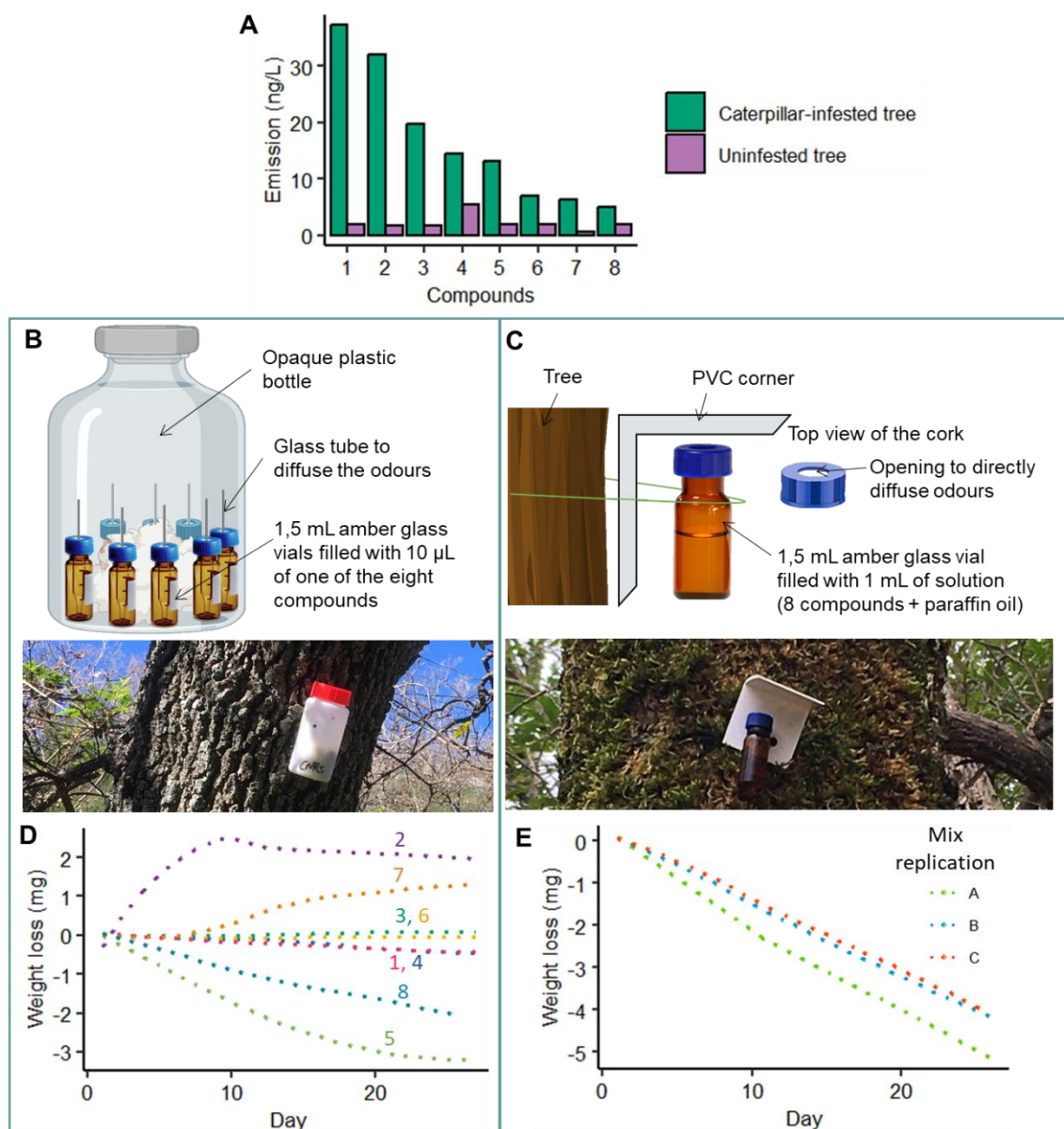


Figure 12: Flasks design and emission rates of the artificial HIPV blends used in the field.

(A) Differences in emissions of eight compounds emitted by caterpillar-infested and uninfested oak buds.

(B) In 2019-2020, flasks were made from eight 1,5 mL amber vials, each filled with 10 μ L of one of the selected compounds, all included in a 150 mL opaque plastic bottle.

(C) In 2021-2022, each flask was a 1,5 mL amber vial filled with 1 mL of a mixture of paraffin oil and a defined amount of each of the eight compounds depending on their chemical properties.

(D) Weight loss, estimating the emission rate, of each vial containing one compound using the method depicted in B. Ocimene (2) and caryophyllene (7) gained weight over time, indicating a possible change of nature of these compounds, potentially changing the odours emitted.

(E) Weight loss, estimating the emission rate, of three replication of the mixture depicted in C. The weight loss is stable and linear over time.

For (A) and (D): 1. Aromadendrene, 2. (E)- β -ocimene, 3. Farnesene, 4. (Z)-hex-3-enyl benzoate, 5. DMNT, 6. α -humulene, 7. (E)-caryophyllene, 8. Methyl salicylate.

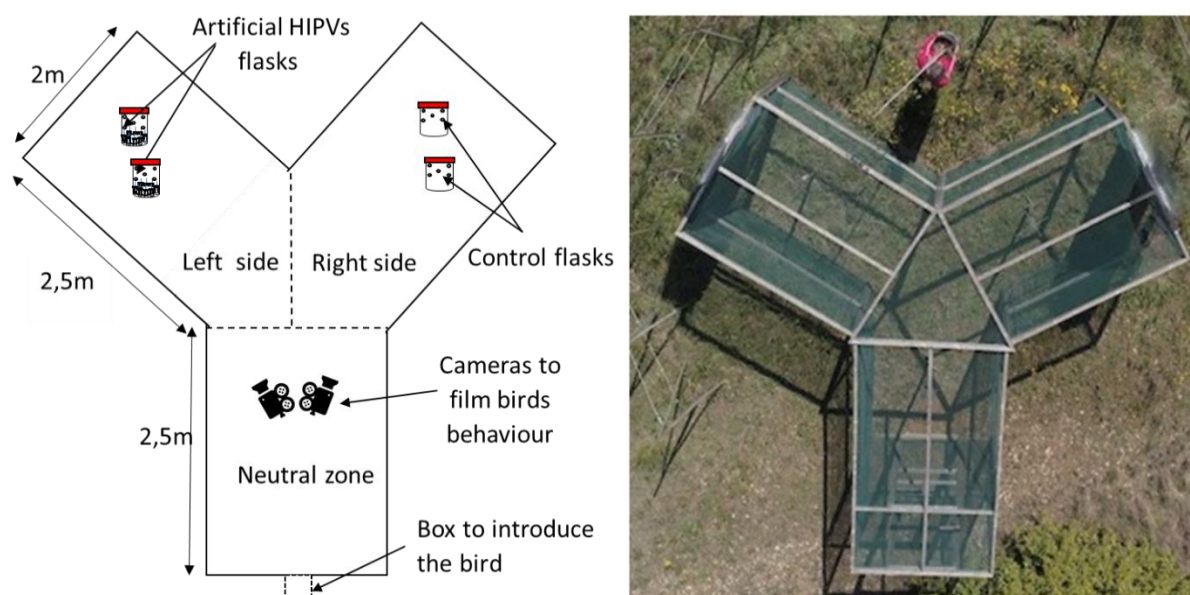


Figure 13: Y-shaped aviary used to test the bird attractiveness to the artificial blend of HIPVs. A small box fixed on the entrance door of the aviary was used to introduce the birds into the aviary. Birds were tested for 30 min. The first side visited, the number of visits and the time spent in each side were measured to evaluate the bird attraction to the artificial blend of HIPVs.

Once I established that birds were able to detect the artificial blend of HIPVs, we deployed the artificial bouquet in natural populations to test whether the presence of these odours in early spring would modify the reproductive timing and investment of birds (**article 6**). This experiment lasted four years and two different emission methods were used. The first emission method, used in the Y-shaped aviary and the first two years (2019-2020) in the field, consisted of 150 mL opaque plastic bottles, each containing eight 1,5 mL amber vials equipped with a thin glass-diffusion tube (**figure 12B**). Each amber vial was filled with 10 μ L of one of the eight selected compounds (**figure 12A**). Three odour diffusion bottles containing artificial HIPVs were placed in a triangle around half of the nest-boxes studied. The other half of the nest-boxes had three control bottles, each containing empty vials. Each flask was hung at the height of the nest-boxes and \sim 5 m from the nest-box.

The results from these first two years of experiments showed that, although the birds did not advance their laying dates, overall more eggs were laid in the areas where nest-boxes were surrounded by HIPVs flasks (see **article 6**). I hypothesised that concentrations of HIPVs, or the duration of the exposure was not sufficient to induce an effect on bird laying dates. I analysed the weight loss of each vial, which reflects the emission rate, to assess the quality of the emissions. A normal emission should show a constant weight loss over time, as for methyl salicylate (8) on **figure 12D**. However, the vials of some compounds, such as caryophyllene and ocimene, gained weight over time (**figure 12D**), suggesting a problem of emission with

these compounds. We noticed that sesquiterpene compounds such as caryophyllene, aromadendrene or farnesene, which are heavy compounds, tended to crystallise in the glass tubes (**figure 12B**). This potentially slowed down the emission rate of these compounds compared to other lighter compounds, leading to imbalances in the emission rates of our artificial bouquet over time. Moreover, one of the compounds, ocimene, tended to change colour as it oxidised. This suggested that this compound had changed in nature, thereby altering the odour emissions. We also did not consider the relative proportions of the different compounds, as each vial was filled with 10 μ L of compound. The ratio between the compounds could be an important cue for birds to identify HIPVs^{263,368}. Consequently, we developed a slightly different method to diffuse the artificial HIPVs for the subsequent two years of the experiment (2021-2022).

A different volume of each compound, reflecting its chemical properties, was mixed with paraffin oil, and 1 mL of this mix was introduced in a single amber glass vial (**figure 12C**). Gathering the eight compounds in a single vial with paraffin oil homogenised the mixture and ensured that the emission rate of each compound only depended on its molar concentration in the mix. By adjusting the volumes of the different compounds added to the mixture, and in particular by adding more of the compounds of lower molecular weight, which evaporate faster, we obtained emissions that were more stable over time (**figure 12E**). The use of paraffin oil also made our mixture hydrophobic, avoiding problems linked to interactions of the compounds with humidity. In addition, the compounds were released directly at the interface between air and paraffin oil, rather than through a glass tube. The larger opening avoided crystallisation in the glass tubes and provided the same exchange surface for all compounds. This method also had the advantage of using only one single vial, which facilitated the implementation of the treatment in the field (**figure 12C**). Lastly, we increased the number of vials around each nest-boxes from three to five, to increase the quantity of HIPVs to which the birds were exposed.

Chapter 1

The role of mate preference on reproductive performance in blue and great tits



Although animals' seasonal behaviours are tied to environmental conditions, their life cycle is also influenced by the social relationships they maintain with their peers³². Concerning reproduction, male sexual traits and courtship behaviours have been shown to modulate female reproductive physiology and investment^{54,61,292,369}. Not only the male's traits or behaviour, but also the degree of female preference for her male can influence her reproductive decisions^{75,76}. Indeed, the motivational or physiological state of the female can be strongly related to the preference for the male with which she is mated⁸⁹. However, many studies on female mate preference have been conducted in the wild^{295–299}, where it is impossible to disentangle the respective roles of the various components involved in mate choice (e.g., competition, mate availability) and their subsequent effects on reproduction^{300–303}. Studies conducted in captivity, on the other hand, often involve experimental manipulations of the male traits, and females generally have access to a restricted selection of males^{304,305,314–320,306–313}. Furthermore, while some studies have looked into the effect of male ornamentation on reproductive success in blue and great tits, none of the studies have examined the effect of female preferences on reproductive decisions in these species. In this first chapter, I investigated how female blue and great tits select their mate, and whether female investment in reproduction is influenced by their degree of preference for the male with which they are paired.

I conducted two distinct experiments with blue tits at the CEFÉ in Montpellier (**article 1**) and with great tits at the NIOO-KNAW in the Netherlands (**article 2**). I first asked female to express their mate preferences for six males in a carousel-shaped six-choice chamber. Then, I investigated whether females modulated their timing, or investment in reproduction according to their level of preference for the male they obtained. With blue tits, I paired females either with the male they preferred during the preference test, or with their most avoided male, in outdoor aviaries. However, with great tits, the main aim of the preference test was to pair females with a compatible male in indoor climate-controlled aviaries to ensure successful breeding of females for the purpose of another experiment (see **Chapter 3, article 5**). All female great tits were consequently paired with their preferred male. Despite this, females spent different amounts of time with their preferred male during the preference test, which allowed me to study the effect of female great tit preference on their reproductive investment. For both species, I monitored reproduction during the breeding season (laying date, egg size, clutch size), with the hypothesis that females showing a stronger preference for a given male would lay earlier, more and larger eggs. In blue tits, I determined egg fertilisation and brood sex-ratio of the embryos, assuming that females paired with a more preferred male would bias the sex ratio

of their brood in favour of sons. In great tits, I investigated the effect of female preference on their reproductive hormonal physiology, expecting them to show higher levels of sex steroids in the blood circulation when paired with a male they more strongly desired.

In blue tits, females preferred males with a blue UV chroma that matched their own, but also with less bright colours of the cheeks and the head crown whereas great tit females spent significantly more time near more exploratory males. In blue tits, I found no evidence for an effect of the pairing treatment on the timing of breeding, or any other aspects of female reproductive investment. Laying dates, clutch sizes, egg sizes and brood sex-ratio were significantly repeatable within blue tit females. In great tits, however, females mated with a highly preferred male laid their first clutch significantly earlier in the season than females mated with a less preferred male. Consequently, this chapter suggests that female reproductive investment in response to her mate preference may be highly species specific.

Contribution: Samuel Caro and I designed the protocol of both experiments. I then conducted the experiments (at the CEFÉ for the blue tits, at the NIOO for the great tits), analysed the data and wrote the first draft of both manuscripts.

Article 1: The manuscript entitled “Whoever their partner, female blue tits breed the same” has been accepted for publication in *Behavioral Ecology* in September 2023.

Article 2: The manuscript entitled “Female great tits (*Parus major*) reproduce earlier when paired with a male they prefer” has been published in *Ethology* in May 2023 (see appendices).

Article 1

Whoever their partner, female blue tits breed the same.

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Abstract

Do females modify their reproductive investment if they do not succeed to pair with a male that matches their preference? In a two-year experiment, we asked female blue tits (*Cyanistes caeruleus*) to select among six males, and then successively paired them with their preferred and their most avoided male. We monitored female reproductive investment through nest building activity, timing of breeding, size and number of eggs, number of fertilised eggs, and brood sex-ratio. We found that females preferred males with a chromatic coloration (blue UV chroma of the head crown) that matched their own, but also that they preferred males with a lower achromatic coloration (less bright color of the cheeks and head crown). Although females paired with their preferred males tended to build heavier nests during the breeding season, we found no evidence for an effect of the pairing treatment on timing of breeding, or any other aspects of female reproductive investment. We however found that laying dates, clutch sizes, egg sizes and brood sex-ratio were significantly repeatable within females between the two years, despite the opposite pairing treatments. These findings show that in female blue tits, the males with which they are paired do not substantially alter their reproductive decisions.

Keywords: reproduction; mate preference; birds; laying date; clutch size

Introduction

Mate choice is a major component of sexual selection in the animal kingdom (Andersson 1994; Cotton et al. 2006; Höner et al. 2007; Locatello et al. 2015). Individuals can derive benefits for themselves in the present generation, or for their offspring in the following generation, from carefully choosing a mate (Andersson and Simmons 2006; Jones and Ratterman 2009; Kokko et al. 2003). They can consequently be expected to evaluate traits indicating partner condition, characteristics and quality. Animals might use physical characteristics to select their mate, like coloration (Baube et al. 1995; Rowland et al. 1995; Pryke and Griffith 2007; Gomez et al. 2010) or size (McKaye 1986; Howard et al. 1998; Shine et al. 2001; Romero-Pujante et al. 2002; Hoefler 2007). They can also use behavioral traits such as song characteristics (Searcy 1992; Wagner and Reiser 2000; Byers and Kroodsma 2009; Riebel 2009; Caro et al. 2010; Iglesias and Hasson 2017), courtship behaviors (Zuk et al. 1990; Sargent et al. 1998; Kallman et al. 2015; Ota et al. 2015), personality (van Oers et al. 2008; Schuett et al. 2011; Bierbach et al. 2015; Chen et al. 2018; Delaitre et al. 2023) or cognitive abilities (Boogert et al. 2011; Kavaliers and Choleris 2017). Even the chooser's own phenotype, such as its condition, coloration or size (Hunt et al. 2005; Holveck and Riebel 2010; Riebel et al. 2010; Rueger et al. 2016; Caro et al. 2021), can play a decisive role at the time they decide with whom to mate. All those criteria can be used separately or in combination (Burley 1981a; Candolin 2003).

Females, historically considered the choosing sex (Jones and Ratterman 2009; Davies et al. 2012), were initially assumed to reach a consensus about who is the most attractive male, and inter-individual variation in female preference and in the optimal male phenotype were therefore expected to be low (Real 1990; Jennions and Petrie 1997). However, in some species, mating preferences seem largely individual-specific, suggesting that they might rather target genetic or behavioral compatibility (Jennions and Petrie 1997; Widemo and Sæther 1999; Brooks and Endler 2001; Forstmeier and Birkhead 2004; Cotton et al. 2006; Rosenthal and Ryan 2022).

Beyond the difficulty of understanding mate selection processes and targets, the impact of partner selection on female reproductive timing, investment, and physiology is also not easy to predict (Burley 1988; Frank 1990; Penn and Potts 1999; Mays and Hill 2004; Gowaty et al. 2007; Bolund et al. 2009). Two main, non-mutually exclusive hypotheses have been proposed to explain why females would invest differently depending on their preference for their partner.

First, the differential allocation hypothesis states that an individual adjusts its investment in its current reproductive effort according to the perceived attractiveness, or overall quality of its partner (Burley 1986; Burley 1988; Sheldon 2000; Haaland et al. 2017). A higher number of offspring and of higher quality are consequently expected when females are paired with a male of higher perceived quality (De Lope and Moller 1993; Gil et al. 1999; Cunningham and Russel 2000; Gowaty et al. 2003; Limbourg et al. 2004; Velando et al. 2006; Anderson et al. 2007; Gowaty et al. 2007; Walling et al. 2010). For example, female zebra finches lay on average 0.5 eggs more when paired with their preferred males, which produce songs of longer duration and faster rate, and are consequently considered higher quality males (Balzer and Williams 1998). In mallards (*Anas platyrhynchos*), females mated with a less-preferred male produce smaller eggs and therefore lower quality offspring (Cunningham and Russel 2000; Bluhm and Gowaty 2004).

Second, the sex-allocation theory predicts that, when the fitness of sons and daughters differs, parents should adjust the sex-ratio of their brood (Charnov 1983; Frank 1990; Booksmythe et al. 2017). Consequently, when sons inherit from their fathers some traits that determine their own attractiveness, females are expected to adjust their brood sex-ratio depending on the qualities of the male with which they are paired (Burley 1981b; Burley 1986; Hardy 2002; Saino et al. 2002; West and Sheldon 2002; Sato and Karino 2010; Bowers et al. 2013; Booksmythe et al. 2017). Females are therefore predicted to produce more sons when mated to a male of higher quality. Great tit (*Parus major*) females have for example been suggested to produce more sons when they are paired to larger males (Kölliker et al. 1999).

Studies investigating the traits used in mate choice and its consequences on reproductive investment have usually been conducted in the wild (Bonneaud et al. 2006; Foerster et al. 2006; Eriksen et al. 2009; García-Navas et al. 2009; Fargevieille et al. 2017). The drawback is that it is often impossible to control, and even know, all the factors that influence mate choice and its subsequent effects on reproduction. The pairings observed in the wild are indeed the result of a series of events mixing mate preferences, competition with other females for males and territories, male availability, and finally mate choice (Jennions and Petrie 1997; Widemo and Sæther 1999; Edward 2015; Scauzillo and Ferkin 2019). Disentangling the respective roles of the different mate choice components may therefore request experiments in controlled conditions, which artificial characteristics may in turn hamper the expression of natural behaviors. In the case of mate preference studies, many have only offered binary choices between two potential partners (Amundsen et al. 1997; Witte et al. 2000; Romero-Pujante et al. 2002; Tomaszycki and Adkins-Regan 2005; Burley and Foster 2006; Rutstein et al. 2007;

Reparaz et al. 2014; Kniel et al. 2015; Caro et al. 2021), or have only manipulated one phenotypic trait and not the overall signaling value of an individual (Clayton 1990; Bennett et al. 1996; Waas and Wordsworth 1999; Caro et al. 2021; Cantarero et al. 2022), which may lead to unnatural contrasts among the sexual signals conveyed (Caro et al. 2021).

In the present study, we adopted an intermediate approach mixing the advantages of wild and captive studies, to test the two hypotheses described above. We first investigated mate preferences of blue tits (*Cyanistes caeruleus*) in captivity by offering females the choice between six, unmanipulated, males. In particular, we evaluated whether male body size, personality and color traits explain the preference patterns expressed. We then paired half of the females with their preferred male and the other half with their most avoided one. We conducted this experiment over two years, so that each female was paired with its preferred male in one year and with its most avoided male in the other. Thus, each female acted as her own control. In line with the differential allocation hypothesis and the sex allocation theory, linking reproductive investment to mate preference, we hypothesised that females paired with their favorite male would lay more and larger eggs, and would produce more fertilised eggs (differential allocation hypothesis), and more sons (sex allocation theory). In addition, we also hypothesised that females paired with their favorite male would build heavier nests and lay earlier in the season. Indeed, nest-building activities of females are stimulated by the presence of a male (Hinde and Steel 1976), and males can stimulate female gonadal development and hormone secretions through social interactions (Erickson and Lehrman 1964; Hinde and Steel 1976; Hinde and Steel 1978; Silverin and Westin 1995; Stevenson et al. 2008; Perfito et al. 2015), which can result in earlier laying (Adkins-Regan and Tomaszycki 2007; Griffith et al. 2011; Crino et al. 2017; Delaitre et al. 2023).

Methods

Ethical note

Blue tits were trapped and maintained under licenses 2018-s-11 issued by the Direction Régionale de l'Environnement, de l'Aménagement et du Logement Languedoc-Rousillon; and 15-XIX-116 issued by the Direction Départementale de la Protection des Populations de l'Hérault. 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#2021120712067677 #34249 v2.

Birds and general procedures

Blue tits originated from the long-term studied population of La Rouvière (43.621°, 3.733°) at Montarnaud near Montpellier. They were born in the wild in 2019, collected as chicks (10 days old) in nest-boxes, and subsequently hand-raised in captivity until independence (35 days old), following the procedure described in Reparaz et al. (2014) and Caro et al. (2021). Birds were kept in large outdoor aviaries (27 m³) as standard housing. They were fed with 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) and mealworms. Food and water were provided *ad libitum*.

Preference test

Experimental subjects and housing

The experiment took place at the CEFE in Montpellier, France, for two consecutive years. We used 24 female and 29 male blue tits the first year and 23 females and 23 males the second year. All birds used in the second year had already been used the first one, in such a way that each female acted as its own control (see below). Birds were moved from aviaries to individual cages (0.8 x 0.4 x 0.35 m) on 8th December 2020 and on 11th January 2022, across 3 indoor rooms. Females and males were housed in separate rooms. Windows in rooms allowed birds to be exposed to natural light, and supplementary artificial lightning was provided from 15min after natural sunrise until 15 min before sunset to respect the natural increase and decrease of light intensity every day. Windows were left open to expose birds to natural fluctuations of temperature.

Experimental setup

Females were tested in a carrousel-shaped six-choice chamber inspired by Zandberg et al. (2017) (figure 1, figure S1) from 12th to 21st December 2020 for the first year and from 19th to 24th February 2022 for the second year. We ran the preference tests a bit later in 2022 compared to 2021 in order to be a bit closer to breeding. All tests took place in a test room (4.0 x 2.4 x 2.5 m) with white walls, and high-frequency fluorescent lights. In the six-choice setup, each female could see all stimulus male birds from the central platform, but when in the choice zone of a stimulus male, she could not see the other males (figure 1). The males were visually isolated from each other. Before being tested, each female was given 15 min to habituate to this

new environment. In the meantime, the six stimulus males were transferred from their home cage to six individual mobile cages (47 x 28 x 26 cm) in an adjacent room. At the end of the 15 min habituation period, each male cage was placed into one of the six compartments of the carousel. Each female was tested once with one group of six stimulus males during 90 minutes. The experimenter left the room at the beginning of the test, which was video-recorded using three GoPro cameras (GoPro, Inc., USA) affixed above the test chamber. After each test, all birds were returned to their individual home cages. Videos were analysed soon after the end of each test and, depending on the treatment to which the female was assigned for the breeding experiment (see below), the male with which she spent the most time (hereafter called the preferred male) or the lowest amount of time (hereafter called avoided), was pulled out of the pool of available males for the next females to be tested. Stimulus males could not belong to the family of the female tested and their positions in the setup were randomised for each test.

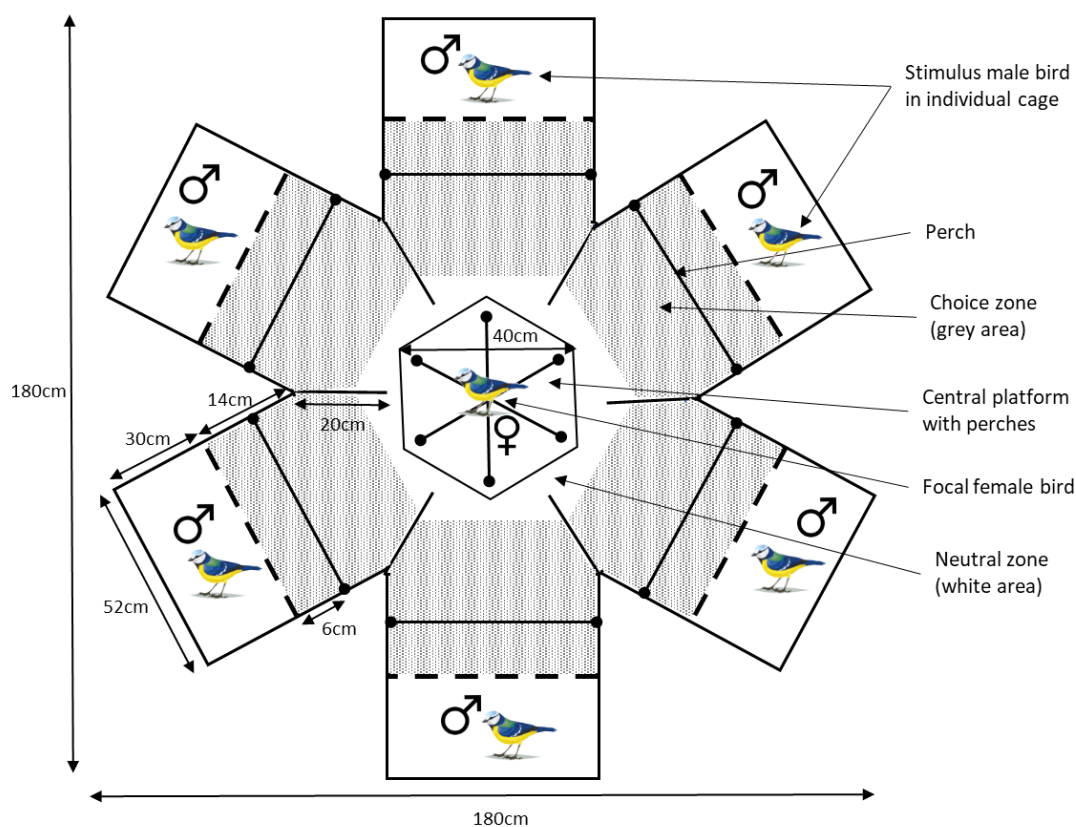


Figure 1. Experimental mate preference test setup. Females were tested for their preference in a six-choice setup for 90 min. From the hexagonal central platform, the focal female could observe all stimulus male birds, whereas only one stimulus male was visible from the perch in the choice zone. The stimulus male birds could not see each other. Time spent in the choice zone of each male was inferred as a proxy for preference. At the end of each test, videos were analysed and, depending on the treatment assigned to the female, the preferred (or avoided) male was pulled out of the pool of available males for the next females tested. Positions of the stimulus male birds were randomised for each test.

Video analysis

Videos were analysed using Behavioral Observation Research Interactive Software (BORIS) (Friard and Gamba 2016) version 7.9.7–14/01/2020 (<https://www.boris.unito.it/>). The time spent by the females in the choice zone of each stimulus male bird was extracted from each video, and used as a proxy for mate-choice (Gonçalves and Oliveira 2003; Witte 2006). All females visited the choice zone of each male at least once during the 90 min of the test. Neither the males nor the females showed any clear solicitation of mating behavior. The time spent by the female in the choice zone of the male was consequently the most appropriate and direct method for estimating female preference.

Phenotypic trait measurements

To identify on which criteria females choose their mate, a set of phenotypic traits was measured on each male and female tested, ahead of the preference tests each year. We measured an achromatic trait, brightness (i.e. the area under the reflectance curve divided by the width of the interval from 300–700 nm, and that can be defined as the total amount of light reflected from the measured surface (Saks et al. 2003)) for the white of the cheek, the yellow of the breast and the UV-blue of the head crown, and a chromatic trait, chroma (i.e. the proportion of the total reflectance falling in the color range) for UV-blue of the head crown ($R_{300-400} / R_{300-700}$) and yellow of the breast ($R_{\max\ 500-700} - R_{450} / R_{300-700}$) (Fargevieille et al. 2017). Higher UV-chroma values correspond to a stronger UV signal, and higher yellow chroma values are associated with higher levels of carotenoids in the plumage (Isaksson et al. 2008). We measured coloration of each bird with a spectrophotometer (AVASPEC 2048, Avantes, Apeldoorn, the Netherlands) equipped with an AVALIGHT-DHS deuterium-halogen lamp (Avantes, Apeldoorn, the Netherlands) and a 200 μm optical probe (FCR-7UV200-2-45-ME, Avantes, Apeldoorn, the Netherlands) whose tip contained a 45° quartz window. Reflectance was calculated in relative terms, via comparison with a black and a white reference (WS1, Ocean Optics, Dunedin FL, USA). We also took morphological measures, including body mass, tarsus, beak and wing lengths. Ahead of the present study, a personality score was derived from exploratory behavior trials in a novel environment, following the protocol described in Charmantier et al (2017) and Dubuc-Messier et al (2017). Briefly, each bird was released separately in a novel environment, which consisted of an exploration cage equipped with 2 perches (0.6 \times 0.4 \times 0.4 m). Once the bird entered the cage, its movements were observed for five minutes. Birds were given an exploration score on a continuous scale based on the total number of movements between the perches, as well as hops up and down and flights. Scores

ranged from 47.5 to 260.5, with higher scores indicating faster exploration, and lower scores indicating slower exploration. This kind of measurement has been used to measure exploration behavior in great tits (Stuber et al. 2013) and blue tits (Mutzel et al. 2013; Charmantier et al. 2017; Dubuc-Messier et al. 2017), and provides repeatable exploratory scores (Dingemanse et al. 2002; Stuber et al. 2013).

Breeding experiment

Pair formation and housing of birds

Before the first preference test, females were randomly distributed across two groups: 12 females paired with the male with which they spent the most time and 12 (11 in 2022) females paired with the male they avoided most. On the second year, we reversed the groups, i.e. a female that had been paired with its preferred male the first year, was paired with its most avoided male the second year. That way each female was used as its own control. Females and males were released simultaneously in outdoor aviaries (2.5 x 3 x 3 m). Pairs were moved in aviaries on 4th January 2021 and on 28th February 2022. Nesting materials consisting of moss and dog, horse and donkey hair were provided from early March.

Reproduction monitoring

Starting from mid-March, we monitored nest building and egg laying activities, first once a week, then twice a week as birds were closer to lay (Dufva 1996). The date the first egg was found was recorded as the pair laying date. Tits usually lay one egg every 24 hours (Perrins 1970), so if more than one egg was found in the nest at the day of checking, we back calculated the laying date. All eggs were measured to the nearest 0.05 mm with a calliper (Ecotone Measy DG), marked with a fine point marker, and replaced in the nest until clutch completion. After at least five days of incubation, nest and eggs were collected, and eggs were candled to identify if an embryo was developing. Eggs were then wiped with paraffin oil and stored at -20°C until further analyses (see below). Nests were dried in an oven at 40°C for 48h and then weighted to the nearest 0.1 g (Precisa, France).

Egg analyses

Germinal disc observation

127 eggs the first year and 177 eggs the second year were collected and preserved in a freezer. Eggs were candled when they were collected, but this method does not enable to distinguish between an unfertilised egg and a fertilised egg in which the embryo died at an early

stage of development. We thus examined the germinal disc (GD) following the method described in Birkhead et al (2008) to identify if an egg was fertilised or not (figure S2). In short, we opened the eggs, discarded the albumen, placed the yolk in a small Petri dish and examined the aspect of the GD under a binocular dissecting microscope (Optika, Italy). Photos of the observed GD was taken with a camera (OPTIKA C-B5), and the OPTIKA PROview software (version x64, 4.11.18914.20210510, <https://www.optikamicroscopes.com/optikamicroscopes/>).

Embryo sexing

107 out of the 127 eggs collected the first year and 117 out of the 177 eggs collected the second year contained an embryo and we succeeded to sex 105 and 114 of those embryos the first and second year, respectively. We sampled between 5 and 20 mg of embryo tissue and extracted the DNA using the DNeasy Blood & Tissue Kit (Ref 69506, QIAGEN). DNA extraction products were analysed by agarose gel electrophoresis (1% in TBE) and visualized under UV light after staining with ethidium bromide, using a molecular size marker of a 1 kb Plus “ladder” type (Quick-Load, N0469S, New England BioLabs Inc.). The sex identification test employs the P8 (5'-CTCCCAAGGATGAGRAAYTG-3') and P2 (5'-TCTGCATCGCTAAATCCTTT-3') primers (Griffiths et al. 1998) and PCR amplification was carried out in a total volume of 20 μ L. For each individual, reaction conditions were as follows: 1 μ L Buffer 10X (Eurogentec, Belgium); 1.5 μ L MgCl₂ 25mM; 1 μ L dNTP Master mix 20mM total (Eurogentec, Belgium); 1 μ L P2 10 μ M (Eurogentec, Belgium); 1 μ L P8 10 μ M (Eurogentec, Belgium); 0.2 μ L 0.15 units of Red diamond Taq DNA polymerase (Eurogentec, Belgium), 12.3 μ L H₂O; 1 μ L DNA. PCR was performed in a thermal cycler (Eppendorf AG, Hamburg, Germany). An initial denaturing step at 94 °C for 5 min was followed by 30 cycles of 48 °C for 45 s, 72 °C for 45 s and 94 °C for 30 s. A final run of 48 °C for 1 min and 72 °C for 5 min completed the program. PCR products were analysed by agarose gel electrophoresis (3% in TBE) and visualized under UV light after staining with ethidium bromide, using a molecular size marker of a 100 bp “ladder” type (Quick-Load, N0467S, New England BioLabs Inc., USA).

Statistical analyses

All the analyses were performed in R version 4.2.2 (R Core Team 2022). Linear mixed-effects models and generalized linear mixed-effect models were conducted using the lme4 version 1.1.31 (Bates et al. 2015) and glmmTMB version 1.1.5 (Brooks et al. 2017) packages. P-values were obtained with the lmerTest package version 3.1.3 in the case of mixed-model

analyses (Kuznetsova et al. 2017). Plots were created with the ggplot2 version 3.4.0 (Wickham 2016) and ggeffects version 1.1.5 (Lüdtke 2018) packages. Models were simplified using backward elimination of the nonsignificant terms, starting with the higher-order interactions (Crawley 2007), we set $\alpha=0.05$. Correlations were calculated and plotted with the Hmisc version 4.7.2 (Harrell 2022) and corrplot version 0.92 (Wei et al. 2017) packages, respectively. Principal component analyses were run with FactoMineR version 2.7 (Husson et al. 2016) and factoextra version 1.0.7 (Alboukadel and Mundt 2017) packages. Repeatability was measured using the rptR package version 0.9.22 (Stoffel et al. 2017).

Preference test

We ran a linear mixed-effect model with the time (in seconds) spent by the female in the choice zone of each male as response variable. Explanatory variables consisted of each male trait, alone and in interaction with the female corresponding trait. These included brightness (white of the cheek, yellow of the breast, UV-blue of the head crown), chroma (yellow and UV-blue), body mass, tarsus, wing and beak size and exploration score. As we had an important number of variables on a relatively restricted number of individuals, we first checked correlations between variables (figure S3). Principal Component (PC) were used for body size to decrease the number of variables to add in the model. It included body weight and tarsus and wing length (figure S4). Identification of the female and year were added as crossed random intercepts, to account for the fact that each female occurred six times in the dataset (once for each six different males) and that females were used in both years of experiment. The response variable (i.e. the time spent in the choice zone of the males) was weighted by the “interest” of the female to allow more statistical weight to the females that spent more time evaluating the males during the test rather than staying in the neutral zone (Reparaz et al. 2014; Caro et al. 2021). “Interest” thus depicts the overall motivation of the female to visit the males during the experiment by indicating the proportion of time that the female spent close to the six stimulus males (i.e. not in the central neutral zone), and was calculated as follows (Reparaz et al. 2014; Caro et al. 2021) :

$$\text{Interest} = \frac{\text{Time spent with (male 1 + male 2 + male 3 + male 4 + male 5 + male 6)}}{\text{Total duration of the test (90min)}}$$

Breeding experiment

Out of the 47 pairs that were formed across both years, 4 pairs had to be removed from the statistical analyses (because a member of the pairs died or was injured before or during the experiment), we thus analysed the reproductive behavior of 43 pairs.

We first used binomial tests to compare the number of females that (i) built a nest and (ii) laid at least one egg, between females paired with their preferred and avoided male, as well as (iii) the total number of eggs laid by all the females on the whole breeding season between the two groups.

We tested the effect of the pairing treatment on female reproductive parameters. We performed linear mixed-effect models on (i) nest weight, (ii) lay date, (iii) egg measures (PC1, see below) and generalized linear mixed-effect models with Poisson distribution on (iv) clutch size and with binomial distribution on (v) the proportion of fertilised egg and on (vi) the proportion of male chicks produced by each female during the breeding season. PC were used for egg measured because egg length, breadth and volume were correlated. The PC1 axis explained 80.6% of the variability in the data (figure S5). In all models, the treatment (preferred vs. avoided) and the year (2021 vs. 2022) were included as explanatory variable. Female ID was included as random effect to account for the fact that the same females were used in both years, and that a same female laid several eggs. As 11 females laid on both years, we measured repeatability of the female identity for each model using the rpt function.

Results

Preference test

Females spent more time close to males with a blue UV chroma of the head crown that was similar to their own ($p = 0.036$, Table 1, figure 2A). That's the only phenotypic traits for which females referred to their own phenotype, all the other interactions between male and female characteristics were non-significant (Table 1). Females also spent more time close to males with a duller blue head ($p = 0.007$, Table 1, figure 2B) and tended to prefer males with duller white cheeks ($p = 0.084$, Table 1, figure 2C). We did not find that females were attracted by any other male phenotypic trait, including body size (PCA body size: $p = 0.544$, beak length: $p = 0.707$, Table 1) and exploration score ($p = 0.514$, Table 1).

Table 1. Results from a linear mixed effect model, exploring the variables that influence the time spent by the females in the choice zone of the males during the 90 minutes of the test ($n = 47$ females). Variables in bold had a significant effect on the response variable ($p < 0.05$), italicized variables depict trends ($p < 0.10$). Variables in light grey were removed during the reduction of the model, and presented in the reverse order in which they were removed.

Explanatory variable	Estimate	S.E	df	F value	p-value
Intercept	213.07	77.21			
PCA Body size male	15.74	25.87	124.11	0.37	0.544
Beak length male	11.95	31.75	125.91	0.14	0.707
Exploration score male	13.88	21.21	121.49	0.43	0.514
<i>White brightness male</i>	<i>-48.04</i>	<i>27.62</i>	<i>123.07</i>	<i>3.03</i>	<i>0.084</i>
Blue brightness male	-64.34	23.42	123.12	7.55	0.007
Yellow brightness male	-17.89	29.12	123.22	0.38	0.540
Yellow chroma male	25.18	26.10	122.01	0.93	0.337
Blue UV chroma male	-29.97	34.67	124.15	0.75	0.389
Blue UV chroma female	-8.35	33.37	38.74	0.06	0.804
Blue UV chroma male*Blue UV chroma female	47.86	22.5	118.13	4.51	0.036
Blue brightness female	-10.68	29.22	71.79	0.13	0.716
Blue brightness male*Blue brightness female	10.96	17.51	117.85	0.39	0.533
Exploration score female	-19.70	42.29	18.42	0.22	0.647
Exploration score male*Exploration score female	-17.49	26.96	105.03	0.42	0.518
Beak length female	-38.65	39.77	18.54	0.34	0.344
Beak length male*Beak length female	11.38	25.26	100.09	0.20	0.653
<i>Yellow brightness female</i>	<i>-53.27</i>	<i>29.79</i>	<i>71.49</i>	<i>3.19</i>	<i>0.078</i>
Yellow brightness male*Yellow brightness female	11.75	26.63	103.70	0.19	0.659
Yellow chroma female	-6.77	43.12	28.08	0.02	0.876
Yellow chroma male*Yellow chroma female	4.53	19.27	97.42	0.06	0.815
White brightness female	36.89	42.08	50.88	0.77	0.385
White brightness male*White brightness female	9.23	34.71	98.74	0.07	0.791
PCA Body size female	22.00	49.44	16.51	0.19	0.662
PCA Body size male* PCA Body size female	1.33	21.69	96.29	0.004	0.951

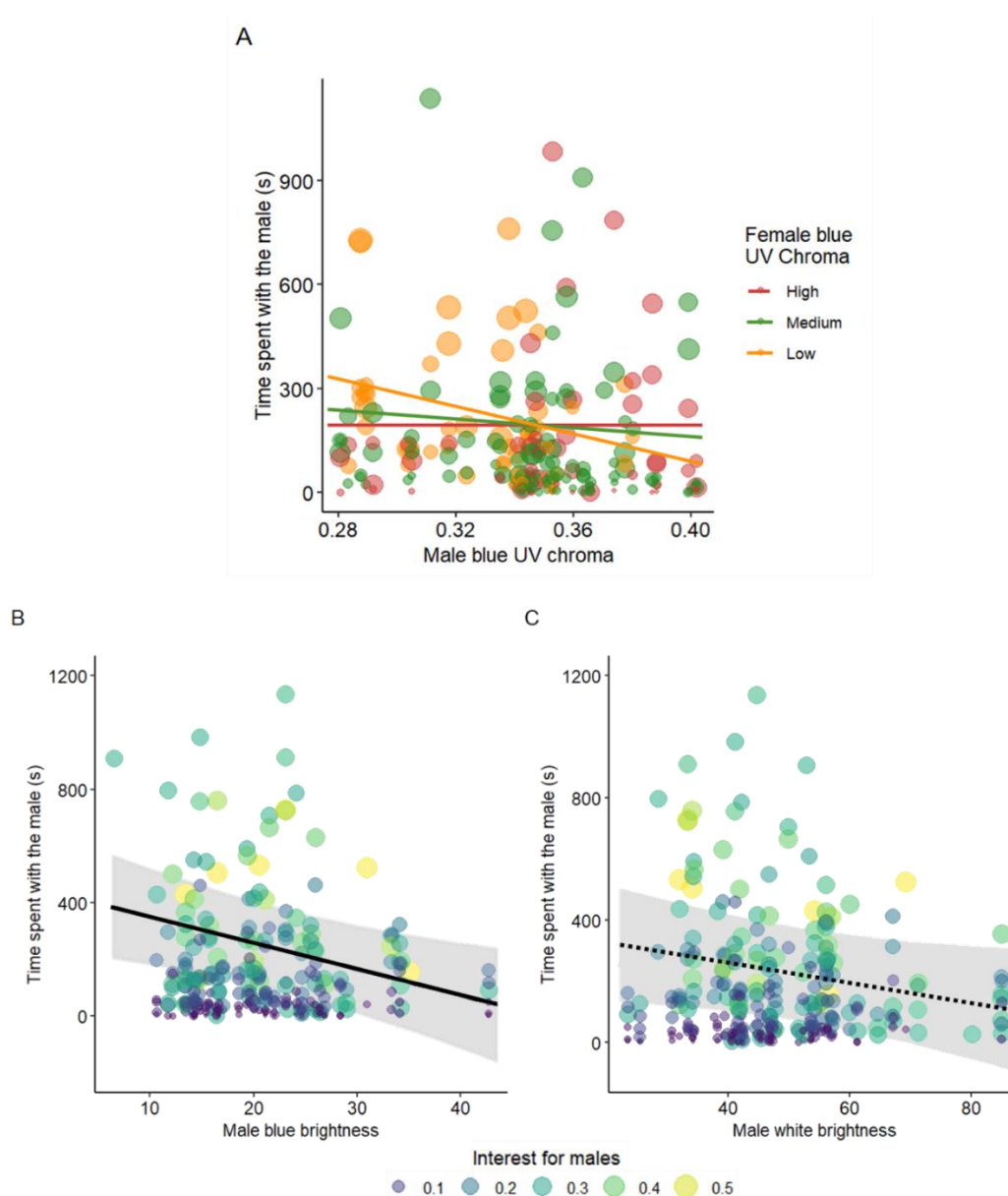


Figure 2. Female preferences for male color traits.

A: Females prefer male with blue UV chroma of the head crown similar to their own ($F_{1,118.13} = 4.51$, $p = 0.036$). The lines represent the predicted values of the model. Although female blue UV chroma was treated as a continuous variable in the statistical model, for graphical purpose they were split into three categories corresponding to the 25% (i.e. low, orange), 50% (medium, green) and 75% (high, red) quantiles. This graphical representation suggests that the interaction could be mostly driven by females with low blue UV chroma preferring males with low blue UV chroma.

Female blue tits prefer males with duller (B) blue head crown ($F_{1,123.12} = 7.55$, $p = 0.007$) and (C) tended to prefer males with duller white cheeks ($F_{1,123.07} = 3.03$, $p = 0.084$). Lines and dotted lines represent the predicted values of the model, with the 95% confidence intervals.

On all graphs, points depict individual data and their size represents the overall interest of females for males, with bigger points depicting a higher interest for males during the preference test.

Breeding experiment

Females built a nest in 36 out of 43 occasions (18 in 2021, 18 in 2022, Table 2), but counts were identical in both treatments (18 in the preferred treatment vs. 18 in the avoided treatment; binomial test, $p = 1$, Table 2). Similarly, the 26 clutch initiations (12 in 2021, 14 in 2022, Table 2) were evenly distributed between the two treatments (13 females in each group; binomial test, $p = 1$, Table 2), and females produced a similar total number of eggs in each treatment (165 eggs in total in the preferred male group vs. 162 eggs in the avoided group, binomial test, $p = 0.91$). However, comparing the total number of eggs between treatments for each year separately, revealed that there were more eggs produced in the preferred than in the avoided group in 2021 ($p = 0.001$, Table 2), while this was the opposite in 2022 ($p = 0.007$, Table 2). As females were exposed to opposite treatments over these two years, this result suggests that it is not the preference for the male that influences the number of eggs laid by the females.

Table 2. Counts of number of nests built, clutch initiations and total number of eggs laid per year and treatment. Each female was used as its own control, i.e. a female that had been paired with its preferred male the first year was paired with its most avoided male the second year. Females laid more eggs in 2021 with the preferred males while they laid more eggs with the avoided one in 2022.

	2021		2022	
	Preferred	Avoided	Preferred	Avoided
Number of nests built	9	9	9	9
Number of females that initiated laying	7	5	6	8
Total number of eggs laid	95	55	70	107

At the individual level, females tended to build heavier nests when paired with their preferred male ($n = 36$, $F_{1,33} = 3.56$, $p = 0.068$, Table 3, figure 3A), but they did not lay earlier ($n = 26$, $F_{1,12.24} = 0.03$, $p = 0.874$, Table 3, figure 3B), nor lay larger clutches ($n = 25$, $z = -0.07$, $p = 0.944$, Table 2, figure 3C). Mean egg size was also similar between groups ($n = 320$, $F_{1,306.78} = 2.23$, $p = 0.137$, Table 3). Finally, we did not find any difference in the proportion of fertilised eggs between the two groups ($n = 282$, $z = -0.03$, $p = 0.977$, Table 3, figure 3D), nor in the proportion of male chicks ($n = 219$, $z = -0.55$, $p = 0.580$, Table 3, figure 3E).

Being paired with a preferred or an avoided male therefore does not seem to make much of a difference in female blue tits. This is further supported by our analyses of the repeatability of the traits we monitored during the breeding season. Laying date ($R = 0.413$, $p = 0.048$, Table 3), clutch size ($R = 0.87$, $p < 0.001$, Table 3), egg size ($R = 0.75$, $p < 0.001$, Table 3) and

proportions of sons ($R = 0.758$, $p = 0.017$, Table 3) were indeed significantly repeatable within females over the two years of experiment, showing that females are relatively consistent in these decisions, whatever the male with which they are paired. Nest weight and proportions of fertilised eggs were not repeatable (Table 3).

Table 3. Results from generalized linear mixed effect models and linear mixed effect models exploring the variables that influence nest weight, laying date, clutch size, egg size and proportion of fertilised eggs and male chicks. Repeatabilities of female identity have been measured with the rpt function. Bold writing depicts significant results ($p < 0.05$), italicized depicts trends ($p < 0.10$). Est.: Estimate, S.E: Standard Error, R: Repeatability, CI: 95% Confidence Interval.

	Nest weight			Laying date			Clutch size		
<i>Fixed effects</i>	<i>Est.</i>	<i>S.E</i>	<i>p</i>	<i>Est.</i>	<i>S.E</i>	<i>p</i>	<i>Est.</i>	<i>S.E</i>	<i>p</i>
Treatment	2.36	1.25	0.068	0.38	2.32	0.874	-0.01	0.15	0.944
Year	1.91	1.25	0.137	-0.52	2.33	0.828	0.19	0.15	0.191
<i>Repeatability</i>	<i>R</i>	<i>CI</i>	<i>p</i>	<i>R</i>	<i>CI</i>	<i>p</i>	<i>R</i>	<i>CI</i>	<i>p</i>
Female identity	0	0- 0.601	1	0.413	0- 0.87	0.048	0.87	0.657- 0.981	<0.001

	Egg size			Prop. fertilised eggs			Prop. male chicks		
<i>Fixed effects</i>	<i>Est.</i>	<i>S.E</i>	<i>p</i>	<i>Est.</i>	<i>S.E</i>	<i>p</i>	<i>Est.</i>	<i>S.E</i>	<i>p</i>
Treatment	-0.16	0.10	0.137	-0.05	1.59	0.977	-0.50	0.91	0.580
Year	-0.38	0.10	<0.001	0.93	1.62	0.568	-0.29	0.91	0.742
<i>Repeatability</i>	<i>R</i>	<i>CI</i>	<i>p</i>	<i>R</i>	<i>CI</i>	<i>p</i>	<i>R</i>	<i>CI</i>	<i>p</i>
Female identity	0.75	0.564- 0.85	<0.001	0	0- 0.826	1	0.758	0.398- 0.975	0.017

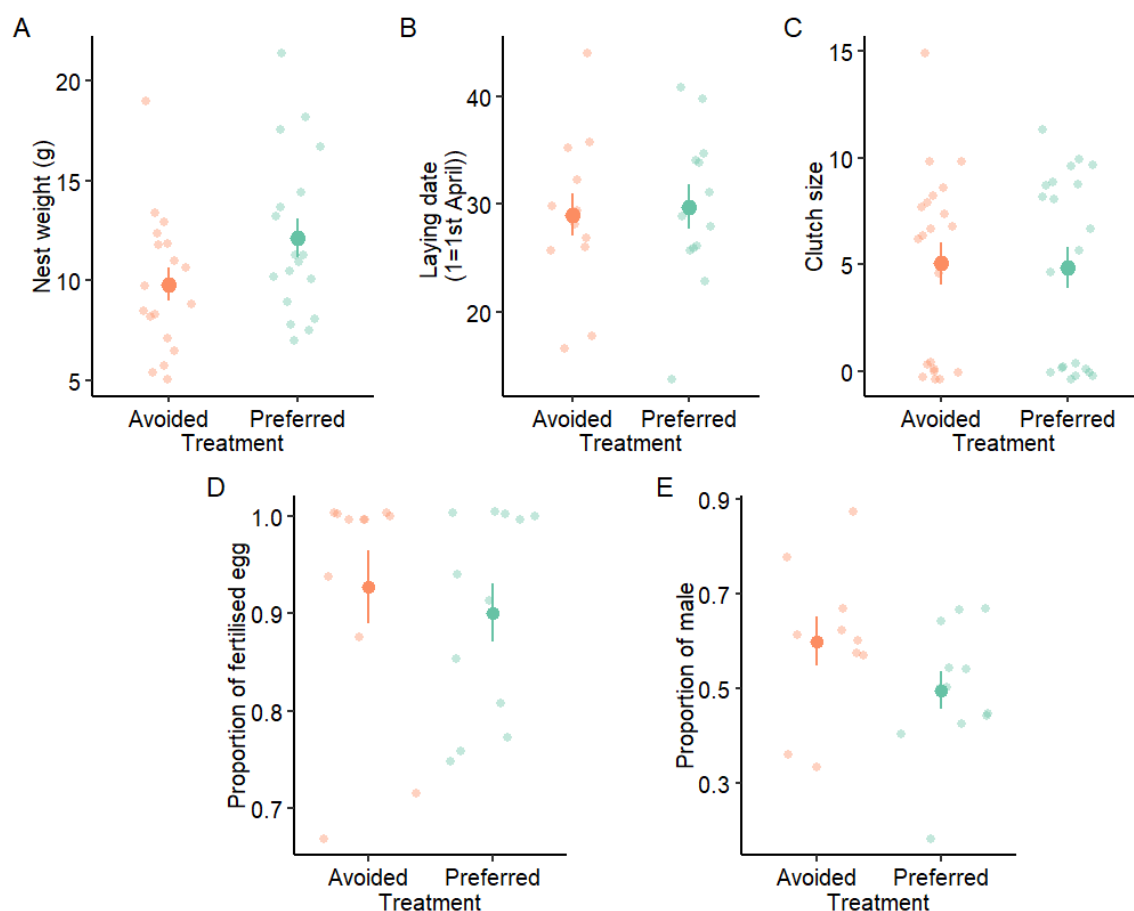


Figure 3. Effect of the preference of the females on their reproductive parameters.

Females tended to build heavier nests when paired with their preferred male ($p = 0.068$; panel A), but they did not lay earlier ($p = 0.874$; panel B) or larger clutches ($p = 0.944$; panel C). They also did not produce more fertilised eggs ($p = 0.977$; panel D) or male chicks ($p = 0.580$; panel E). Large dots and error bars represent mean \pm SE, smaller dots depict raw data.

Discussion

We found assortative preference of female blue tits for blue UV chroma of the head crown, a relationship that might have mostly been driven by females with low blue UV chroma selecting males that also presented low levels of blue UV chroma (figure 2A). We also found that females expressed a general preference for males with duller blue head crown and tended to prefer males with duller white cheeks. Contrary to our hypotheses however, we found no effect of female preference on reproductive timing and investment. Although females paired with their preferred male tended to build heavier nests, they did not lay earlier, more or bigger eggs, and they did not produce more fertilised eggs or more sons, compared to females paired with the male they avoided during the preference test. Females however showed significant

repeatability for laying dates, clutch sizes, egg sizes, and egg sex-ratio, despite the fact that they each had been paired with both their preferred and most avoided males.

A first finding of our experiment is that female blue tits expressed preferences for males that had values of blue UV chroma similar to their own, but at the same time they also preferred males with duller blue heads and tended to prefer males with duller white cheeks. All three signals (blue UV chroma, blue and white brightness) have been suggested to be linked to individual quality (Pärt and Qvarnström 1997; Keyser and Hill 2000; Mennill et al. 2003; Alonso-Alvarez et al. 2004; Ferns and Hinsley 2004; Siefferman and Hill 2005; Delhey et al. 2006; Galván and Sanz 2008; Rémy et al. 2010; Midamegbe et al. 2011; Doutrelant et al. 2020; White 2020). For example, in the blue Grosbeak (*Guiraca caerulea*), blue brightness indicated male ability to hold large territories with more resources (Keyser and Hill 2000), and in the great tit (*Parus major*), males with more immaculate white cheek patches had significantly greater access to feeding sites and produced heavier chicks (Ferns and Hinsley 2004). UV coloration has also been suggested to reflect genetic quality (Brown 1997; Foerster et al. 2003; Kempenaers 2007; García-Navas et al. 2009; Ferrer et al. 2015). Female preference for males with similar blue UV chroma to their own could therefore select a male with the same level of quality than themselves (Jennions and Petrie 1997; Tregenza and Wedell 2000; Cotton et al. 2006). This is in line with several studies showing assortative mating in blue tits in the wild (Andersson et al. 1998; Hunt et al. 1999; Fargevieille et al. 2017). A closer look at figure 2A suggests that it is particularly females with low blue UV chroma that prefer males with low blue UV chroma. In captivity, more colored individuals might be more aggressive (Senar 2006), and pairing with an aggressive partner may lead to reproductive failure (Caro et al. 2007). High blue UV chroma males might therefore be more aggressive toward females. Consequently, females with higher blue UV chroma levels, assumed to be more aggressive, would be less choosy because they would be better able to defend themselves, contrary to low blue UV chroma females, which could explain why low blue UV chroma females seem to express clearer preference than higher blue UV chroma females. Females also surprisingly preferred males with duller, and not brighter, blue heads and white cheeks. Caro et al (2021) also found preference for individuals with paler chest plumage in captive blue tits. Consequently, our females would select duller individuals based on blue and white brightness to avoid aggressive individuals in a restricted environment (Caro et al. 2021).

Surprisingly, besides nests that tended to be bigger with their preferred males, female blue tits did not adjust any other reproductive traits to the male with which they were paired. If

females were to match their reproductive decision to the differential allocation hypothesis (Burley 1986; Burley 1988; Sheldon 2000; Haaland et al. 2017), which predicts that male attractiveness should modulate female reproductive investment (Cunningham and Russel 2000; Gowaty et al. 2003; Limbourg et al. 2004; Anderson et al. 2007; Walling et al. 2010), we expected females to lay earlier, more and larger eggs and more fertilised eggs when paired with their preferred male. For example, female zebra finches and barn swallows (*Hirundo rustica*) paired with an attractive male lay larger clutches (De Lope and Moller 1993; Balzer and Williams 1998), and we recently showed that female great tits lay earlier when they are paired with a male they prefer (Delaitre et al. 2023). None of this happened with our blue tits, which therefore do not seem to match the differential allocation hypothesis. Alternatively, we hypothesized that if females were to match the sex-allocation theory, those paired with their preferred male would produce more sons (Charnov 1983; Frank 1990; Booksmythe et al. 2017). For example, the proportion of sons increases with the intensity of the sexual characters of the mate in barn swallows (Saino et al. 2002). In tits, brood sex-ratio has been related to male quality, with females producing more sons when paired with a high-quality male (Svensson and Nilsson 1996; Kölliker et al. 1999; Sheldon et al. 1999; Griffith et al. 2003; but see Limbourg et al. 2013). However, our female blue tits were significantly repeatable in their brood sex-ratio (see below), whatever the pairing treatment. They therefore did not produce more sons when paired with their preferred male, which does not support the sex-allocation theory. Consequently, none of the two hypotheses has been verified.

Females thus express preferences for certain males without adjusting their breeding decisions according to whether or not their pairing matched their preferences. One possibility is that females may have expressed a social, instead of a sexual preference in our preference testing. Females were indeed not fully photostimulated at the time of testing, and therefore not sexually active yet (Dawson et al. 2001). Stimulating females with long photoperiods, or exogenous oestradiol capsules, would have ensured sexual responses to males (Byers and Kroodsma 2009; Reparaz et al. 2014; Caro et al. 2021), but at the cost of interfering with the reproductive experiment that followed the preference testing. Furthermore, in the wild, tits are often seen in pairs as early as February (Caro, pers. obs.) and can be found roosting in nest-boxes close to the one in which they will reproduce much later in the season (Salis et al. 2019). Finally, using the same mate preference testing apparatus, we recently showed that the more time female great tits had spent close to the male with which they were then paired in aviaries, the earlier they reproduced (Delaitre et al. 2023). This strongly suggests that we effectively captured sexual preferences during the mate preference tests, and that our experimental set-up

cannot itself explain the absence of an effect of the female preferences on their reproductive decisions.

We must also remember that we used captive birds with unlimited food access. Consequently, environmental constraints might not be harsh enough for females to adapt reproductive decisions with respect to the quality of their mate the same way they would have done it in the wild. Captivity can however not be the only explanation for the results obtained here, given that among the studies described above to illustrate each of the hypotheses, many were similarly conducted in captivity (Burley 1981b; Balzer and Williams 1998; Gil et al. 1999; Bolund et al. 2009). In fact, another hypothesis seems to emerge from our results, supporting that female blue tits are consistent in their main reproductive decisions, whatever their preference for the male with which they are paired.

Males seem to play a minor role in the major breeding decisions in blue tits. Females were indeed consistent in several of their reproductive decisions across the two years, despite the fact that they were paired with males that were at the opposite ends of their preference rankings. We found relatively high repeatability for laying date, clutch size, egg size, and proportion of sons, suggesting that females were consistent whatever the pairing treatment to which they were assigned. For example, in 2021, females paired with their preferred male laid more eggs (Table 2). While this was totally in line with our initial hypothesis, in 2022, we found the opposite pattern, with more eggs laid in the group of females paired with the male they avoided most. Since we reversed the treatment for each female between the two years, each female was acting as its own control. Females that laid numerous eggs in 2021 with the preferred male were thus the same females that laid numerous eggs in 2022 with the avoided male. This result was confirmed by the significant repeatability of laying dates and clutch sizes within females. This accessorially also shows the importance of repeating experiments over multiple years to avoid confounding effects of unidentified environmental variables, which in our case seem to be that the preferred group of 2021 contained females investing more in reproduction, regardless of the treatment applied to these females. There are other passerine species, like the collared flycatcher (*Ficedula albicollis*) (Sheldon et al. 2003) or the European starlings (*Sturnus vulgaris*) (Cornell et al. 2017), in which male quality does not influence female laying dates or clutch sizes. In zebra finches, a study found no evidence of a difference in reproductive timing and investment between females that were force-paired and those that were free to choose their partner (Griffith et al. 2017). Earlier studies on blue tit populations from the Mediterranean region have shown that males play little role in reproductive phenology. In Corsica, male blue tits from two distinct populations indeed start developing their

reproductive system at the same time in late winter, despite the fact that their females lay more than one month apart (Caro et al. 2005; Caro et al. 2006). Further quantitative genetic analyses revealed that the timing of breeding was solely driven by the females, with no evidence for an additive genetic male effect on laying dates (Caro et al. 2009). Finally, reproductive experiments crossing males and females from different populations suggested that the timing of laying matched the origin of the females and not the origin of the males (Caro et al. 2007). Interestingly however, although there is evidence for a higher heritability and repeatability of laying dates in female than male great tits (van Noordwijk et al. 1981; Van Der Jeugd and McCleery 2002; Noordwijk et al. 2002), we recently found that female great tits paired with a male they preferred during a preference test laid their first clutch earlier (Delaitre et al. 2023). Contrary to the present study however, all those female great tits were paired with one of their favorite males, and none with the male they avoided most. In any case, our results highlight the fact that female investment in reproduction might be species, and potentially even population-specific.

To conclude, this study suggests that although females have expressed preferences for some ornamental traits in males, based in part on their own phenotype, the male with which they are paired does not alter their reproductive decisions. The fact that females lead most reproductive decisions has already been shown in earlier studies in the wild on Mediterranean blue tits (Caro et al., 2007; 2009). In this species, males might thus be primarily used for providing genes and for helping rearing offspring. Consequently, females breeding with a male that does not match their selection criteria, either because their preferred male was already mated with another female, or because they only encounter less-preferred males, will not prevent them from breeding. This could be an adaptive strategy in such a short-living bird that, on average, has only two breeding seasons over its lifetime (Olioso 2017).

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Data availability

Analyses reported in this article can be reproduced using the data provided by Delaitre (2023).

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Article 2

Female great tits (*Parus major*) reproduce earlier when paired with a male they prefer.

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Abstract

Mate choice is a key component of reproductive biology. Females often prefer certain males but do females modulate their reproductive investment depending on whether they are mated with their preferred partner? We investigated this question in great tits (*Parus major*) where we subjected 36 females to a six-choice mate preference test. Male morphological traits and the female's own characteristics did not influence the preference females expressed. We however found that females spent more time near more exploratory males. We then paired females with one of the males in indoor aviaries, and subsequently monitored their reproductive investment (through measurement of plasma 17 β -oestradiol concentrations, first egg date, clutch size, and egg size). Females that were mated with a male for which they had a strong preference laid their first clutch significantly earlier in the season than females paired with a male they less preferred. Our results show that mate preference influences reproductive investment in great tits, thereby linking mate choice to bird reproductive decisions.

Keywords: reproduction; mate preference; birds; laying date

Introduction

Individuals can derive direct (for themselves and their offspring in the present generation) and indirect benefits (for their offspring in the following generation) from carefully choosing their mates (Andersson & Simmons, 2006; Jones & Ratterman, 2009; Kokko et al., 2003). Indeed, female preference for a male ornament might be beneficial if this ornament reflects the ability of the male to provide high-quality territory, nutrition, parental care or protection for example (Andersson & Simmons, 2006). Individuals consequently need reliable signals to evaluate potential partner quality. Criteria used by animals to choose their mate can be extremely different from one species to another. Birds might use morphological characteristics to select their mate, like plumage coloration (Hill, 2006; Siitari et al., 2002) or ornament size (Price, 1984; Romero-Pujante et al., 2002; Sheldon et al., 1997). They can also use behavioural traits such as song characteristics (Byers & Kroodsma, 2009; Searcy, 1992b), courtship (Ota et al., 2015; Zuk et al., 1990), personality (Schuett et al., 2011; van Oers et al., 2008) or even cognitive abilities (Boogert et al., 2011). Additionally, all these criteria may be used separately or in combination (Burley, 1981; Candolin, 2003).

For long, it has been assumed that individuals always prefer the highest quality partners and that mate preferences are absolute, meaning that individuals assess each potential partner independently and assign a fixed value to a certain trait (Jennions & Petrie, 1997; Zandberg et al., 2020). Under these circumstances little inter-individual variation is expected both in female preference and in male phenotype. However, individual females vary substantially in their mate preferences (Cotton et al., 2006; Jennions & Petrie, 1997), for example depending on their experience (Caro et al., 2010), their diet (Hunt et al., 2005), or their own quality (Holveck & Riebel, 2010). Condition-dependent preferences have for example been shown in zebra finches, where females that had been raised in enlarged brood, and therefore considered to be lower quality females, preferred lower quality males (Holveck & Riebel, 2010). Such kind of self-reference phenotype matching has been suggested in birds (Andersson et al., 1998; Caro et al., 2021; Fargevieille et al., 2017; Zandberg et al., 2017), reptiles (Sacchi et al., 2018), mammals (Farrell et al., 2011), fishes (Verzijden & Ten Cate, 2007) and insects (Malausa et al., 2005).

Besides the challenge of understanding complex mate choice mechanisms, the effect of partner choice on reproductive timing, investment and physiology remains poorly understood. Females have classically been considered the choosing sex (Jones & Ratterman, 2009; Wong & Candolin, 2005), and the ones that drive several reproductive decisions such as onset of

reproduction or clutch size (Caro et al., 2009). However male sexual traits (Limbourg et al., 2004; Walling et al., 2010) and social solicitations can also modulate female reproductive decisions and investment (Hinde & Steel, 1976). For example, female mallard (*Anas platyrhynchos*) lay larger eggs and produce better quality offspring after copulating with a preferred male (Cunningham & Russel, 2000) and female zebra finches incorporate higher level of testosterone in their eggs when mated with a preferred male (Gil et al., 1999). Gonadal development and hormone secretions in females are also under the influence of male behaviour (Hinde & Steel, 1978; Lehrman et al., 1961; Perfito et al., 2015; Stevenson et al., 2008). Having a non-preferred partner could thus lead to a reduction in the motivational state or to physiological stress altering reproductive performance (Bolund et al., 2009; Griffith et al., 2011). On the contrary, females could opt for a compensatory strategy in which females would invest more in reproduction to compensate for the lower quality of, or at least the lower preference for, its male partner (Bolund et al., 2009; Gowaty, 2008; Gowaty et al., 2007). Therefore, we predict that the deviation between the male traits a female prefers and the traits of the male with which the female is effectively mated, can influence its investment in its current reproduction.

In this study, we tested female preferences for a range of male traits, and how well its reproductive decisions and physiology are influenced by the deviation between its mate preference and its actual mate, using the great tit as our model. Previous work has suggested that females rely on characteristics of the male to decide with whom to mate, suggesting that mate choice could play a role for reproductive success in this species (Norris, 1990, 1993; Patrick et al., 2012; Zandberg et al., 2017). First, we investigated the characteristics used by females for mate choice. We then tested if this choice has an impact on reproductive parameters of the female. We hypothesised that females showing a stronger preference for a given male would show a stronger reproductive physiological development when mated to this male. We measured this through 17β -oestradiol (E_2) level, a key reproductive hormone that links brain neuroendocrine mechanisms to downstream mechanisms in the liver and thus stimulates the production of yolk precursors (Caro et al., 2019; Williams, 2012). This would, furthermore, imply that females mated to a more preferred mate would lay earlier, and produce more and larger eggs (Balzer & Williams, 1998; Cunningham & Russel, 2000).

Methods

Ethical note

All ethical permits requested for this experiment have been provided by the Animal Welfare Body of NIOO-KNAW (IVD - NIOO 20.09 AVD8010020209246/IVD 1556a).

Preference test

Experimental subjects and housing

The experiment took place at the Netherlands Institute of Ecology (NIOO-KNAW) in Wageningen, the Netherlands. We used 36 female and 54 male great tits born in 2020, and hand-reared at the NIOO following the procedure described in previous studies (Drent et al., 2003). Fully-grown birds were fed daily with a mixture of minced beef heart, canary egg-food (CéDé, Evergem, Belgium), proteins, vitamins, minerals and trace elements (Carnizoo 2%, Avian, Raalte, The Netherlands, and Calci-Lux 1,5 %, Versele-Laga, Deinze, Belgium), *ad libitum* dry food (Canary egg food, CéDé, Evergem, Belgium, Uni patee, Versele-Laga, Deinze, Belgium), a piece of apple three times a week, and water for drinking and bathing.

On 20th January 2021, the 54 males were moved from outdoor aviaries (same-sex groups) to individual indoor cages (0.9 x 0.4 x 0.5 m) in two different rooms. Females were moved to individual cages in three successive groups of 12 females. They were housed in a separate room from the males, and returned to outdoor aviary once tested. Windows in rooms allowed birds to be exposed to natural light, and supplementary artificial lighting was provided from natural sunrise until sunset. Sunrise and sunset times were determined using the ptaff.ca data base (<http://ptaff.ca/soleil/>) for Nijmegen, the Netherlands.

Experimental setup

Females were tested in a carousel-shaped six-choice chamber inspired by Zandberg et al. (2017) (figure 1, figure S1) from 22nd to 31st January 2021. The carousel was placed in a room (4.0 x 2.4 x 2.5 m) with white walls, and high-frequency fluorescent lights. In the six-choice setup, each female could see all stimulus male birds from the central platform, but when in the choice zone of a stimulus male, she could not see the other males. The males were visually isolated from each other. Before being tested, each female was given 15 min to habituate to this new environment. In the meantime, the six stimulus males were transferred from their home cage to six individual mobile cages (47 x 28 x 26 cm) in an adjacent room. At the end of the 15 min habituation period, each male cage was placed into one of the six compartments of the

carrousel. Each female was tested once with one group of six stimulus males during 90 minutes. The experimenter left the room at the beginning of the test, which was video-recorded using a central camera (Panasonic WV-CP500) affixed above the test chamber (figure S1B). After each test, all birds were returned to their individual home cages. Males were returned to the pool of available males from which to select the six males to enter the next test. Each group of six males was selected randomly among the 54 males available, with the constraint that each male could not be tested more than four times, and that the males could not belong to the family of the female tested. The positions of the males in the device were randomised for each test.

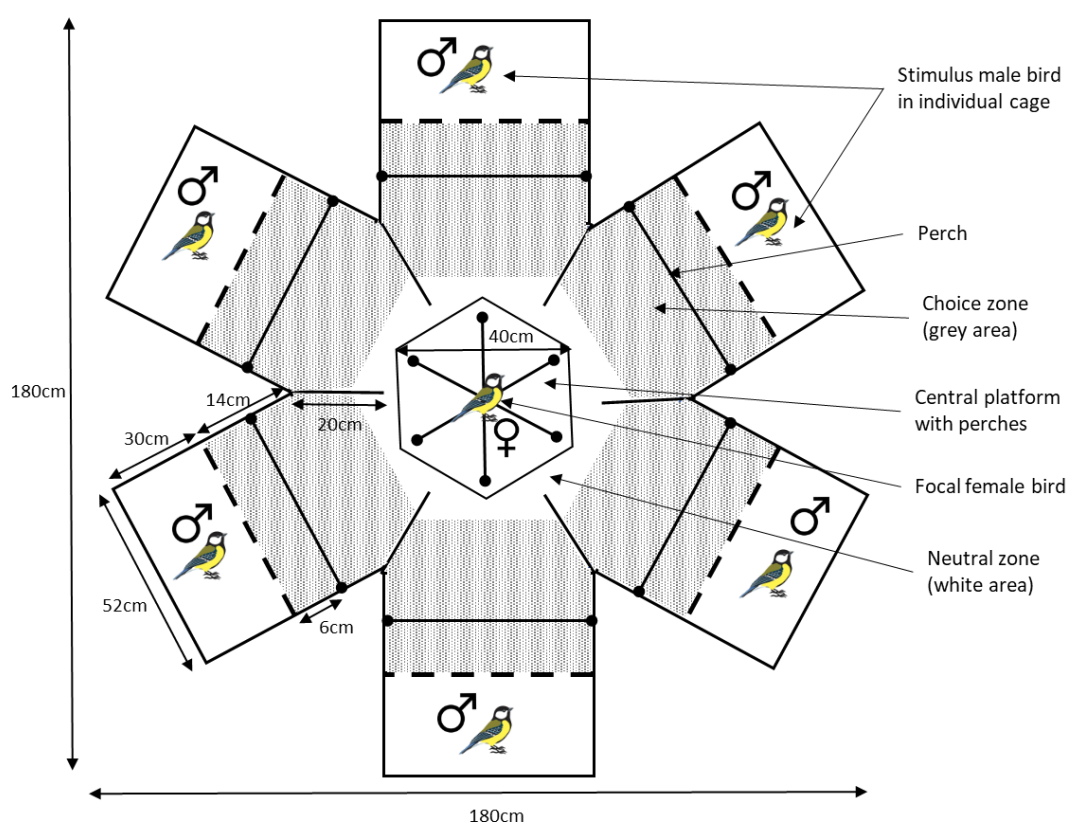


Figure 1. Experimental setup for mate preference test.

Females were tested for their preference in a carrousel-shaped six-choice chamber for 90 min. The stimulus male birds could not see each other. From the hexagonal platform, the focal female could observe all males, whereas in the choice zones, only the stimulus male was visible.

Video analysis

Videos were analysed using EthoVision XT software (Noldus Information Technology, Wageningen, The Netherlands). The time spent by the females in the choice zone of each stimulus male bird was extracted from each video, and used as a proxy for mate preference. In numerous species, time spent in a choice zone has been shown to predict courtship behaviours or pair formation (Caro et al., 2021; Clayton, 1990; Dechaume-Moncharmont et al., 2011;

Drickamer et al., 2003; Hill, 1990; Jeswiet & Godin, 2011; Lehtonen & Lindström, 2008; Mays & Hill, 2004; Senar et al., 2013; Witte, 2006). For example, in zebra finches, the time females spent in front of a male correlates positively with the number of solicitation displays directed to that male, therefore linking mate preference measured by courtship displays with preference measured through the time a female spends with a male (Witte, 2006).

Phenotypic trait measurements

To identify on which criteria females choose their mate, a set of phenotypic traits was measured on each male and female that was used in the mate choice trials. We measured breast stripe width from photographs using GIMP software (version 2.10.22, <https://www.gimp.org/>). We collected five yellow breast feathers, placed them on top of each other, and measured brightness, UV chroma and hue using a spectrophotometer (AVASPEC 2048, Avantes, Apeldoorn, the Netherlands) equipped with an AVALIGHT-DHS deuterium-halogen lamp (Avantes, Apeldoorn, the Netherlands) and a 200 μm optical probe (FCR-7UV200-2-45-ME, Avantes, Apeldoorn, the Netherlands) whose tip contained a 45° quartz window, which guaranteed a constant distance between the light and the sample and highly repeatable measurements. Reflectance was calculated in relative terms, via comparison with a black and a white reference (WS1, Ocean Optics, Dunedin FL, USA). We also took morphological measures, including body mass, tarsus, beak and wing lengths and beak depth. Exploration score in a novel environment was measured following methods described in Dingemanse et al (2002). Briefly, each bird was released individually, without handling, in an observation room (4.0 \times 2.4 \times 2.3 m) that was equipped with five artificial wooden trees. Exploration score was calculated as the sum of movements (flights and hops) performed by each bird during the first two minutes after entering the room. Scores ranged from 0 to 43, with higher scores indicating faster exploration. Exploratory behaviour is repeatable and this exploration test is a well-validated test in passerine birds (Reparaz et al., 2014; van Oers & Naguib, 2013).

Reproduction

Pair formation and bird housing

The preference test was a preliminary test to ensure that birds would breed in aviaries for the purpose of another experiment. All females were therefore preferentially paired with one of the males with which they spent the most time. If several females selected the same male (eight males were selected more than once), we paired the female that had the smallest “interest” for males (see below) with her second choice ($n = 10$ out of 36 females), i.e. with the male for

which she spent the second most time. Preliminary analyses showed that being paired with the first or second choice male did not affect their reproductive traits (data not shown). Despite this, females differed in the total time they spent with their preferred male during the mate choice test, thus we had variability in the strength of female preference. From early February, we housed each 36 opposite-sex pairs in 36 indoor aviaries (2 x 2 x 2.25 m) under an artificial light regime mimicking a natural daylight pattern. Sunrise and sunset times were determined using the ptaff.ca data base (<http://ptaff.ca/soleil/>) for Nijmegen, the Netherlands. The main source of light consisted of three high frequency fluorescent light tubes. An additional 7W incandescent light bulb mimicked dawn and dusk five minutes before natural sunrise and five minutes after sunset to respect the natural increase and decrease of light intensity every day. Temperatures of the next eight days in aviaries were set to mimic the temperatures of the past eight days in the wild. Hourly temperatures were determined using The Royal Netherlands Meteorological Institute (KNMI) data base for the weather station at Deelen (<https://www.knmi.nl/home>). A perch and an artificial tree were installed in the aviaries so that birds could perch and hide. Three nest-boxes were available in each aviary. Pairs had no visual or acoustic contacts with the other pairs.

Reproduction monitoring

Nesting material consisting of moss and dog hair was provided from 8th March 2021. From mid-March, we monitored nest building and egg laying activities, first once a week, and then twice a week as birds were closer to lay (Dufva, 1996). The date that the first egg was found was recorded as the pair laying date. All eggs were weighted (to the nearest 0.01 mg with an electronic balance, Eidyer), and measured (to the nearest 0.05 mm with a calliper, Ecotone Measy DG). Egg volume was calculated as follows: $\text{egg volume} = 0.4673 \cdot \text{length} \cdot \text{breadth}^2 + 0.042$ (Dufva, 1996). After the birds laid the last egg, the clutch size was determined.

STRANGE statement

The birds used in this study could be considered STRANGE as defined by (Webster & Rutz, 2020) due to their rearing history and acclimation and habituation. Indeed, birds were collected from the wild but hand-reared, which may have affected their development as well as their exploration, activity, or personality traits. However, all of the subjects used in this study were of the same age and none had ever experienced a breeding season. Therefore, none of the birds could have been influenced by their previous experience in choosing a mate or in deciding

on their investment in reproduction. We therefore considered the STRANGE character of our sample to be low.

Physiological measures

Blood samples

Females were caught in the aviary with a net and transported in bags to a laboratory room, where blood sampling was performed from the jugular vein (max. 150 μ l). Each bird was always sampled at the same time of day to control for daily rhythms in hormone levels. Blood was transferred to a 0.5 mL heparinised tube. Plasma was separated by centrifugation (14800 rpm, 10 min), kept on ice until the last blood sample of the day was collected, and stored in freezers (-80°C) until assayed for hormones. After blood sampling, females were released in their aviary. We blood sampled half of the birds (i.e. 18 females) every week, so that each bird was sampled every two weeks. In total seven blood samples were performed on each bird, between 18th March (before first laying) and 17th June 2021. Mean time between catching and blood sampling was 3 min 22 s \pm 46 s.

Estradiol assay

Hormonal assays were conducted at the Institut de Génomique Fonctionnelle in Montpellier, France. Plasma 17 β -estradiol (E_2) was measured using a commercially available double-antibody ^{125}I - E_2 radioimmunoassay (DSL-4800, Ultra-sensitive Estradiol RIA, Beckman Coulter, Brea, CA, USA) that was modified to increase the sensitivity of the assay (Caro et al., 2019; Charlier et al., 2010). Steroids were first extracted from 30 μL of plasma using solid phase extraction with C18 columns (100 mg C18 material, Sep-Pak Vac 1 cc, 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). Recovery value after extraction was estimated at 91.5%. Concentration was adjusted for samples that did not have 30 μL of plasma available ($n = 10$ of 250, no plasma for 2 samples). Resuspended samples were then assayed in duplicate and tubes were counted on a gamma counter (Automatic Gamma Counter, Perkin Elmer, Waltham, MA, USA). Concentrations of E_2 were obtained using a linear regression with the log-transformed concentrations of the standards provided in the assay kit. Samples were run in seven assays, and the intra- and inter-assay coefficients of variation, as estimated by assaying one high and one low concentration E_2 standard in duplicate, were 4.27% and 12.27%, respectively. Assay sensitivity, defined as the highest point on the standard curve whose standard deviation did not overlap that of the blank standard (Wingfield & Farner, 1975),

was 0.65 pg/mL. 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).

Statistical analyses

All the analyses were performed in R version 1.3.1093 (R CoreTeam). Linear mixed-effects models and generalized linear mixed-effect models (for details see below) were conducted using the lme4 package (Bates et al., 2015). P-values were obtained with the lmerTest package in the case of mixed-model analyses (Kuznetsova et al., 2017). Plots were created with the ggeffects package (Lüdtke, 2018). Principal component analyses were run with FactoMineR (Husson et al., 2016) and factoextra (Alboukadel & Mundt, 2017) packages. Models were simplified using backward elimination of the nonsignificant terms, starting with the higher-order interactions (Crawley, 2007). We set $\alpha = 0.05$.

Preference test

We first ran three principal component analyses (PCA) on (i) body size, (ii) feather coloration and (iii) beak size for males and females separately, as these variables were represented by measures that were correlated. PCA for body size included body mass, wing and tarsus lengths (Freeman & Jackson, 1990; Wiklind, 1996) (figure S2 A-B), PCA for feather coloration of the breast included yellow brightness, as well as yellow and UV chroma and hue (figure S2 C-D) and the PCA for beak size included beak depth and length (figure S2 E-F). Score of the PC1 for each PCA were then used in the statistical models. For colouration, we also used PC2 scores as this axis was well represented by three variables of feather colouration (figure S2 C-D). Detailed results of the PCAs are available in supplementary materials (figure S2).

To identify the criteria used by female great tits during the preference test to choose their mate, we ran a linear mixed effect model using the time (in seconds) spent by the female in the choice zone of individual males as the dependent variable. The model included the following male characteristics: breast stripe size, PC1 and PC2 of colouration, PC1 of beak size, PC1 of body size and exploration score as explanatory variables, as well as one female*male interaction for each characteristic assessed. ID of the female was added as random intercept to account for the fact that each female occurred six times in the dataset (once for each six different males). The time spent in the choice zone of the males was weighted by the “interest” of the female to give more statistical weight to the females who spent more time evaluating the males during the test rather than staying in the neutral zone (Caro et al., 2021; Reparaz et al., 2014).

“Interest” depicts the overall motivation of the female to visit the males during the mate choice test, defined as the proportion of time that the female spent close to the six stimulus males (i.e. not in the central neutral zone), and was calculated as follows (Caro et al., 2021; Reparaz et al., 2014):

$$\text{Interest} = \frac{\text{Time spent with (male 1 + male 2 + male 3 + male 4 + male 5 + male 6)}}{\text{Total duration of the test (90min)}}$$

Reproduction

First, we ran a PCA on egg size including weight, length, breadth and volume of the eggs, as these variables were correlated (figure S3). We tested the effect of the intensity of the female preference for a male (i.e. the time the female had spent close to the male with which she was subsequently paired, as explanatory variable) on her reproductive parameters. Specifically, we performed (i) a linear model on lay date, (ii) a linear mixed-effect model on egg size (iii) a generalized linear model with Poisson distribution on clutch size. In the mixed effect model, female ID was included as a random intercept to account for the fact that each female laid several eggs. For reproductive hormone analyses, data were first log transformed to achieve normality. In order to assess if a female paired with a more preferred male would increase E_2 levels earlier in the breeding season, we ran a linear mixed effect models on all E_2 levels measured during the season, with sampling date, time spent with the male, and their interaction as explanatory variables. Female identity was added as a random intercept to account for the fact that each female occurred seven times in the dataset (seven sampling points). We also ran a linear model on the E_2 level measured closest to the laying date of the first egg ($n = 17$ sampled before the first egg, $n = 14$ sampled during the laying period, $n = 1$ after laying. For two additional females, we used the average of two successive samples since the first-egg date was exactly in between) with time spent close to the male with which the female was paired included as explanatory variable. Linearity of the data, normality of the residuals, homoscedasticity and independence of residuals have been checked for all models.

Results

Characteristics selected during the preference test

All the 36 females tested visited the choice zone of all six males at least once. Time spent in the choice zone of males ranged from 24.84 to 1240.88 seconds (mean $325.42 \pm \text{SD } 186.51$). We found no evidence for an assortative mating as none of the interaction terms between corresponding male and female characteristics were significant (Table S1). We found

that females showed a stronger preference for more exploratory males ($F_{1,206.71} = 3.87$, $p = 0.050$, Table S1, figure 2). None of the quality measures (i.e. body size, beak size, tie size or coloration) were clearly preferred by the females.

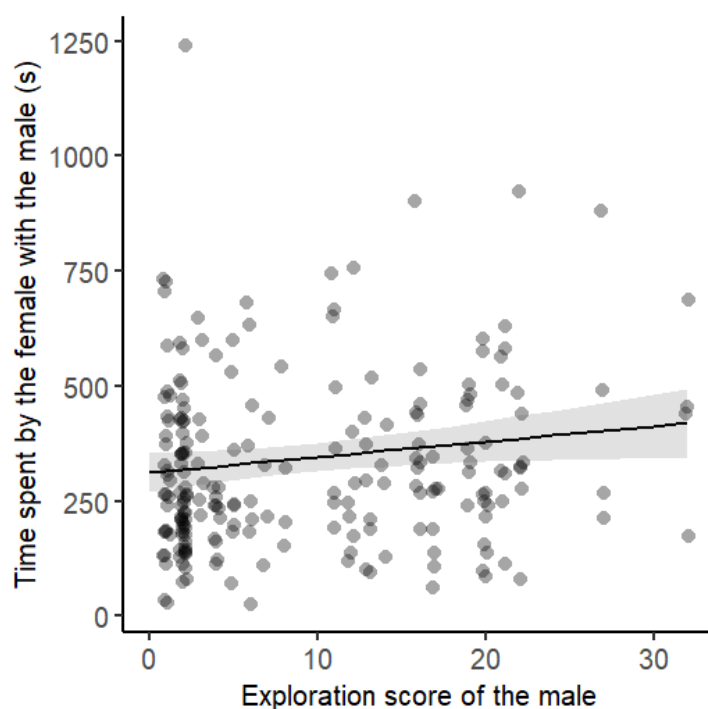


Figure 2. Females show a stronger preference for more exploratory males.

Females spent more time in the choice zone of more exploratory males ($F_{1,206.71} = 3.87$, $p = 0.050$). Points represent raw data ($n = 216$) and the line represents the predicted values of the model with 95% confidence interval.

Reproduction

35 out of the 36 pairs laid eggs during the breeding season. Females that had shown a stronger preference for the male with which they were paired laid earlier than females that had shown a lower preference for their male ($F_{1,32} = 10.31$, $p = 0.003$, figure 3). Females showing stronger preference for their male also laid larger clutches ($z_{1,32} = 2.69$, $p = 0.007$, figure S4A). However, one female was an outlier in our dataset with 28 eggs laid. When this female was removed, the effect of preference intensity on clutch size was no longer significant ($z_{1,31} = 0.14$, $p = 0.891$, figure S4B). We did not find an effect of the female preference for its mate on egg size ($F_{1,31.55} = 2.80$, $p = 0.104$) and they did not show a faster increase in E_2 level when paired with a more preferred male (date*time spent with the male interaction: $F_{1,15.12} = 0.51$, $p = 0.484$, figure S5A), nor a higher concentration of E_2 close to laying ($F_{1,31} = 0.29$, $p = 0.589$, figure S5B).

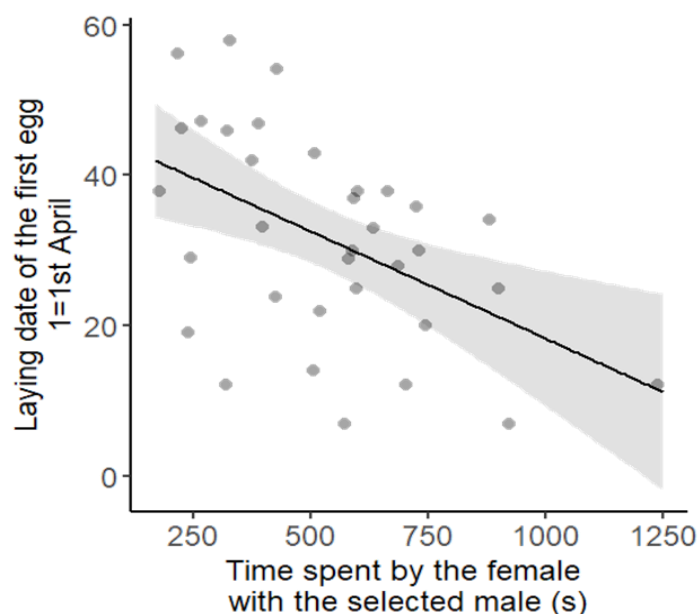


Figure 3. Females showing a stronger preference for their male lay earlier.

Females laid earlier when paired with a more preferred male ($F_{1,32} = 10.31$, $p = 0.003$). Each point represents a female ($n = 34$) and line represents the predicted values of the model with 95% confidence interval. Laying date: 1=1st April.

Discussion

We found that female great tits seem to express a stronger preference for more exploratory males. Male size, colours, tie size and beak size did not reliably influence female preference. Subsequently, we found that females that had shown a stronger preference for the male with which we paired them reproduced earlier. We did not find statistically significant effects of female preference strength on clutch size, egg size or E₂ levels during the breeding season.

One of the main findings of our study is that females seem to spend more time close to more exploratory males ($p = 0.050$). This is in accordance with a previous study in great tits (Carere et al., 2000); and Wang et al (2022) who found similar attraction for more exploratory males without assortative mating in Java sparrow (*Lonchura oryzivora*). Exploratory behaviour has been well studied in birds and is known to be representative of animal personality, referring to consistent differences between individuals in behaviour across time or contexts (Groothuis & Carere, 2005; Sih et al., 2004). This trait is also highly repeatable and heritable, both in wild populations (Dingemanse et al., 2002; Quinn et al., 2009) and under controlled laboratory conditions (Drent et al., 2003; van Oers et al., 2004). The 'fast-slow exploration' personality axis thus contrasts fast individuals that are more exploratory, or more likely to take risks (van

Oers et al., 2005), with 'slow' individuals that are less exploratory, more risk averse and more responsive to changes in their environment (Groothuis & Carere, 2005). In tits, it was shown that fast explorers were more aggressive (Verbeek et al., 1996), competitive (Cole & Quinn, 2012), and showed more foraging flexibility (Coomes et al., 2021). Consequently, being a fast explorer male could be advantageous for birds and may reflect their individual quality (Réale et al., 2007), in terms of territory defence capabilities (Amy et al., 2010) or in the number of offspring that they are able to produce (Dingemanse et al., 2004; McCowan et al., 2014), but see (Barnett et al., 2012; Wischhoff et al., 2018). With such a relationship between exploratory behaviour and fitness (Dingemanse & Réale, 2005), female great tits may have expected to obtain a high-quality male by selecting a more exploratory male.

We did not find any evidence that females preferred males of higher quality, as indicated by body size, beak size or feather coloration. Female preferences were thus not related to morphological characteristics of the males. While in the United Kingdom, longer bills have been associated with increased fitness in great tits (Bosse et al., 2017), this is not the case for Dutch great tit populations (Bosse et al., 2017), from where our birds originates. It may therefore not be surprising that females do not reliably choose their mate based on beak size. Although UV reflectance of the plumage has been involved in mate choice in different bird species (Doutrelant et al., 2012; Siitari et al., 2002), and that in the wild the intensity of the yellow coloration is often considered an honest indicator of the quality of an individual and of its territory (Casagrande et al., 2006; Isaksson & Andersson, 2007; Weaver et al., 2018), the great tits that we used were hand-reared captive birds, which diet is relatively poor in carotenoids, the pigments responsible for their yellow plumage coloration (Brush, 1990; Ferns & Hinsley, 2008; Isaksson et al., 2008). As a consequence, there was some inter-individual variation in the reflectance spectra among our males but all birds involved in the present study had a paler chest than what is observed in wild great tits (figure S6). It may therefore no longer carry any relevant signal to choose among individuals.

Another important finding of the study is that females laid significantly earlier when they had spent more time close to (i.e. expressed a stronger preference for) the male with which we subsequently paired them. This result is in accordance with our initial hypothesis of a higher reproductive investment when females are paired with their more preferred male. Indeed, egg production is both nutritionally and energetically costly for females (Nilsson & Råberg, 2001; Vézina & Williams, 2002; Visser & Lessells, 2001) and these costs might constrain the start of reproduction (Perrins, 1970). Laying earlier therefore represents an additional investment because females start laying at a time when food resources and temperatures are not favourable

yet. In the literature, there are several examples where mating with its preferred male increases reproductive success and offspring performance. Female house mice (*Mus domesticus*) mated with their preferred partners have higher reproductive success and better progeny performance than individuals mated with non-preferred partners (Drickamer et al., 2003). In zebra finches, females display a reduced readiness to copulate and males reduce parental care if they are force-paired (Ihle et al., 2015), while pairs that resulted from free mate choice achieve higher reproductive success (Balzer & Williams, 1998; Ihle et al., 2015). These results could be explained by synchronization between partners, for example. Ihle and colleagues (2015) found that individuals of freely-chosen pairs stayed closer together and behaved more synchronously than those of non-chosen pairs. Many studies show that pair coordination and bond duration is important for breeding success (Griggio & Hoi, 2011; Leniowski & Węgrzyn, 2018; Van De Pol et al., 2006) and that incompatible pairs take longer to breed than compatible ones (Griffith et al., 2011). In our case, great tit females expressing higher preference for their male may have been more stimulated by their partner and more synchronized with it, leading to earlier reproduction. One could however argue that females expressing stronger preferences for males were closer to reproduce than the others, explaining why those females laid earlier. However, contrary to males that become ready to reproduce early in spring (Caro et al., 2006; Dawson et al., 2001), females do not reach sexual readiness until a few days before they actually start laying, in April (Caro et al., 2009; Williams, 2012). This is supported by the fact that 17β -estradiol (E_2) did not start to increase until April 10 (figure S5A). At the time of preference testing, in January, all females were thus still far from reproducing.

Female pairing did not seem to affect their reproductive hormonal physiology, nor their clutch size. This goes against our initial predictions. We expected females showing stronger preferences for their males to have higher levels of E_2 and to lay larger clutches. Indeed in zebra finches, females lay on average 0.5 eggs more when paired with their preferred male (Balzer & Williams, 1998) and barn swallows tend to lay larger clutches when paired with an attractive male (De Lope & Moller, 1993). E_2 has been shown to enhance female sexual behaviours and has even been used to promote pre-copulatory displays in avian mate-choice experiments (Searcy, 1992a). E_2 has also been associated with nest-building behaviour, oviduct growth and yolk synthesis (Hunt & Wingfield, 2004; Williams, 2012). However, other studies showed that elevating E_2 in female do not change their preferences (Enstrom et al., 1997) and that reproductive hormones do not always predict laying dates (Schaper et al., 2012). Schwabl and colleagues also found in female red-backed fairy-wrens (*Malurus melanocephalus*) that E_2 levels varied with reproductive stage, but that these stage-dependent patterns were not affected

by the male phenotype with which they were paired (Schwabl et al., 2014). Finally, Bottoni and colleagues showed that plasma luteinizing hormone (LH) levels, the hormone regulating gonadal steroidogenesis and initiating ovulation (Williams, 2012), did not differ between pairs that had freely chosen themselves and those that had been forced-paired (Bottoni et al., 1993), which is consistent with what we find here with E₂.

In conclusion, this study highlights the importance of female preference on reproductive timing. We have shown that partner preference is not only based on morphological characteristics (Andersson & Simmons, 2006), but may also depend on consistent behavioural trait. This provides some potential for selection on behavioural phenotype in the wild, as suggested in other studies (van Oers et al., 2008). Taken together, our results suggest that there could be major fitness consequences of mate preferences in socially monogamous species.

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Data availability statement

The datasets needed to reproduce the analyses are available in supplementary materials.

Conflict of interest statement

None of the authors have a conflict of interest to disclose.

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Chapter 2

Study of phenological synchrony between tits reproduction and vegetation phenology using high-resolution satellite imagery



In the first chapter of this thesis, I examined how the social environment, through mate preference, can influence female reproductive decisions. However, reproduction is influenced not only by social factors, but also by environmental conditions ²⁹. Identifying the factors affecting the seasonal timing of animals is essential to understand how they respond to environmental constraints. In the oak-caterpillar-passerine food chain that I study in this thesis, the optimal breeding season for birds is defined by the period when caterpillar abundance reaches a peak, as the growth and survival of nestlings depend on their availability ^{170,361,371}. Caterpillars depending entirely on tree leafing for their development, it has been suggested that vegetation phenology could be a reliable cue for birds to properly predict the timing of the food peak, and by extension, to appropriately time their breeding season.

In this chapter, I adopted a descriptive approach to study whether reproductive decisions and success of wild blue and great tits can be predicted from the phenology of the vegetation around their nest-boxes. A classic way to determine vegetation development is to visually assess bud stages on multiple trees directly in the field ^{186,187,197}, which is a time-consuming activity that can hardly be adopted over large surfaces and on the long term. An alternative way is to use a vegetation index derived from satellite imagery. Previous studies investigating the link between avian reproduction and vegetation phenology using satellite imagery, have done so at spatial resolutions that did not allow to link individual bird reproductive traits with vegetation development at the scale of each bird territory ^{195,321}. Using high spatial-resolution satellite images, I calculated the Normalised Difference Vegetation Index (NDVI), an indicator of vegetation primary productivity, around nearly 150 nest-boxes and over five consecutive years in one of our long-term studied populations, close to Montpellier, and related this measure to variation in individual reproductive phenology and performance of blue and great tits. I predicted that later vegetation development would correspond to later laying dates, and inversely. Additionally, I hypothesised that individuals would have a greater reproductive success if their breeding phenology was more synchronised with that of the local trees.

For each studied year, I first calculated the NDVI values around each nest-box for every exploitable satellite image over the period November to August. I then modelled the change in NDVI values over time around each nest-box. From these models, I estimated the date when the first leaves appear, which I called “the date of vegetation green-up”. I subsequently calculated the difference between the bird laying dates and the vegetation green-up dates at each nest-box. This measure reflects the degree of synchrony between the bird reproduction and the vegetation development. I finally estimated the effect of this synchrony on bird fitness-

related traits (clutch size, reproductive success, chick mass and tarsus length). I did not find that individual bird laying dates were related to vegetation phenology, although there seem to be a correlation at the population level. In addition, apart from a positive effect of the synchrony between bird laying dates and plant phenology on great tit chick tarsus length, I found no other clear effect of the degree of synchrony with the local vegetation.

The results of this chapter seem to contradict some previous studies suggesting that birds could rely on vegetation development to adapt their reproductive investment to local environmental conditions^{186,187,195}. However, this correlation has been found in studies conducted at the population level, i.e. at lower spatial and/or temporal resolutions compared to what we did. Studies examining the correlation between tree phenology and bird reproduction at a scale more relevant to individual birds struggled to find evidence that bud burst was a significant determinant of avian breeding timing and success^{187,197}. To conclude, this chapter highlights that birds might use vegetation development as a cue for reproduction, but that the scale is an important feature when considering this relationship. Moreover, this chapter also underlines that, although vegetation index derived from satellite imagery has great potential to investigate the influence of vegetation phenology on bird reproduction, these studies remain descriptive and experimental approaches manipulating vegetation-related cues are needed to test the causal relationships between vegetation phenology and bird reproduction.

Contribution: Samuel Caro designed and collected the first data. I processed the images with Cyril Bernard. I participated in the monitoring of the bird reproduction in the field. I then analysed the data with Jean-Yves Barnagaud and I wrote the first draft of the manuscript.

Article 3: The manuscript entitled “Predicting individual reproductive decisions of passerine birds using a satellite-derived vegetation index” is in preparation.

Article 3

Predicting individual reproductive decisions of passerine birds using a satellite-derived vegetation index.

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In preparation.

Abstract

Identifying the factors affecting the seasonal timing of animals is essential to understand how they respond to environmental heterogeneity. High-resolution satellite imagery now enables to estimate habitat variation and phenology of entire animal populations at spatial resolutions sometimes relevant to one single individual. In the well-studied tree-caterpillar-passerine food chains, vegetation phenology has been suggested as a cue that birds use to predict the phenology of their main insect food source, and by extension their individual timing of reproduction. Here, we studied the relationship between the phenology of oaks and the reproductive parameters of a Mediterranean population of blue and great tits, at the level of the individual territories. We used the Normalized Difference Vegetation Index (NDVI) calculated from high-resolution satellites imagery as a proxy for local spring development of vegetation. We monitored birds' laying dates, compared them to the estimated vegetation green-up dates over five consecutive years, and tested the effect of their synchrony on fitness-related traits (clutch size, chick mass, chick tarsus length, reproductive success). Vegetation green-up did not explain variation in laying dates at the individual level, and the degree of synchrony showed no clear impact on bird fitness-related traits, besides a positive effect of synchrony on chick tarsus length in great tits. Our results suggest that, contrary to what has previously been described at lower spatial resolutions in other populations, insectivorous birds may not always use the spring development of vegetation on their own breeding territories as a cue to time and adjust their reproductive effort.

Keywords: Normalized Difference Vegetation Index; satellites images; vegetation phenology; bird laying date; reproductive success; phenological mismatch

Introduction

Many organisms have shifted their phenology in response to current climate change (Parmesan and Yohe 2003; Root et al. 2003; Cohen et al. 2018). In a meta-analysis, spring-related events of 203 species' lifecycles advanced by 2.8 ± 0.35 days per decade across the northern hemisphere (Parmesan 2007), but recent studies updated this estimation to 4 ± 1.5 days per decade since the early 80's (Kharouba et al. 2018). The direction, magnitude and timing of phenological change also vary among taxonomic and trophic groups (Kharouba et al. 2018). In terrestrial habitats, phenology of primary consumers often changes more quickly than in other trophic levels (Both et al. 2009; Poloczanska et al. 2013; Sunday et al. 2015; Thackeray et al. 2016). For example, terrestrial invertebrates shifted their phenology faster (4.1 days per year) than terrestrial vertebrates (2.6 days per decade) (Thackeray et al. 2016). These different rhythms in phenological change can result in phenological mismatches between the organisms forming a food chain, causing demographic consequences and biotic interaction disturbances (Edwards and Richardson 2004; Kudo and Ida 2013; Renner and Zohner 2018; Visser and Gienapp 2019; Simmonds et al. 2020; Iler et al. 2021; Youngflesh et al. 2023).

In the well-studied tree-caterpillar-passerine food chains (Perrins 1970; Visser et al. 1998), the optimal breeding time for birds is defined by the peak period of caterpillar abundance. Nestlings of several insectivorous bird species almost entirely depend on the availability of caterpillars for their growth and survival (Dias and Blondel 1996; Naef-Daenzer and Keller 1999; Thomas et al. 2001; Visser, Holleman, and Gienapp 2006). Nevertheless, birds have advanced their phenology slower than other trophic levels in response to temperature increases (Both and Visser 2001; Cresswell and McCleery 2003; Both et al. 2009; Visser et al. 2012), which leads to important mismatches with their prey (Visser et al. 1998; Sanz et al. 2003) and strong selection for earlier breeding in some model species (Visser et al. 1998; Charmantier et al. 2008; Charmantier and Gienapp 2013; Marrot et al. 2018). The extent to which birds match their shift in breeding phenology with the advancement of their food source is however highly variable among populations and habitats (Goodenough et al. 2010). For example, great tits (*Parus major*) have not advanced their laying dates as much as caterpillar advanced their emergence dates on the Hoge Veluwe in the Netherlands (Visser et al. 1998). This mismatch negatively affected chick and parent fitness (Reed et al. 2013). Inversely, in the Wytham wood population (United Kingdom), individual adjustment of breeding behaviours in response to environmental changes has enabled the birds to track the food peak very closely (Charmantier et al. 2008).

The development of caterpillars entirely depends on the leafing of some tree species, such as oaks, because newly-hatched larvae can only feed on young shoots (Du Merle and Mazet 1983; Buse and Good 1996; van Asch and Visser 2007; van Asch et al. 2012). Vegetation type and phenology have therefore been suggested to be integrative and reliable cues for birds to properly predict the future abundance of their food source, and by extension to fine-tune their breeding period (Blondel et al. 1999; Bourgault et al. 2010; Hinks et al. 2015; Matthysen et al. 2021). For example, blue tits (*Cyanistes caeruleus*) breed at different times in different types of habitats in Corsica. In evergreen oak forests characterized by late bud burst, blue tits lay about one month later than in the earlier-bursting deciduous forests, enabling them to synchronize the feeding period of chicks with the peak of caterpillar abundance in their local habitat (Dias and Blondel 1996b; Lambrechts et al. 1997; Blondel et al. 1999; Blondel et al. 2006; Charmantier et al. 2016).

Classical methods to measure vegetation phenology are based on visual assessment of the phenological stages of buds on several trees around a point of interest, making it a relatively time-consuming activity that can hardly be applied to an entire forest (Bourgault et al. 2010; Morellato et al. 2010; Hinks et al. 2015; Matthysen et al. 2021). Remote sensing has proven as an efficient and reliable alternative to field collection data in studies on phenology (Schwartz et al. 2002; Fisher et al. 2006; O'Connor et al. 2012; Ali et al. 2016; de la Torre Cerro and Holloway 2021), widely used in animal ecology for population-level analyses (Pettorelli et al. 2005; Tveraa et al. 2013; Hurley et al. 2014). Satellite-derived vegetation indices now offer the potential to study phenological timing simultaneously on large surfaces and at high spatial resolutions, closer to that of the environment actually perceived by an individual (Turner et al. 2003; Wilkin et al. 2007; Skurikhin et al. 2013; Szulkin et al. 2015; Park et al. 2019; Pu and Landry 2020). The Normalized Difference Vegetation Index (NDVI) is a satellite-derived indicator of vegetation primary productivity used to determine the length and the peak of the growing season (Pettorelli et al. 2005). This index is already widely used to link vegetation phenology with animal distributions or breeding phenology and performance at large spatial scales, using satellites that offer moderate spatial resolutions, i.e. between 240 m and 8 km (Sanz et al. 2003; Wittemyer et al. 2007; Soudani et al. 2008; Hamel et al. 2009; Cole et al. 2015; Stoner et al. 2016; Uyeda et al. 2017; Funghi et al. 2020; Leveau et al. 2020). High-resolution satellite imagery has already been shown to provide reliable and ground-validated information on bird habitat heterogeneity (Szulkin et al. 2015), but whether such imagery is suited to describe bird phenological variability at the individual level remains to be explored.

The aim of this study was twofold. First, we investigated whether the individual reproductive phenology of a blue and great tit population in the Mediterranean region was correlated with the development of the vegetation, using a satellite-derived vegetation index. For that purpose, we calculated the NDVI extracted from images of two high-resolution satellites, Pléiades and Sentinel-2, estimated the vegetation green-up date, and linked that estimation to the laying dates of blue and great tits, over five consecutive years. We predicted that later vegetation green-up dates would be associated with later laying dates, and conversely. Second, we studied if the degree of synchrony between the bird laying dates and the local tree phenology modulated bird breeding success. We hypothesised that birds that better synchronized their breeding phenology with that of the local trees would have a higher reproductive success.

Methods

Study area

Blue and great tit reproduction was monitored across five successive years from 2016 to 2020, as part of a long-term monitoring program of 227 nest-boxes located in a forest dominated by deciduous downy oaks (*Quercus humilis*) (Charmantier et al. 2016) near Montpellier, France (La Rouvière wood, Montarnaud, 43.621°, 3.733°, 2.4 km long × 1.4 km wide, 300 hectares, figure 1). Nest-boxes are approximately 50 m apart.

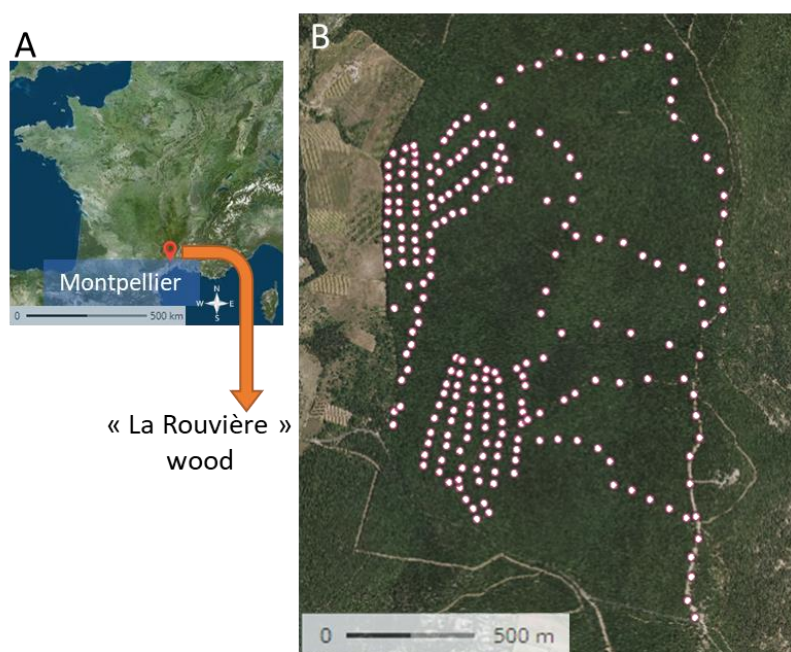


Figure 1. Study area

A: Location of the study site. B: spatial arrangement of the 227 nest-boxes located on the study site.

Breeding monitoring

Nest-boxes were monitored every year from March to July to collect bird reproduction data on a weekly basis. Brood monitoring began prior to nest-building and ended after the last nestling had fledged. The exact date the first egg was laid (lay date), the number of eggs laid (clutch size), and the number of nestlings were observed during nest-box field visits. Nest-box were also visited 21 days after hatching to check the number of fledglings. When nestlings were 9-15 days old, parents were captured to be uniquely identified with metal rings provided by the CRBPO (Centre de Recherches sur la Biologie des Populations d'Oiseaux, Museum National d'Histoire Naturelle, Paris) and measured (age, body mass, tarsus, wing and tail length). At 15 days of age, nestlings were also ringed, weighed and their tarsus measured (Blondel et al. 2006; Charmantier et al. 2017).

Nest-boxes selection

The nest-boxes are surrounded by both deciduous (downy oak) and evergreen trees (mainly Cane-apple bush *Arbutus unedo* and Holm oak *Quercus ilex*). The increase in NDVI in early spring mainly reflects the appearance of first leaves on trees. In areas dominated by evergreen trees however, the NDVI is constantly high throughout the year and varies much less than in areas dominated by deciduous trees. As our aim was to study whether the breeding season of birds was synchronised with vegetation development, we excluded from our study the areas dominated by evergreen vegetation because NDVI variation is much harder to quantify in these habitats, leading to larger uncertainties in our proxy of the maximal green-up date (see below). Based on the species identification of 30 trees around each nest-box (Pascal Marrot, unpublished data), we therefore removed the nest-boxes for which the combined proportions of holm oak and cane-apple bush was equal to, or greater than 50% ($n = 85$ nest-boxes). We also removed one nest-box surrounded by 98% of pine trees, as well as two nest-boxes in close proximity of artificial ponds. Our study therefore included 139 nest-boxes (figure 2A).

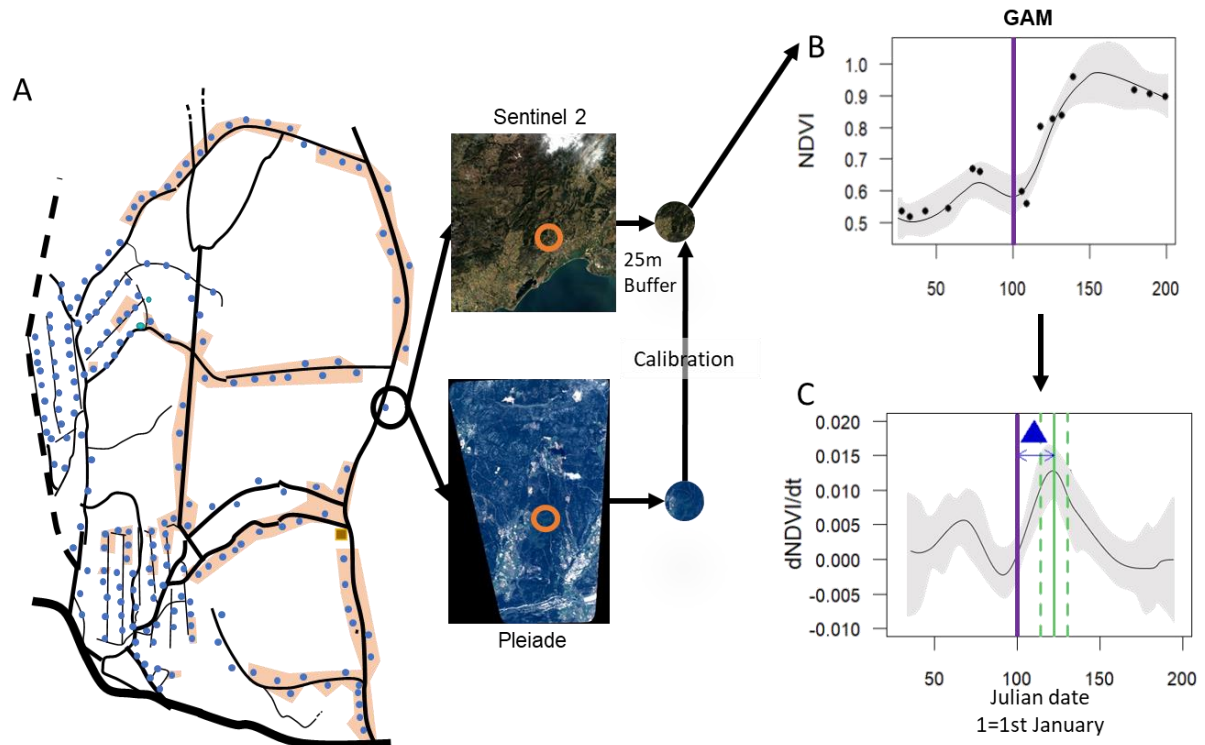


Figure 2. Nest-box selection, NDVI data, vegetation green-up date and synchrony with bird reproduction.

A: Map of the study area. Nest-boxes (blue dots) highlighted by orange bands ($n = 88$) were excluded from the analyses due to the high proportion of evergreen trees, or the presence of artificial ponds. We collected images from Sentinel-2 and Pléiades satellites for the five studied years, then calibrated Pléiades images based on Sentinel-2 images thanks to invariant areas (quarries, buildings, bare ground) on both images, and finally calculated a NDVI value in a 25 m radius zone around each nest-box on each of the image collected.

B: Annual NDVI time series were modelled by a Generalized Additive Model (GAM). The grey area depicts the 95% confidence interval of the model, and the purple line represents the laying date of the bird for this specific nest-box.

C: Derivative of the GAM used to model the NDVI time series. The grey area depicts the 95% confidence interval of the derivative. The purple line represents the laying date of the bird for this specific nest-box. The green line depicts the date of the inflexion point, estimated by the maximum of the derivative curve, and that we defined as the date of the vegetation green-up. The two dotted green lines represent the 95% confidence interval around this estimated date. The blue delta (Δ) and arrows represent the degree of synchrony between the bird laying date and the vegetation green-up date. Delta was calculated as the bird laying date minus the vegetation green-up date.

Figure layout inspired by (Cole et al. 2015).

Image Data Acquisition and Processing

We collected 113 satellite images generated spanning five years from Pléiades (Gleyzes et al. 2012) and Sentinel-2 (Drusch et al. 2012). Images with more than 35% of cloud cover over the study site were excluded from the processing and analyses. We finally used 98 images on 92 days across the study period (2016: 16 days, 2017: 20 days, 2018: 17 days, 2019: 21 days, 2020: 18 days). Details on dates and numbers of images collected per year and per satellite are described in figure S1.

Satellite images

Pléiades is a constellation of two very-high-resolution satellites (70 cm for panchromatic spectral mode and 2.8 m for multispectral mode) launched in 2011 (17th December 2011 for Pléiades 1A and 2nd December 2012 for Pléiades 1B) by the French Space Agency (CNES), capable of acquiring imagery of any point on the globe in less than 24 hours (Gleyzes et al. 2012). We obtained panchromatic and multispectral Pléiades images from ISIS program (Airbus Defense & Space GEO SA, Toulouse, France) for 2016, 2017 and 2018. From the raw images, we performed three corrections. First, using multispectral images with the ENVI software (version 5.4, <https://www.l3harrisgeospatial.com/Software-Technology/ENVI>) and the *RPC Orthorectification with Reference Image* tool, we applied a geometric correction (i.e. orthorectification) to properly superpose satellite images relative to an IGN reference image. The resulting images were projected in UTM31N with a resolution of 2 m. Second, we performed a radiometric correction, by calculating the Top Of Atmosphere (TOA) reflectance in the ENVI software with the *Radiometric correction* tool, in order to conceal the brightness change due to the sensor features, or the seasons. Third, cloudy areas on the images were masked. Clouds and their shade were drawn manually by photo-interpretation and digitized using QGIS software (<https://www.qgis.org/fr/site/>).

The Copernicus Sentinel-2 mission is a program of the European Space Agency (ESA) based on a constellation of two identical satellites in the same orbit, phased at 180° to each other, launched in 2015 (23rd June 2015 for Sentinel-2A and 7th March 2017 for Sentinel-2B) with the European launcher VEGA. The orbital swath width is 290 km and the spatial resolution is 10 m. The satellites sample 13 spectral bands from visible to near infra-red (four bands at 10 m, six bands at 20 m and three bands at 60 m spatial resolution) and have a revisit time of five days (Drusch et al. 2012; Wang et al. 2016). We downloaded the level-2A Sentinel-2 products corrected for atmospheric effects provided by the French space centre (Centre National d'Etudes Spatiales, CNES) through the THEIA thematic center (<https://www.theia-land.fr/>)

using the *aria 2* software (<https://aria2.github.io/>) for the five years studied. Level-2A images are provided with geometric and radiometric corrections for atmospheric effects and a cloud mask. Sentinel-2 images covered a much larger area than Pléiades images. Therefore, we extracted the study area from Sentinel-2 images for the four spectral bands needed to calculate the NDVI (2: 490 nm Blue, 3: 560 nm Green, 4: 665 nm Red, 8: 842 nm Near Infra-Red (NIR)).

We identified several features of these satellites that might lead to different NDVI values between the two satellites. First, the spatial resolution of each satellite is different (10 m for Sentinel-2, 2 m for Pléiades). Second, Pléiades theoretical revisit time is shorter (24 h) than the one of Sentinel-2 (5 days). In theory, we could thus have acquired more images from Pléiades than from Sentinel, but Pléiades image captures of the globe surface were not systematic, but programmed and prioritised according to rules that we did not control. Third, whereas Sentinel-2 provides images with radiometric correction, Pléiades does not. Consequently, the radiometric correction method that we used on Pléiades images might be slightly different from that used for the Sentinel-2 images, which could lead to slightly different NDVI calculations between both satellites. We therefore calibrated the images from the two satellites (see below).

NDVI calculation and satellite images calibration.

NDVI was calculated for a circular surface of 25 m radius (buffer zone) around each nest-box as follows: $(\text{NIR}-\text{R}) / (\text{NIR}+\text{R})$, where NIR and R are the near infra-red and red reflectance, respectively. This index generates values between -1 and 1. Negative values mostly represent clouds, water and snow, while values close to zero are due to rocks, bare soil or sand. Higher values therefore depict denser vegetation. NDVI increase with the growth of new leaves.

A first analysis of the NDVI time series calculated from Pléiades and Sentinel-2 images showed that both satellites were producing the same curve shape of NDVI across time, but with absolute values that varied slightly between the satellites. We thus calibrated Pléiades images to match those of Sentinel-2. For this, we identified invariant areas on Sentinel-2 and Pléiades images, and took those invariant areas as a reference to calibrate the NDVI values of Pléiades images on NDVI values of Sentinel-2 images. We proceeded in 4 steps: (i) From the Sentinel-2 level-3A images downloaded on the THEIA platform, which correspond to a monthly synthesis of images, we extracted the studied area on the spectral band 2,3,4,8, saved the coloured composition, overlaid all images from all years and calculated for each pixel the standard deviation of the reflectance. (ii) We determined polygons on the images where reflectance did not significantly change (quarries, buildings, bare ground), using QGIS. We manually drew the invariant polygons in a new shapefile with QGIS drawing tools. (iii) We

calculated the mean reflectance on the invariant polygons for the bands 4 and 8 (Red and NIR) for both Sentinel-2 level 3A and Pléiades images. (iv) We matched each Pléiades image to a reference Sentinel-2 image (the closest in time). For each pair of Pléiades/Sentinel-2 images, we computed two linear regressions based on Red and NIR reflectances of the invariant polygons. We then applied the two coefficients extracted from the linear regressions to the two Pléiades Red and NIR bands. Finally, we calculated the NDVI with the calibrated Pléiades and Sentinel-2 level 2A images, with a mask for clouds, buildings, roads and fields, to prevent NDVI to be calculated on these non-forest areas. When both Pléiades and Sentinel-2 images were available for one day (6 days, figure S1), NDVI value of this day was the mean of the two NDVI values extracted from both satellite images.

NDVI validation with field data on oak bud development

To assess whether NDVI values calculated from satellite images reliably matched oak leafing in the field, we compared the seasonal variation of the mean NDVI value calculated around 30 nest-boxes with the stages of development of buds of 150 oaks around the same 30 nest-boxes (i.e. five trees visually assessed around each nest-box), in 2017. The NDVI profile paralleled the ground data observations, with the NDVI increasing approximately 20 days later than the start of bud development (figure 3). This time-gap between bud burst and NDVI is to be expected given that the NDVI represents the photosynthetic activity of the vegetation (Soudani et al. 2008). NDVI therefore only starts to increase when leaves have started growing, meaning at a later stage than bud burst that many studies use as a reference point for comparing vegetation development to bird phenology (Nilsson and Källander 2006; Bourgault et al. 2010; Matthysen et al. 2021).

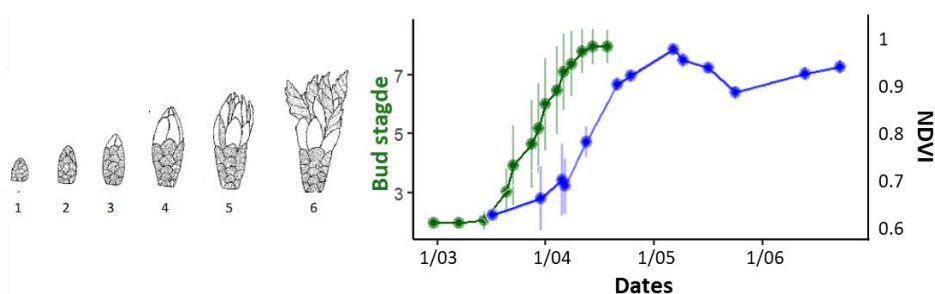


Figure 3. Parallel variations of bud development and mean NDVI in 2017.

The left panel depicts the first six stages of development of oak buds (Du Merle and Mazet 1983). Stages 7 and 8 (fully mature leaves) are not depicted. On the right panel, the green line depicts the spring development of deciduous oak buds based on ground data measurements (average scores of 150 trees around 30 nest-boxes at each time point). The blue line depicts the variation of the mean NDVI in a 25 m buffer around those same 30 nest-boxes. Vertical bars on each curve depict standard deviations.

Estimating vegetation green-up dates and the degree of synchrony of bird laying dates with vegetation development.

Following (Soudani et al. 2008), we took the point of maximum rate of change, i.e. the inflexion point of the curve fitted to a vegetation index, as an indicator of the vegetation green-up. To define this point, we modelled the NDVI time series of each nest-box as a generalized additive model (GAM), with date as a thin-plate spline smoother (figure 2B). We defined the date of vegetation green-up for each nest-box as the day on which the derivative of this function was maximized (Cole et al. 2015). To account for the uncertainty associated with the estimated parameters of the GAMs, we sampled 1000 posterior distributions of each GAM for each year and each nest-box, retrieved the 1000 corresponding maximum derivatives, and use their mean and 95% confidence intervals as indicators of green-up date on each nest-box (Wood 2001) (figure 2C).

In order to determine the relationship between bird reproduction data and nest-box-specific green-up each year, we calculated the degree of synchrony (Δ) between each bird laying date and each vegetation green-up date estimated from the derivative function of the GAM model as follows:

$$\Delta (\text{degree of synchrony}) = \text{bird laying date} - \text{vegetation green up date}.$$

Thus, a $\Delta < 0$ indicate a bird that laid before the vegetation green-up. For each year, we retrieved 1000 Δ values corresponding to the 1000 GAM posterior distribution samples to quantify annual synchrony between laying date and vegetation green-up.

Statistical analyses

All the analyses were performed in R version 3.6.1 (R CoreTeam). Linear mixed-effects models and generalized linear mixed-effects models were conducted using the lme4 package (Bates et al. 2015). P-values for linear mixed models were obtained with the lmerTest package (Kuznetsova et al. 2017). GAMs were conducted using the mgcv package (Wood 2001).

Synchrony of bird laying dates with vegetation green-up dates

In order to test the influence of vegetation green-up on bird laying date, we ran a linear mixed effect model with laying date as a Gaussian response variable. The interaction between linear effects of vegetation green-up date and year was included in the model because we expected that the degree of synchrony could vary from year to year. Nest-box identifier was added as a random intercept to account for repeated measurements across time. We also

investigated the relationship between population-scale mean laying dates and mean vegetation green-up dates per year with a Pearson's correlation test.

As an alternative to using the inflexion points of the vegetation green-up trajectories, we modelled bird laying dates as a function of the raw NDVI values taken at several successive dates (i.e. satellite images). We fitted a generalised additive model (GAM) with the laying date as response variable and a time-varying effect of NDVI as a thin-plate spline smoother, with six time intervals (six periods of 15 days: from early March (1st-15th March) to late May (15th-31st May)). Nest-box identifier and year were added as random intercepts.

Effect of the degree of synchrony on bird reproductive traits.

We studied the effect of the degree of synchrony between the bird laying dates and the vegetation green-up on four reproductive traits: (i) clutch size (Poisson distribution), (ii) breeding success (defined as the number of fledglings relative to the number of hatchlings, binomial distribution), (iii) nestling mass at 15 days post-hatching (Gaussian distribution) and (iv) tarsus length (Gaussian distribution). In all models, we included delta (the degree of synchrony) as a 2nd order polynomial explanatory variable, and nest-box identifier and year as crossed random effects. We ran each model with three values of delta: the mean delta and the two delta of the high and low bounds of its 95% confidence interval.

We re-fitted our models with the median (instead of the mean), quantile 5% and quantile 95% (instead of the low and high bound of the 95% confidence interval) of delta (figure S2) as a sensitivity analysis. We also ran the analyses only on 2019 and 2020 data for which NDVI values originate only from Sentinel-2 images (figure S3), to evaluate the impact of uncertainty on the NDVI values added by our calibration method of Pléiade images on Sentinel-2 images. For these two analyses, we processed the same way to calculate the vegetation green-up date and delta, and ran the same models previously described, with the mean, high and low bound delta of its 95% confidence, as well as the median, quantile 5%, and quantile 95% delta. The results from the sensitivity analysis will not be presented in the results but figures are available in supplementary materials (figure S2-S3).

Results

Mean reproductive traits and vegetation green-up

Birds laid on average the 7th April across the five years with 2017 being the earliest year (31st March) and 2018 the latest (17th April) (Table 1). Blue tits laid on average 10.1 eggs against 9.5 eggs for great tits (Table 1). Approximately one chick out of two fledged for blue tits while the reproductive success of great tit was a bit higher (mean reproductive success for blue tit: 0.53 and great tit: 0.65, Table 1). Chicks were heavier with longer tarsus in 2018 (Table 1). Mean vegetation green-up date across the five years studied was the 13th April, with 2020 being the earliest year and 2018 being the latest.

Table 1. Breeding data across the five years for blue (BT) and great tits (GT), and estimated vegetation green-up dates.

Mean and 95% CI are depicted. For dates: 92 = 1st April, 100 = 9th April.

Year	Green-up dates (n = 294)	Species	Laying date (n = 301)	Clutch size (n = 304)	Number of fledglings per clutch (n = 307)	Reprod. Success (n = 306)	Chick weight (n = 1965)	Chick tarsus length (n = 1857)
2016	104.24	BT	94.6±1.45	10.92±0.43	5.42±1.08	0.54±0.11	9.73±0.11	16.48±0.08
	±0.55	GT	101.4±2.64	9.92±0.58	3.00±2.45	0.31±0.25	15.21±0.43	19.53±0.17
2017	101.46	BT	89.62±1.58	10.63±0.47	3.72±1.01	0.41±0.11	9.81±0.17	16.41±0.08
	±1.91	GT	93.50±2.34	9.15±0.76	5.55±1.49	0.67±0.18	15.72±0.29	19.57±0.12
2018	112.78	BT	107.40±2.21	9.51±0.66	7.06±1.05	0.80±0.10	11.05±0.12	16.82±0.06
	±2.20	GT	109.14±2.63	9.71±0.56	7.24±1.67	0.76±0.17	17.06±0.19	19.71±0.09
2019	106.42	BT	94.93±1.59	10.05±0.49	3.61±1.04	0.39±0.11	10.24±0.14	16.59±0.08
	±1.51	GT	100.75±2.69	10.19±0.74	5.19±1.89	0.54±0.19	15.00±0.33	18.98±0.16
2020	93.78	BT	96.44±4.09	9.37±0.41	5.45±1.15	0.65±0.13	10.58±0.13	16.70±0.06
	±1.69	GT	102.00±6.47	8.82±0.58	6.71±1.16	0.83±0.13	16.10±0.30	19.52±0.10

Synchrony of bird laying dates with vegetation green-up dates

On average, blue and great tits respectively laid 8.43 and 3.47 days before the vegetation green-up date (Table 1). The year 2020 was characterised by an early green-up in the season (Table 1, figure 4 A-B). At the individual level, we found no effect of green-up dates on laying dates ($F_{1,270.41} = 0.17$, $p = 0.67$). If, instead of the individual data, we consider the annual mean laying dates and green-up dates of the population, as in some earlier studies (e.g. Bourgault et al. 2010), we also found no relationship (Pearson correlation coefficient = 0.59, $t = 1.29$, $df = 3$, $p = 0.29$), unless 2020 is removed, in which case there was a positive relationship between

laying dates and green-up dates (Pearson correlation coefficient = 0.99, $t = 8.83$, $df = 2$, $p = 0.013$) (figure 4B).

As an alternative to inferring the vegetation green-up dates from inflexion points, we tested the effect of the raw NDVI values on bird laying date. The NDVI values in early March (1st-15th March) had a significant effect on bird laying dates ($F = 4.818$, $p = 0.028$), with birds laying later when NDVI was high in early March (figure 4C). None of the other NDVI periods had an effect on laying dates (all $p > 0.05$).

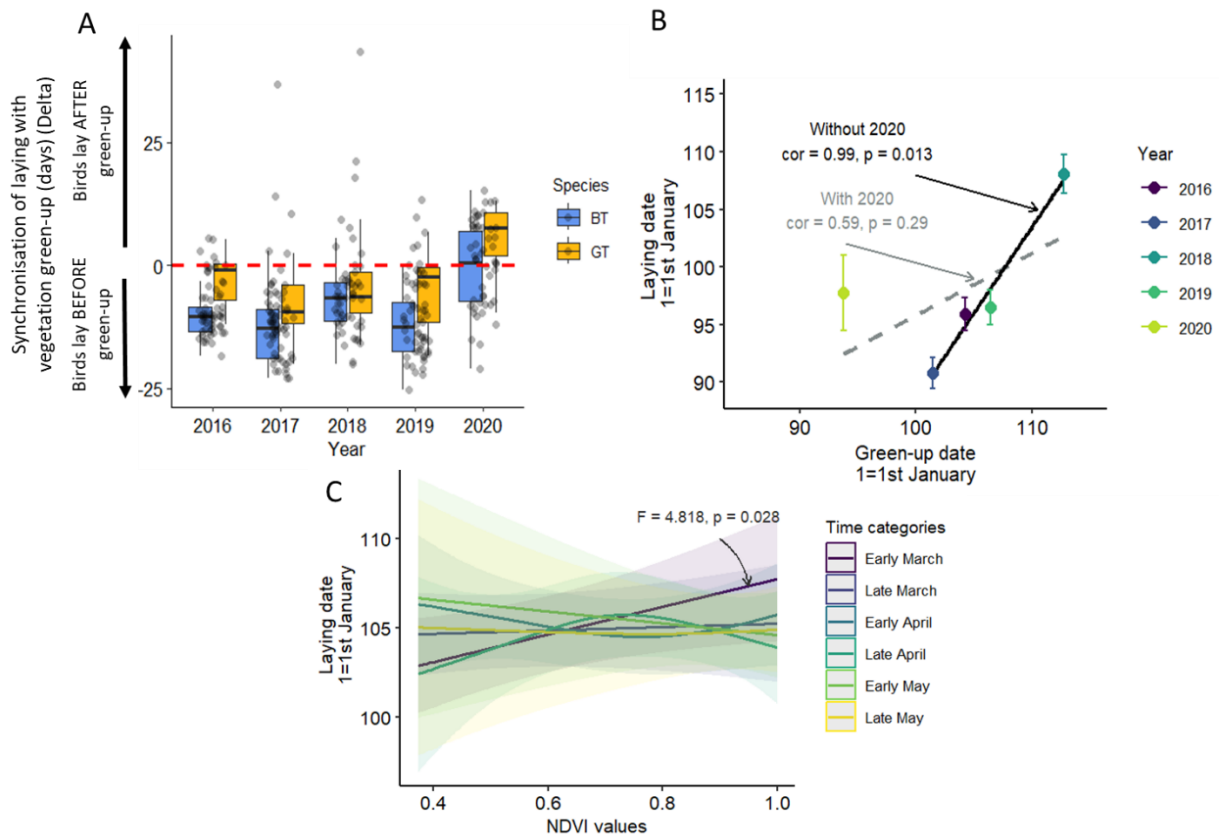


Figure 4. Synchronisation of bird laying with vegetation green-up.

A: Synchronisation per year and per species. On the y-axis, the red dashed line depicts the perfect synchronisation between green-up and lay dates; negative/positive values depict birds that lay before/after vegetation green-up, respectively. On average, blue and great tits laid before the vegetation green-up, except in 2020. GT: Great tit, BT: Blue tit. Grey points depict raw individual data.

B: Laying date as a function of vegetation green-up date, for each studied year. Mean laying date and mean green-up date are correlated, but only after removing the 2020 data ($p = 0.013$). Dots and error bars represent mean \pm CI. Black and grey dotted lines depict the correlation between mean laying date and mean green-up date per year with (grey dotted) or without (black) 2020 data.

C: Effect of NDVI values on bird laying dates. Time intervals was used as a categorical variable, with six intervals of 15 days: from early March (1st-15th March) to late May (15th-31st May). Birds lay later when NDVI values in early March are high ($p = 0.028$). Predicted values of the GAM model and 95% confidence interval are depicted.

Effect of the degree of synchrony on bird reproductive traits.

We ran separate analyses for blue and great tits as they differ in their breeding traits, and because we collected more data for blue tits than great tits over the five years (blue tits: 218 first clutches, great tits: 83 first clutches).

Although we showed a seasonal decrease in clutch size in blue tits, we did not observe an effect of the synchrony of laying with vegetation green-up on clutch size in any species (figure 5A-B). In great tits, we observed one individual with a delta of 40. This female laid the 25th April 2018, for an inflexion point occurring on the 12th March. It is possible that the inflexion point corresponding to that nest-box has been overestimated, especially since the vegetation green-up date of the neighbouring nest-boxes occurred on the 23rd April. Removing this outlier from the data did not change the clutch size results, however (figure 5B).

Reproductive success seemed to increase during spring for great tit when removing the outlier, but independently of the synchrony with vegetation green-up in both species (figure 5D). Reproductive success first decreases if birds laid too early compared to the vegetation green-up (between 20 and 10 days before, so approximately at the time of budburst (figure 3)), then increased if bird started laying from 10 days before vegetation green-up date (figure 5C-D), but this effect remains small given the wide confidence intervals observed.

Chicks mass showed opposite patterns for blue and great tits. Indeed, blue tit chicks were heavier if laid later in the breeding season and after vegetation green-up (figure 5E), while it is the opposite for great tits (lighter if laid later and after vegetation green-up). When removing the outlying great tit, chick mass remained constant over the season, independently of the synchrony of laying with vegetation green-up (figure 5F).

In blue tits, we did not detect an effect of the synchrony of laying with vegetation green-up on chick tarsus length (figure 5G). For great tits, however, chick tarsus length of great tits reached a maximum when birds were perfectly synchronized with vegetation green-up (figure 5H). The same pattern persisted with and without the outlier (figure 5H).

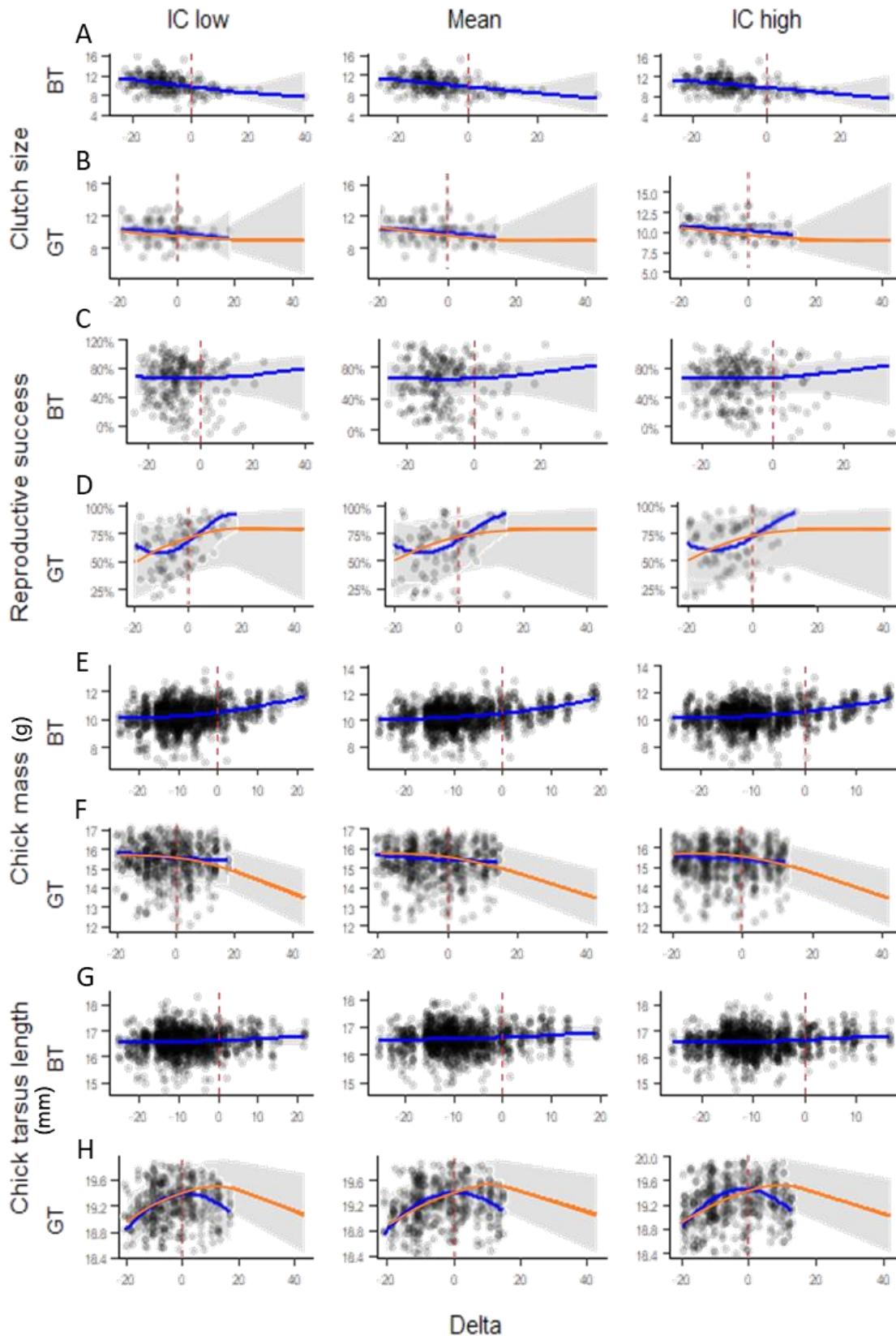


Figure 5. Effect of the synchrony of bird laying date with vegetation green-up date on their breeding data.

We used three values of delta to estimate the effect of the synchrony of bird laying with vegetation green-up on their breeding traits: the mean delta and the two delta of the high and

low bound of its 95% confidence interval. Results of each model ran for breeding traits and delta estimations are depicted on the graphs. Points represent residuals and lines depict the predictions of the models with a 95% confidence interval. For great tits, orange lines depict the predictions of the models for all individuals and blue lines depict the predictions of the models when removing an outlier with a delta of 40. *Delta* is defined as *bird laying date* – *vegetation green-up date*. Therefore, $\text{delta} < 0$ represents birds that laid before the vegetation green-up. Red-dotted lines indicate a delta equal to 0, i.e. birds that synchronised their laying with vegetation green-up. GT: great tits, BT: blue tits.

Discussion

While we found that mean vegetation green-up date explains mean laying date of birds in most years at the population scale, we found no convincing evidence that the green-up date around each nest-box was related to the reproductive phenology of the birds' pair occupying this nest-box, in our long-term studied Mediterranean populations of blue and great tits. We also found no clear evidence for an effect of the synchronisation of laying dates with vegetation development on reproductive performances, besides an increase in chick tarsus length in great tits that lay close to the green-up.

Our results do not support the hypothesis of a spatial synchrony between laying dates and tree leafing at the level of the individual territories. At first, these results seem to contrast with several previous studies (Bourgault et al. 2010; Cole et al. 2015; Hinks et al. 2015). Bourgault et al. (2010) found a strong correlation between oak phenology and laying dates of blue tits in Corsica. However, their correlation was built on the annual means of laying dates and budburst across habitats, while we estimate the same relationship at the level of individual bird pairs. When upscaling our data to the annual means of green-up and laying dates, we found some support for a relationship as found in Bourgault et al. (2010) (figure 4B). Temporal and spatial scales therefore matter for estimating the link between bird breeding and plant phenology. On an evolutionary point of view, selection at the population level may operate differently than at the individual level, where sensitivity to small-scale environmental variation may be more prominent (Garant et al. 2007). Hinks et al. (2015) and Matthysen et al. (2021) approached the topic a bit differently and analysed the relationship between laying dates of tits and oak budburst (directly assessed in the field on individual trees) over three years in forests respectively located in the United Kingdom and Belgium, at smaller spatial scales (15-20 m), relevant to individual birds. If Hinks et al. (2015) results show that intra-annual variation in oak timing close to the nest is more influent in modulating great tit laying dates than at a wider scale, oak phenology remained overall a relatively poor predictor of individual great tit timing

of breeding in this three-year period. Matthysen et al. (2021) on their side found absolutely no support for a relationship between budburst timing and blue and great tit timing at the level of the bird territories. Finally, Cole et al. (2015) used a similar method to ours, with vegetation green-up estimated from an index derived from satellite imagery, on a 13-year dataset in the Wytham wood forest in the UK. Although they found that the onset of egg laying of great tits (but not blue tits) was correlated to local intra-annual variation in vegetation green-up, they used imagery from MODIS satellites that have a spatial resolution of 240 m, when our study uses Pléiades and Sentinel-2 satellites, with spatial resolutions of 2 and 10 m, respectively. This means that Cole's et al. (2015) green-up was estimated on surface units of circa 5.8 ha. As the average territory size in Wytham wood is 1.25 ha (Wilkin et al. 2006), their estimation of one single vegetation green-up was applied to 4.6 pairs on average, which is less accurate than our individual-based models. Furthermore, the relationship between vegetation green-up and timing of breeding became more evident, particularly for blue tits, when the spatial and temporal resolutions of the data were further reduced to one single mean green-up and lay date per year, i.e. back at the population level (Cole et al, 2015). Altogether, ours and earlier studies therefore seem to converge to the conclusion that the effects of vegetation phenology on bird seasonal timing of reproduction are more evident at the population than at the individual level. This suggests that the influence of vegetation development on individual bird timing might be less than expected, or that it comes about via other variables, like temperature for example.

Birds might respond to signals that are themselves influencing local tree bud-burst, like microclimate fluctuations. Experiments in aviaries have indeed shown that increasing temperatures, but not leafing trees, trigger earlier laying in great tits (Visser et al. 2009; Schaper et al. 2011; Schaper et al. 2012). Synchronization observed in other studies between bird laying and tree phenology may therefore have been caused by microclimatic variations that impact both bird and tree phenology (Shutt et al. 2019; Matthysen et al. 2021). In a recent study, Shutt et al (2019) found that mean night-time temperature in early spring was the strongest predictor of lay date in a blue tit population in Scotland. In this population, birch budburst phenology significantly predicted lay date, but additionally to temperature (Shutt et al. 2019). Another possible cue that could be suggested from previous studies is the local vegetation composition of birds' breeding territories, instead of the phenology of the trees surrounding nest-boxes. Indeed, recent studies have found a better synchrony between bird breeding phenology and vegetation phenology and better reproductive success in nest-boxes surrounded by more deciduous oaks (Wilkin et al. 2007; Cole et al. 2015; Amininasab et al. 2016; Dekeukeleire et

al. 2019; Matthysen et al. 2021). In the Corsican population of blue tits, the correlation between tree and bird breeding phenology found by Bourgault et al. (2010) was largely driven by the fact that birds breed in very contrasting habitats, with populations breeding in evergreen forests having late phenologies, and populations breeding in deciduous habitats having early phenologies. Szulkin et al. (2015) confirmed this observation finding that, in the population we studied, the number of evergreen oaks, but not deciduous, within a 50 m radius of the nest-box was positively associated with later breeding dates in blue tits. In our analyses, we excluded nest-boxes surrounded by evergreen vegetation (see method) to test whether there was a relationship between tree and bird breeding phenology when only deciduous vegetation was examined, but we found no relationship at the individual territory level. This seems surprising given that oaks usually harbour higher densities of caterpillars compared to other tree species (van Asch and Visser 2007; Wilkin et al. 2007; Wilkin and Sheldon 2009; Shutt et al. 2019) and that caterpillars are the preferred prey of tits to feed their nestlings (Banbura et al. 1999). However, the Rouvière forest that we studied has been shown to be very poor in caterpillars. For example, blue tit pairs breeding in deciduous downy oak forest (*Quercus pubescens*) in Corsica raise their young on a caterpillar peak almost 25 times higher than that available to pairs breeding in the Rouvière forest (the peak caterpillar abundance is identified as the maximal abundance of frass, in milligrams per square meter per day ($\text{mg}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$); mean peak caterpillar frass fall: $1474 \text{ mg}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$ in Corsica *versus* $61 \text{ mg}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$ in the Rouvière forest) (Tremblay et al. 2003). Birds living in this caterpillar-poor forest then have no choice but to diversify their diet to meet the needs of their chicks. Selection for the synchrony between the onset of breeding and the peak of caterpillar abundance, and therefore with vegetation green-up, could therefore be relaxed, as birds would feed their offspring on many other insect species. Consequently, the diet of nestlings in our population could not be limited to caterpillars, but could include a wider diversity of prey, such as spiders, as it has been shown in other tit populations in the Mediterranean regions (García-Navas and Sanz 2011; Pagani-nunez et al. 2011; Navalpotro et al. 2016; Serrano-Davies and Sanz 2017).

The absence of a convincing effect of the vegetation green-up on bird breeding traits could also be due to technical limitations linked to the proxies we used, rather than to biological reasons. First, the noise generated by the mostly evergreen shrub layers could prevent accurate estimations of green-up dates from NDVI data. La Rouvière forest, where we carried out our analyses, is a relatively young forest, composed of small-trunked trees and a dense shrub layer dominated by evergreen arbutus (Estornell et al. 2011; Estornell et al. 2012; Helman et al. 2015;

Chang and Shoshany 2016). Moreover, in this forest, June NDVI values for deciduous tree areas were shown to be 58% higher than in February, while in evergreen tree areas, NDVI values increased by only 8% (Szulkin et al. 2015). Similarly, a study conducted in a deciduous forest in Korea, in which the authors measured the NDVI at four different canopy heights using light-emitting diode (LED) sensors, found that the understory vegetation determined the onset of green-up in that area (Ryu et al. 2014). The understory canopy developed leaves seven days earlier than the overstorey canopy but the sensors did not detect the difference in phenology between the two canopy layers, resulting in an estimate of the green-up date eight days earlier than the date observed from ground data (Ryu et al. 2014). In our study area, understory evergreen arbutus could therefore generate high NDVI values even before the deciduous canopy starts to leaf. Although we removed from our analyses the nest-boxes that were not predominantly surrounded by evergreen vegetation (see methods), we still observed several NDVI curves that did not show the characteristic NDVI sigmoid curve of a classical deciduous forest in spring (figure S4), which could distort our estimate of vegetation green-up date for some nest-boxes. Therefore, estimating vegetation green-up from NDVI time series in Mediterranean habitats may be more challenging than in higher latitude forests, where the vegetation budburst is more distinct in spring (Maselli 2004; Pettorelli et al. 2005).

Second, the mathematical method used to estimate the vegetation green-up date from the NDVI time series might be a source of errors. A wide array of methods exists to calculate spring vegetation green-up of deciduous trees (Jönsson and Eklundh 2003; Jönsson and Eklundh 2004). For example, methods use the date of the maximum rate of change in a specific vegetation index (Zhang et al. 2003; Soudani et al. 2008; Busetto et al. 2010), the averaged value of a vegetation index in a time window (Sanz et al. 2003) or the date of the half-maxima of a sigmoid curve fitted to the vegetation data (Fisher et al. 2006). Cole et al. (2015) used the maximum of the derivative of the sigmoid curve fitted to the enhanced vegetation index 2 (EVI2) time series (Jiang et al. 2008), on surface units regrouping more than 4 breeding pairs. We therefore decided to use the same method in order to be able to compare our results to this previous study. As we were working at a finer scale, i.e. at the level of a single breeding pair, using the same method as Cole et al. (2015) allowed us to rule out a potential source of variation that could have explained why our results were different. However, we cannot exclude the possibility that using one of the other methods mentioned above would not have resulted in a better relationship between vegetation and bird breeding phenology.

Third, the use of images from several different satellites can lead to variations in the estimation of NDVI. As satellite images are not collected on a daily basis, using only images

from a single satellite can result in long periods without images, making it impossible to model an accurate NDVI time series, and consequently introducing uncertainties in the estimate of the green-up date by a few days. To minimise the length of the periods without data, we used images from two satellites. However, this method involves calibrating the images from one of the two satellites to the images from the other satellite (see method), implying an additional level of data manipulation, that may increase the uncertainties in the estimates derived from these data. To analyse whether our calibration method reduced the quality of our estimates of the green-up dates and affected the quality of our results, we made a sensitivity analysis using only Sentinel-2 images. In this analysis, we used the same processing as for both satellites to estimate the vegetation green-up date from NDVI time series and the effect of the degree of synchrony of bird breeding with vegetation green-up (see methods). This analysis shows similar results to those obtained with the two satellites (figure S3). The use of images from two satellites instead of one and the method of calibrating the two satellites therefore did not appear to be the cause of the lack of effect of vegetation phenology on bird reproduction.

In summary, we did not highlight a strong impact of vegetation green-up on blue and great tit individual breeding traits, despite the relatively large sample size allowed by modern remote sensing techniques. If this may seem to contradict some earlier studies, this is mostly true for those conducted at a lower spatial and/or temporal resolution (Nilsson and Källander 2006; Bourgault et al. 2010; Wesolowski and Rowiński 2014; Cole et al. 2015; Youngflesh et al. 2023), because those that focussed on variation at scales relevant to individual birds similarly struggle to find convincing evidence that budburst is a decisive predictor of avian timing of breeding and performance (Germain et al. 2015; Hinks et al. 2015; Matthysen et al. 2021, this study). Our study therefore raises the question of the scale at which birds could use vegetation development as a cue for their reproduction (Hinks et al. 2015), if they use it at all. It also highlights the fact that the type of vegetation constituting a forest, and the local dynamics of insect abundance (and therefore of selection pressures) within a given habitat, might interact with the local plant phenology in shaping bird breeding decisions.

We are the first to use a vegetation index derived from satellite images to investigate the effect of vegetation development on bird reproductive traits at a level relevant to a single breeding pair. The use of this method has great potential for future studies, as satellite imagery can be collected retrospectively, and so used with long-term datasets to examine trophic relationships over time. Advances in new technologies, and in particular the development of Unmanned Aerial Vehicle (UAV), could also improve the temporal resolution of remote

sensing data (Martinez et al. 2021). As selection acts at the individual level, such level of precision is needed to clarify whether birds might really rely on vegetation development to time their reproduction. If further improving spatial and temporal resolutions of remote sensing methods is still very much needed, one should however not forget that these studies will always remain descriptive, and that experimental approaches testing the causal relationships between vegetation phenology and the reproduction of birds (e.g. Schaper et al. 2011; Voigt et al. 2011; Caro et al. 2023) will sooner or later become critical. Experiments under controlled conditions manipulating cues linked to vegetation will indeed be central for further understanding the effect of plants on avian reproduction, its underlying physiological mechanisms, as well as the sensory channels through which birds perceive and use the information gathered by developing plants in spring. Together, these studies are likely to shed light on which environmental cues are really important for animal reproduction, which is central in ecological studies.

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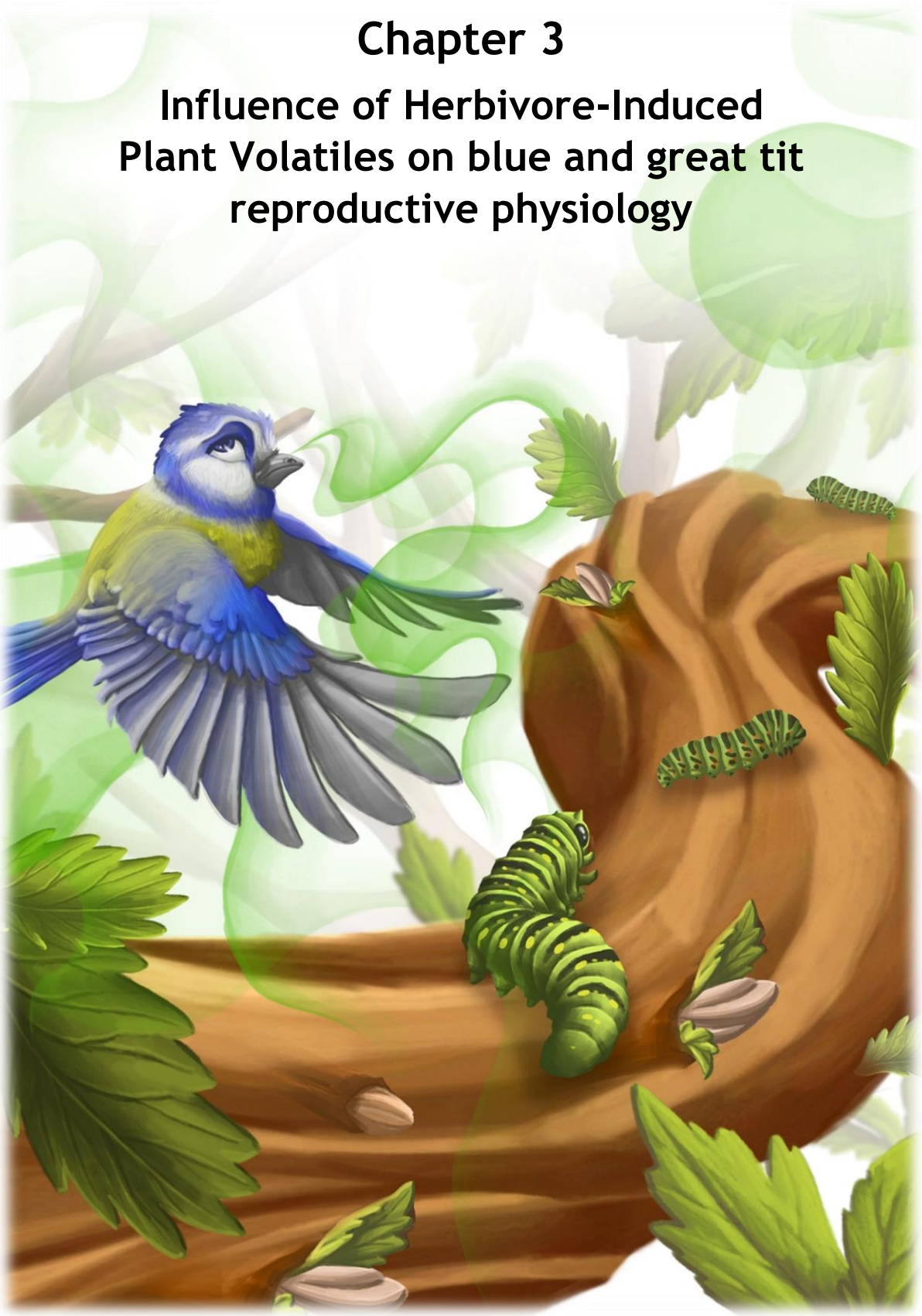
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Chapter 3

Influence of Herbivore-Induced Plant Volatiles on blue and great tit reproductive physiology



Even though the relationship between bird reproduction and vegetation development I described in **Chapter 2** was less evident than I first expected, it still highlighted that the way birds could use vegetation as a source of information may depend on the spatial scale and the habitat considered, and that there may be some benefits in using this information, as revealed by the positive effect of synchrony on great tit tarsus length. The descriptive approach used has however its limits and does not tell anything about how vegetation might impact bird behaviours. In the following chapters, I have therefore adopted a more experimental approach to explore one of the mechanistic pathways through which trees might influence bird reproduction.

If birds were to use cues originating from plants to orchestrate their reproduction, the sensory pathway through which those cues would exert their influence remains elusive. The effect of vegetation phenology on bird breeding phenology could occur through various sensory channels, including vision, gustation, or olfaction. According to previous experiments, visual detection of developing buds does not seem to be enough to trigger reproduction in birds^{203,337,338}. However, birds could assess vegetation development through another possible sense, that is olfaction. Although birds have been considered anosmic for a long time^{219–221}, many avian species are equipped with a functional olfactory system that they use in a wide variety of contexts²⁴⁴. Plants produce Herbivore-Induced Plant Volatiles (HIPVs) when damaged by insect herbivores to defend themselves and attract their predators (“cry for help hypothesis”)²⁸³. They represent good candidate odours for birds to use in a reproductive context, because HIPV emissions reflect the timing of emergence and the abundance of caterpillars in the environment^{211–213}. Several studies have already shown that birds can use HIPVs to discriminate between caterpillar-infested and uninfested trees, but that was exclusively done in a foraging context^{229,259,260}. None has ever studied whether birds detect and use HIPVs at an earlier stage, when tree buds are just beginning to develop and caterpillars have recently hatched and are therefore too small to be considered food items.

In this chapter, I have investigated whether HIPVs emitted by caterpillar-infested buds elicit a reproductive physiological response in birds, and whether females fine-tune their reproductive decisions in response to this cue. This chapter consists of two manuscripts, referring to one experiment on blue tit gonadal development (**article 4**), and one on great tit endocrine concentrations and onset of reproduction (**article 5**). In both cases, we exposed birds to HIPVs emissions induced by manually infesting oak tree buds with freshly hatched caterpillars. Birds could smell, but not see the trees. In the blue tit experiment, conducted at the

CEFE, pairs of birds were housed in airtight compartments in which the air came from enclosures containing oak trees infested with caterpillars. In the great tit experiment, conducted in climate-controlled aviaries at the NIOO, birds were housed together with infested trees, but trees were hidden behind opaque screens, out of birds' sight. If HIPVs exert an effect on the birds' reproduction, sex steroid hormones and gonads were predicted to increase faster in birds exposed to infested trees, and eggs were predicted to be laid earlier and in greater number, relative to controls. Therefore, birds' reproduction was monitored and great tits were blood sampled to analyse sex hormone levels, while blue tits were laparotomised to study gonadal growth.

In blue tits, while males and females grew their gonads at the same rate in both odour treatments, more exploratory females had larger ovarian follicle sizes when exposed to the HIPVs than to the control air. In great tits, I did not find that great tit females exposed to HIPVs advanced their laying dates, adjusted their clutch size or increased their oestradiol levels, but males exposed to HIPVs had higher testosterone concentrations during the egg-laying period. These results suggest that HIPVs may well play a role in reproductive physiology in insectivorous birds. However, detecting an effect on females reproductive timing or investment might require experiments carried out over longer periods, or with larger sample sizes, than those we could afford in our study.

Contribution: The experiment with great tits in the climate-controlled aviaries of the NIOO was conducted during the Dutch lockdown caused by the Covid19 pandemic. Samuel Caro and I designed the protocol, I carried out the experiment, performed the hormonal assays, analysed the data and wrote the first draft of the manuscript. For the experiment with blue tits, I contributed to the analyses of the data and the writing of the manuscript. I should have participated in the data collection on the second year of the experiment, but could not due to one of the French lockdowns.

Article 4: The manuscript entitled “The influence of plant odours on sexual readiness in an insectivorous songbird” has been published in *Journal of Experimental Biology* in March 2023 (see appendices).

Article 5: The manuscript entitled “Odours of caterpillar-infested trees increase testosterone concentrations in male great tits” has been submitted to *Hormones and Behavior* in August 2023.

Article 4

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

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Abstract

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

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

Introduction

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

Vegetation development, and more specifically timing of bud burst in trees, has also received attention as a possible reproductive cue, particularly in insectivorous birds. Relying on bud development to decide when to start breeding could be a good proxy for predicting the optimal timing for breeding in several bird species because buds and young leaf shoots constitute the exclusive food of the caterpillars that these birds provide to their chicks (Du Merle and Mazet, 1983; Naef-Daenzer and Keller, 1999; van Asch et al., 2013). Many studies have indeed found significant correlations between tree phenology and laying date in wild populations of insectivorous birds (Bison et al., 2020; Bourgault et al., 2010; Cole et al., 2015; Hinks et al., 2015; Nilsson and Källander, 2006; Shutt et al., 2019; Slagsvold, 1976). However, bud burst is tightly linked with temperature and only a handful of studies have experimentally tested the effect of vegetation cues independently from temperature cues on bird reproduction (Schaper et al., 2011b; Visser et al., 2002; Voigt et al., 2007; 2011). To complicate things further, the effect of plants on seasonal timing could occur through various sensory channels, which include visual, gustatory, tactile and olfactory pathways (Ettinger and King, 1981; Voigt et al., 2011).

Plants have been shown to defend against herbivores by releasing volatile alarm signals (Herbivore-Induced Plant Volatiles: HIPV) that attract predators. This ability to attract

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

The goal of this study was to determine if insectivorous songbirds use HIPVs emitted by tree buds in response to initial caterpillar infestation in the spring as a cue to prepare for breeding. We exposed breeding pairs of blue tits (*Cyanistes caeruleus*) in captivity to the odors of caterpillar infested trees or a control odor, over two consecutive years using a complex experimental setting in which we controlled the air birds breathed. While photoperiodic and temperature cues were identical to all breeding pairs, we only exposed birds to the tree odors so any potential visual, gustatory or tactile cues provided by vegetation would not be present (Amo et al., 2013). We monitored breeding pairs for gonadal development and hypothesized that pairs exposed to odors from caterpillar infested trees would develop their gonads more quickly than pairs in the control group.

Gonadal development might not only differ between the experimental and the control groups, but also among birds exposed to the odor treatment. Exploring variance among individuals is central to understand ecological and evolutionary processes, and it has long been neglected by endocrinologists (Williams, 2008). One way individuals differ reliably is in their temperament or personality, which in songbirds is often measured through exploratory behaviors (Réale et al., 2007). Several of these behaviors have indeed been shown to be repeatable, heritable, and to covary with one another, like exploration of new environments, new objects and aggression (Dingemanse et al., 2002). Interestingly these temperaments also

covary with physiological traits and environmental perception (van Oers et al., 2011). In great tits for example, ovarian follicle volumes differ between selection lines for personality, with fast exploring females having larger ovarian follicles (Caro et al., 2019). We also recently showed that more exploratory male blue tits are more attracted to HIPVs than less exploratory males (Delaitre et al., unpubl. results). Exploration behavior thus seems to be linked to both HIPV sensitivity and reproductive mechanisms in tits, and therefore constitutes a potential source of variation in how individuals integrate and respond to HIPV signals.

Methods

Ethical note

Blue tits were trapped and maintained under licenses 2018-s-11 issued by the Direction Régionale de l'Environnement, de l'Aménagement et du Logement Languedoc-Rousillon; and 15-XIX-116 issued by the Direction Départementale de la Protection des Populations de l'Hérault. These experiments were carried out under license D34-172-11 from the Direction Départementale de la Protection des Populations de l'Hérault, and approved by the animal experimentation ethical committee of the French Ministry of Higher Education and Research (APAFIS#8608-2017012011062214 v4).

Animal Housing

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

see each other inside the nest box. *Ad libitum* food and water were replaced three times per week.

Terrariums were housed indoors to remove any potential visual cues provided by vegetation. We housed the birds in three different rooms with four breeding pairs per room. Pairs were visually isolated from other pairs and the terrariums also strongly reduced the ability of the pairs in the same room to hear each other. One terrarium in each room received air flow from one of four outdoor enclosures, so that each room had two treatment and two control terrariums. A window allowed natural light into the room. We additionally supplemented artificial light starting 15 min post-sunrise, until 15 min pre-sunset, so that birds were exposed to the natural increase and decrease in light intensity from the relatively small window (55 x 42 cm) (Fleissner and Fleissner, 2002). Extra light aimed at increasing and homogenizing light intensity in the room during the day. Sunrise and sunset times were determined using the United States Naval Observatory data base (www.usno.navy.mil/USNO).

Equipment design

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

Air could also be diverted through a center enclosure (made of glass in 2019, aluminum container in 2020) shared by all 12 terrariums (see 9 on figure 1). When trees needed to be watered or changed out, birds continued to receive filtered, uncontaminated air while the treatment and control enclosures were open. A third pump (not shown on figure 1) was used to flush contaminated air out of the enclosures for several hours before resuming airflow from the

enclosures. Due to high humidity levels inside the terrariums in 2019, particularly later in the season, several changes were made to the system in 2020 (figure S1). First, the tubing leading from the small pumps to the terrariums was run through a cooling system to remove water. The condensed water vapor was collected in glass jars below the cooling system before the air entered the terrarium. These jars were emptied at least once per week. Second, the center enclosure described above was filled with silica gel. Two times per week at the time trees in the other enclosures were manipulated, air was diverted through this silica gel for approximately 24 hours to dry air in the terrariums (Yu et al., 2001).

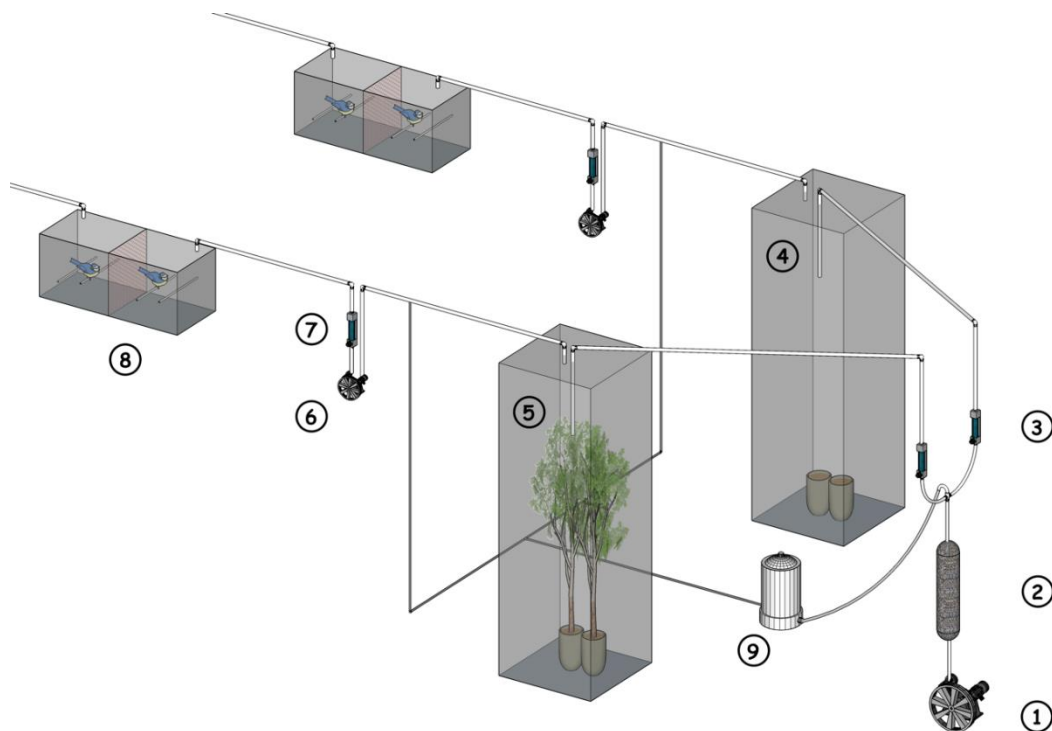


Figure 1. Diagram of the experimental setup.

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

Infestation of Trees

Green oak tortrix moths (*Tortrix viridana*) were collected from Corsica in June 2018 and 2019 and placed in large nets on oak branches to lay eggs. Branches were returned to Montpellier and kept outside at ambient temperature to synchronize hatching of eggs with oak bud burst. Green oak tortrix caterpillars are oak specialists and hatching needs to be closely

timed with elongation and development of oak buds so caterpillars can perforate the bud and eat young developing leaves inside the bud (Du Merle, 1999; Ivashov et al., 2002). Thus, we utilized downy oak trees (*Quercus pubescens* Willd.) of approximately 2.5 meters high, grown in pots. To spread out timing of bud burst over the season, we housed 20 trees on the south side of a building, 20 trees on the north side, and 40 trees at higher elevation to delay bud burst. We began to expose birds to HIPV air as soon as bud burst and caterpillar hatching occurred simultaneously.

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

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

Even if this was beyond the scope of the present study, a small number of air samples reaching the terrariums were pulled into cartridges containing an adsorbent to collect volatile compounds. These compounds were subsequently separated over a Gas Chromatograph - Mass Spectrometer coupled system (GC-MS) using a semi-standard non-polar column. Mass spectrum and linear retention index comparison enabled us to identify the compounds present. Those samples revealed that (*E*)- β -ocimene, (*E*)-4,8-dimethyl nona-1,3,7-triene (DMNT),

methyl salicylate, (*E*)-caryophyllene, aromadendrene, α -humulene and (*E,E*)- α -Farnesene, all known HIPVs (Röse and Tumlinson, 2004, Graham et al. unpublished data), were found in the terrariums of the HIPV treatment group (figure 2).

Reproductive measures

Three laparotomies were conducted each year to measure gonadal growth in all birds. Initial laparotomies were conducted when photoperiod was 11 hours of light (11L:13D) to obtain a pre-breeding measure of the gonads and confirm the two treatment groups did not differ. Two subsequent laparotomies were performed at four-week intervals (photoperiods 12.5L:11.5D and 14L:10D). Individuals were unilaterally laparotomized under isoflurane anesthesia. Left testis length and width were recorded in males and diameter of the largest developing ovarian follicle was measured in females. Measures were collected using the scale engraved in the ocular of a binocular microscope (Optika SZM-4) to the nearest 0.1mm. Testis volume was calculated as: $V = \frac{4}{3}\pi\alpha^2\beta$ where α is half the testis width and β is half the testis length. Follicle volume was calculated as: $V = \frac{4}{3}\pi\alpha^3$ where α is half the follicle width (Visser et al., 2011). The observer was blind to the treatments. Throughout the experiment, birds were provided with moss to build nests and monitored for possible nest building and egg laying.

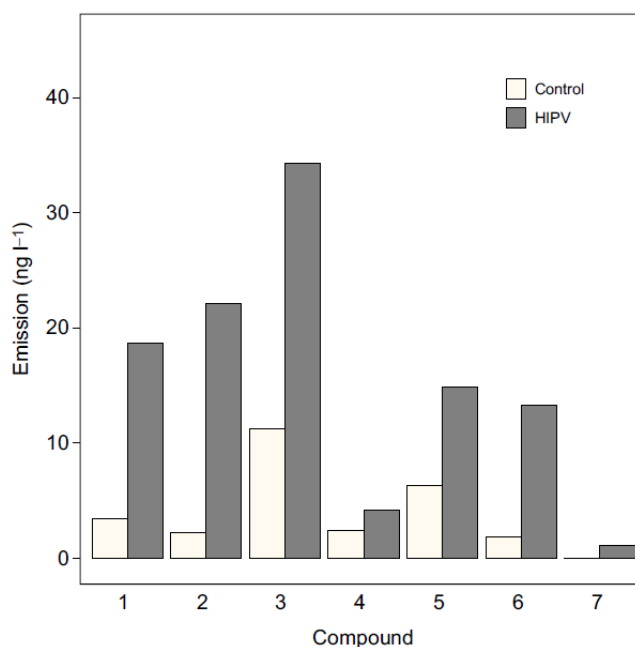


Figure 2. Concentrations of the compounds detected in the terrariums.

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

Exploration scores

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

Statistical Analysis

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

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

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

Gonadal volume was log transformed before analysis to account for exponential growth. Linear mixed effects models included treatment (factorial), time point (continuous), exploration

score (continuous), body mass (continuous), treatment by exploration score interaction, and treatment by time point interaction, as fixed factors. Individual ID was included as a random intercept to account for repeated measures. Non-significant interactions were removed from the models. Since the HIPV treatment was only provided after T_0 , we also ran the analyses described above, restricting data to T_1 and T_2 . Results were very similar to those obtained with all three time points (data not shown). Not enough individuals built nests (7 out of 24; 3 in the HIPV group, 4 in the control group) or laid eggs (6 out of 24; 3 in each group), thus no statistical analyses were performed on breeding data.

Results

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

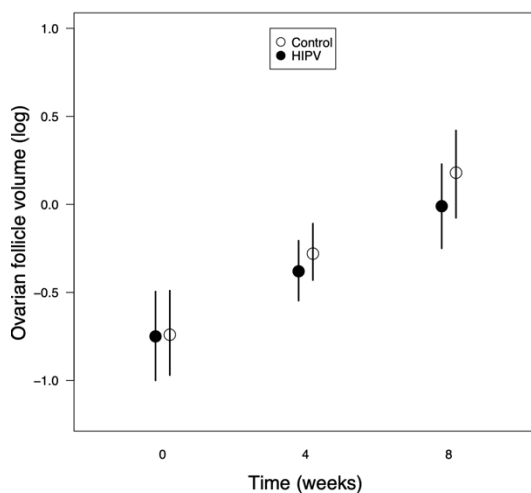


Figure 3. Ovarian follicle growth in blue tits. While there was a significant increase in follicle size over time (LMM, $F = 36.83$, $p < 0.001$), they grew at the same rate in both treatments (LMM, $F = 0.45$, $p = 0.505$). Points indicate mean \pm SEM; $n = 24$, 12 birds per treatment.

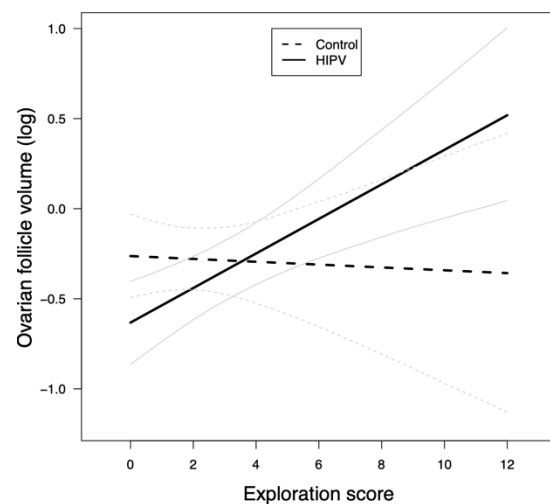


Figure 4. Effect of odour treatment and personality on ovarian follicle size in female blue tits. Fast explorers had larger ovarian follicles when exposed to air containing HIPVs than to control air (LMM, $F = 5.04$, $p = 0.037$). Lines depict model estimates and 95% CI, $n = 24$, 12 birds per treatment.

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

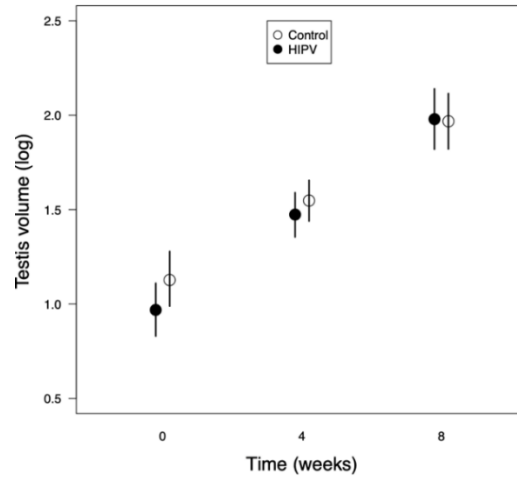


Figure 5. Testes growth in blue tits.

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

Table 1. Analysis of the variables that potentially influence the volume of the gonads of female ($n = 24$) and male ($n = 24$) blue tits.

Eliminated interactions (in grey) are presented in the reverse order in which they were removed from the model. Intercept includes Treatment = control. Volumes are log-transformed.

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

Discussion

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

Experiments manipulating non-photoc cues related to the initiation of reproduction in strictly seasonal breeding birds rarely result in striking differences between groups, owing to the fact that those cues are fine-tuning cues that only modulate the powerful effect of photoperiod (Wingfield and Kenagy, 1991). The case study of the influence of temperature cues on reproduction in great tit, highlighted in introduction, is probably the best example (Schaper et al., 2011a; Visser et al., 2009). Experimental evidence for an effect of temperature on great tit timing of reproduction in an ecologically relevant context requested nearly 10 years of experiments in captivity. Furthermore, temperature was never found to influence some of the main physiological mechanisms underlying egg-laying, which includes gonadal size, luteinizing hormone, and prolactin concentrations (reviewed in Caro et al., 2013b). Only patterns of gene expression related to seasonal timing were found to differ between temperature treatments (Laine et al., 2019), suggesting that the substrate for temperature-induced changes in reproductive physiology is there, but hard to detect. It is possible that the same complexity applies to environmental cues linking vegetation to timing of breeding, as suggested here by the absence of an effect of HIPVs on gonadal development, except for a subset of females (see below). Only a few previous studies have manipulated vegetation cues in captivity in tits (mainly through the addition of cut branches to the aviaries where the birds were), and they found no effect on timing of reproduction, or at least not in the predicted direction (Schaper et al., 2011b; Visser et al., 2002). In both cases however there was evidence for a positive effect of the leafing branches on luteinizing hormone, a central reproductive hormone, which called for more investigation on the topic. Other studies on other bird and mammal species sometimes found stronger evidence. In meadow voles (*Microtus montanus*), adding sprouted wheatgrass, or one of its constituents (6-methoxybenzoazolinone: 6-MBOA), triggered reproduction and modulated sex-ratio (Berger et al., 1987; Negus and Berger, 1977), while in white-crowned sparrows (*Zonotrichia leucophrys*), adding wheat sprout leaves to the diet accelerated ovarian development (Ettinger and King, 1981). In canaries (*Serinus canaria*) the presence of

vegetation in the aviaries advanced egg-laying by several weeks (Voigt et al., 2007; Voigt et al., 2011). While the number of experimental studies on the possible influence of vegetation development on bird reproduction is already very limited, the sensory pathways through which the plant stimuli were provided varied tremendously and mixed in various proportions tactile, gustatory, olfactory and visual signals. The drawback is that in several of those earlier studies, animals from the experimental groups could physically interact with the plants, while the control animals received none, which introduces environmental enrichment as a confounded factor. Disentangling pathways by carefully controlling sensory modes of perception of plants, like Voigt et al. (2011) did for example, will be key for clarifying whether and how vegetation cues potentially affect reproduction in vertebrates. In our case, the only possible sensory mode of perception of infested oak trees was olfaction, and we show that more exploratory females responded to these HIPVs. Future studies will need to determine whether combining olfactory with other carefully selected cues (either visual or gustatory cues from plants, or other environmental cues like photoperiod or temperature) could enhance the results obtained here.

More exploratory females grew their ovarian follicles more rapidly when exposed to HIPVs. This is not the first time that a link between a behavior related to personality and HIPVs is discovered. In a recent study testing whether blue tits can innately detect and orient to a blend of artificial HIPVs mimicking an infestation of oak buds by caterpillars, we found that the preference for the side of a Y-maze containing the HIPVs was driven by fast exploratory males (Delaitre et al., unpub. results). The results of both experiments suggest that fast explorers might be more sensitive to HIPVs. Through which mechanisms this link operates is still unclear at present, but we know that high sensitivity to HIPVs in male blue tits is associated with testosterone (Graham et al., 2021). We also know that testosterone is locally produced in the olfactory epithelium in rats (Horie et al., 2017; Lupo et al., 1986), providing a possible mechanistic link between sex steroids and olfactory sensitivity for HIPVs. On the other hand, in female great tits we have recently shown relationships between exploration behavior, ovarian follicle sizes and plasma concentrations of 17β -estradiol, the main sexual steroid in female. Females from a line artificially selected for fast exploration more rapidly increased 17β -estradiol after a GnRH challenge, and had larger ovarian follicles than females from the line selected for slow exploration (Caro et al., 2019). This interplay between sex steroid hormones, HIPV sensitivity and personality clearly needs further investigation, in particular to understand if the enhanced olfactory sensitivity of fast explorers is driven through the action of steroid hormones.

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

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

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Article 5

Odours of caterpillar-infested trees increase testosterone concentrations in male great tits.

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Abstract

Trees release Herbivore-Induced Plant Volatiles (HIPVs) into the air in response to damage inflicted by insects. It is known that songbirds use those compounds to locate their prey, but more recently the idea emerged that songbirds could also use those odours as cues in their reproductive decisions, as early spring HIPVs may contain information about the seasonal timing and abundance of insects. We exposed pre-breeding great tits (*Parus major*) to the odours of caterpillar-infested trees under controlled conditions, and monitored reproduction (timing of egg laying, number of eggs, egg size) and two of its main hormonal drivers (testosterone and 17 β -estradiol in males and females, respectively). We found that females exposed to HIPVs did not advance their laying dates, nor laid larger clutches, or larger eggs compared to control females. This absence of an effect of HIPVs on female reproduction was confirmed by similar 17 β -estradiol concentrations between experimental and control birds. However, males exposed to HIPVs had higher testosterone concentrations during the egg-laying period. Our study supports the hypothesis that insectivorous songbirds are able to detect minute amounts of plant odours. The sole manipulation of plant scents was not sufficient to lure females into a higher reproductive investment, but males increased their reproductive effort in response to a novel source of information for seasonal breeding birds.

Keywords: Birds; herbivore-induced plant volatiles; olfaction; reproduction; reproductive hormones; seasonal timing

Introduction

The timing of reproduction of most animal species is tightly linked to the phenology of other organisms (Durant et al. 2005; Post and Forchhammer 2008). Consequently, many species try to adjust their reproductive decisions to the surge of resources they need to feed their offspring (Dias and Blondel 1996; Maillard and Fournier 2004; van Asch et al. 2010; Cadby et al. 2010; Dunn et al. 2011; Ljungström et al. 2015; Neuheimer et al. 2018; Peláez et al. 2020). Being too early or too late can have fitness consequences; having the offspring hatching sometimes just a few days away from this food peak can negatively affect offspring growth or survival (Thomas et al. 2001; Reed et al. 2013; Renner and Zohner 2018). In the case of predators, they need to predict the date at which their prey will reach a peak of biomass to initiate their breeding at the appropriate time, which is often weeks before this peak. They therefore need to use cues present early in their environment to predict the annual phenology of the lower trophic levels (Wingfield and Moore 1987; Di Bitetti and Janson 2000; Rubenstein and Wikelski 2003; Visser et al. 2004; Grant et al. 2009; Visser et al. 2009).

Photoperiod and temperature have been the most studied environmental cues for breeding phenology in a wide range of taxa (Nelson 1985; McAllan and Dickman 1986; Nelson 1986; Wingfield et al. 2003; Dawson 2008; Visser et al. 2009; Schaper et al. 2012; Rani and Kumar 2014; Nakane and Yoshimura 2019). However, changes in photoperiod are the same every year, meaning that photoperiod alone can not predict yearly variation in the optimal timing of breeding. Similarly, while temperature has been shown to exert an influence on the seasonal timing of reproduction (Crick et al. 1997; Visser et al. 2009; Schaper et al. 2012; Chambers et al. 2013; Williams et al. 2015; Wegge and Rolstad 2017), several studies failed to show such a causal link with phenology and its underlying physiological mechanisms (Dawson 2005; Perfito et al. 2005; Visser et al. 2011; Caro et al. 2013; Verhagen et al. 2020).

In the well-studied tree-caterpillar-passerine food chains (Perrins 1970; Visser et al. 1998; Both et al. 2009), nestlings of several insectivorous bird species almost exclusively depend on the availability of a few caterpillar species (mainly the winter moth - *Operophtera brumata*, and the green oak tortrix - *Tortrix viridana*) (Dias and Blondel 1996; Naef-Daenzer and Keller 1999; Thomas et al. 2001; Gienapp and Visser 2006). Caterpillars on the other hand entirely depend on the leafing of a few tree species for their development (Du Merle and Mazet 1983; Buse and Good 1996; van Asch and Visser 2007; van Asch et al. 2013). Plant phenology has therefore been suggested to be one of the supplementary cues that birds could use to predict the phenology of their food source, and thereby their timing of reproduction. Many studies have

indeed found correlations between tree phenology and laying dates in wild populations of insectivorous birds (Nilsson and Källander 2006; Bourgault et al. 2010; Cole et al. 2015; Hinks et al. 2015; Szulkin et al. 2015), but whether there is a causal relationship between the two, and through which sensory path this relationship could come about, is not well understood yet (Visser et al. 2002; Schaper et al. 2011; Voigt et al. 2011).

Trees respond to insect grazing by releasing Herbivore-Induced Plant Volatiles (HIPVs) (Dicke and van Loon 2000; Arimura et al. 2005; Dicke and Baldwin 2010) that carnivorous arthropods and parasitoids use to locate their insect prey (Turlings et al. 1990; Kugimiya et al. 2010; Fontana et al. 2011; Giunti et al. 2016). HIPV release thus provides indirect protection to the plant against small herbivores (Kessler and Baldwin 2001; Kant et al. 2009; McCormick et al. 2012). Although birds have been considered anosmic for a long time, they have a functional olfactory system allowing them to detect odours at minute concentrations (Caro and Balthazart 2010) and in different contexts (Bonadonna et al. 2003; Hagelin et al. 2003; Bonadonna and Nevitt 2004; Amo et al. 2008; Caro et al. 2015). Foraging birds can discriminate between caterpillar-infested and uninfested trees only by smell (Amo et al. 2013; Mrazova et al. 2019; Graham et al. 2021; Sam et al. 2021), and recognise and orient to an artificial odour mimicking HIPVs (Mäntylä et al. 2014; Rubene et al. 2022). In a recent study using artificial HIPVs, the authors found an innate attraction to those odours in blue tits (*Cyanistes caeruleus*), and birds increased their reproductive investment by producing more nestlings when artificial HIPVs were added in their breeding territories (Delaitre et al, in prep.). HIPVs thus represent a promising cue that birds could use to infer the phenology of their prey and modulate their reproductive investment accordingly.

Sexual behaviours and reproductive control result from a cascade of physiological reactions involving steroid hormones produced by the gonads (Wingfield 1994; Ball and Balthazart 2009). In males, increases in testosterone (T) concentrations is an important indicator that they are preparing to breed (Kempnaers et al. 2008; Zhang et al. 2017), as it stimulates territorial defence (Silverin 1980; Chandler et al. 1994), aggressive behaviour (Wingfield et al. 1987; Beletsky et al. 1990), as well as courtship and mating behaviours (Silverin 1980; Ketterson et al. 1992; Enstrom et al. 1997; Hill 1999). In females, oestrogens promote courtship behaviour. For example, the removal of the ovary in ring doves leads to the disappearance of wing flipping that are normally shown by females in response to male courtship (Cheng and Lehrman 1975), but treating those females with oestrogens restore their normal sexual behaviours (Adkins and Alder 1972). Moreover, estradiol is a good predictor of laying, as it

stimulates the production of yolk precursors and peaks just prior females lay their first egg (Bluhm et al. 1983; Rehder et al. 1986; Sockman and Schwabl 1999; Williams 2012).

If HIPVs exert an effect on bird reproduction, we should observe effects of this olfactory signal on the mechanisms coordinating reproduction, i.e. on their reproductive hormone levels. Supporting this hypothesis, Graham et al. (2021) recently found that blue tit males with higher testosterone concentrations spent more time close to a tree infested with caterpillars compared to an uninfested tree. Gonads, which produce sex steroids, also developed faster in more exploratory (a personality trait) female blue tits exposed to HIPVs (Caro et al. 2023). Since HIPV emissions reflect the timing of emergence and the abundance of caterpillars in the environment (Horiuchi et al. 2003; Girling et al. 2011; McCormick et al. 2012; Miresmailli et al. 2012), by detecting HIPVs in early spring, songbirds could adjust their onset of breeding to synchronize the feeding period of their chicks with the timing of maximal caterpillar biomass (Marciniak et al., 2007), and adjust their clutch size to match the predicted food availability in their breeding territory (Hussel and Quinney 1985; Marciniak et al. 2007, Delaitre et al, in prep).

The purpose of this study was to determine experimentally if HIPVs emitted by caterpillar-infested oak buds induce behavioural and hormonal responses in an insectivorous songbird in terms of reproductive physiology, timing and investment. For this, we used 36 pairs of great tits housed in climate-controlled aviaries and induced the release of HIPVs by infesting oak buds (*Quercus robur*) with freshly hatched winter moth caterpillars. We hypothesized that birds exposed to HIPVs would advance and increase their reproductive hormone concentrations (i.e. testosterone and 17β -estradiol), advance their laying date, and increase their reproductive investment (i.e., clutch size and/or egg size), compared to control birds.

Methods

Ethical note

Ethical permits requested for this experiment have been provided by the Animal Welfare Body of NIOO-KNAW (IVD - NIOO 20.09 AVD8010020209246/IVD 1556a).

Birds and experimental set-up

Birds

The experiment was carried out at the Netherlands Institute of Ecology (NIOO-KNAW) in Wageningen, the Netherlands. 72 great tits (36 females and 36 males) born in 2020 were used in this experiment. Birds were hand-reared at the NIOO following the procedure described

in a previous study (Drent et al. 2003). Adult birds were fed daily as described in Delaitre et al (2023), with mixture of minced beef heart, canary egg food proteins and vitamins, dry food, apple and water for drinking and bathing.

Housing in climate-controlled aviaries

To enhance the chances that birds successfully breed in climate-controlled aviaries, a preference test to pair females with one of their preferred males was conducted (Delaitre et al. 2023). In short, females could visit six males in a carousel-shaped six-choice chamber for 90 minutes. Mate choice was inferred from the amount of time females spent close to each male.

From early February, birds were housed by opposite-sex pairs in 36 climate-controlled chambers (2 x 2 x 2.25 m) as described in Delaitre et al (2023) (figure 1). Briefly, photoperiod and temperature were set to mimic natural conditions prevailing outside at the time of the experiment. Three 58 W high frequency fluorescent tube lights served as the main source of lighting. Before sunrise and after sunset, a second 7 W incandescent light bulb simulated dawn and nightfall for five minutes each. From a logistical point of view, it was not possible to adjust the aviary temperatures to match the outside temperatures on a daily basis. As a result, the simplest logistical solution was to set the temperatures of the next eight days in aviaries based on the temperatures of the previous eight days in the wild. Hourly temperatures were determined from The Royal Netherlands Meteorological Institute (KNMI) database. Perches, an artificial tree and three nest boxes were available in each aviary. In order to hide the potted oak trees, a screen (0.85 x 2.25 m, figure 1, figure S1) made of an opaque white fabric was installed in the back-left corner of each aviary. No visual or acoustic interactions were possible between the pairs.

Oak Trees

160 common oak trees (*Quercus robur*; approx. 2 m high, and 0.5 m of diameter) grown in pots were used as sources of odour. From early January, trees were maintained at different locations to spread out timing of bud burst over the season. A group was maintained in an open area fully exposed to sun ($n = 60$), one was placed in the corner of a building in the shade ($n = 45$), and the last group was kept on the east side of a building with partial exposure to sun ($n = 55$). The oaks were considered ready to use in the experiment when the buds began to elongate and to lose their scales (stage 4, figure S2). Caterpillars are indeed unable to perforate the protective scales and penetrate into the buds prior to this stage of development (Du Merle and Mazet 1983). Out of the 160 trees, 108 were used in the experiment.

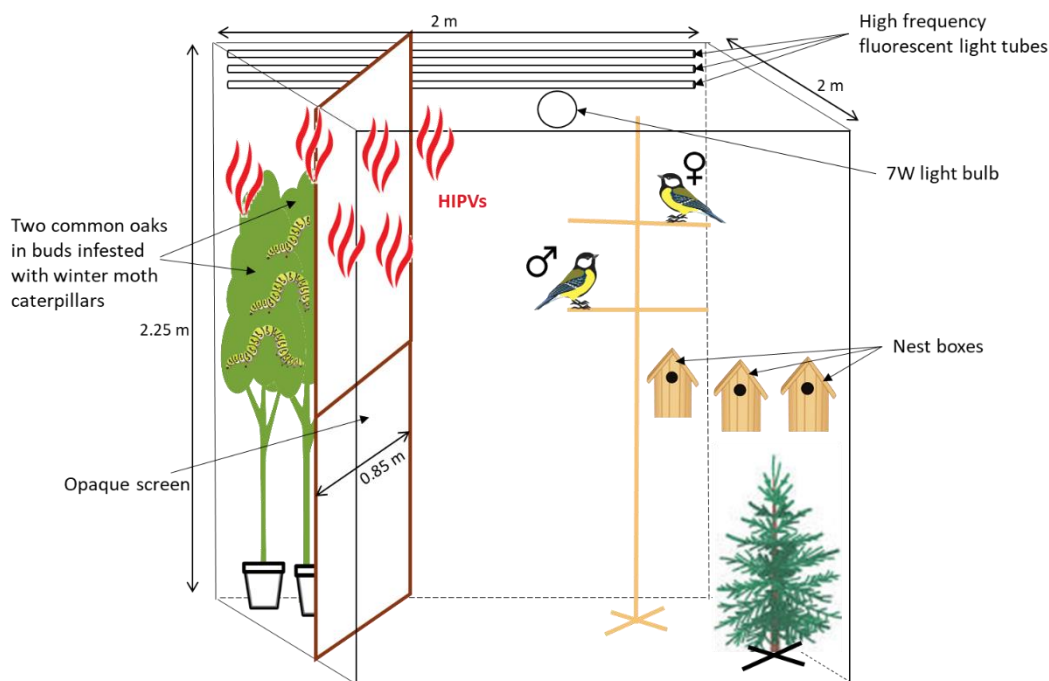


Figure 1. Housing of birds in climate-controlled aviaries.

Each of the 36 opposite-sex pairs of great tits was housed in a climate-controlled aviary under an artificial light regime mimicking a natural daylight pattern using three 58W tube lights and one 7W light bulb. Temperatures were weekly set to mimic the outside temperatures of the week before. Birds had *ad libitum* access to food and water. Perches and an artificial tree were provided to the birds to perch and hide. Three nest boxes were installed in each aviary. An opaque screen was installed in the back-left corner to hide the two caterpillar-infested trees for the experimental group, or the two pots filled with soil for the control group. The experimental birds could thus only smell the HIPVs released by the trees but could not have access, or see the oak trees and caterpillars.

Caterpillars

The main caterpillar species infesting the common oak in the Netherlands is the winter moth (Visser et al. 2006). Adult winter moths were collected in Doorwerth, the Netherlands, between November and December. Adults were kept in 50 mL tubes with paper strips on which the females laid their eggs. Eggs were kept outdoor protected from rain and direct sunlight. Caterpillars and trees were thus experiencing similar temperature conditions.

Bud infestation

Once caterpillars began to hatch, oak trees with buds at an appropriate stage were infested with caterpillars using a small paintbrush (Graham et al. 2021). Two oak trees were placed behind the opaque screen in 18 experimental aviaries, whereas two pots filled with the same soil were placed behind the opaque screen in 18 control aviaries. Every week, one of the two trees was replaced by a new one with earlier bud stages than the one that had just been

removed. When experimental aviaries were opened to change a tree, control aviaries were also opened to ensure all birds experienced similar disturbances. Between 20 and 50 caterpillars (depending on caterpillar availability, on average 36 caterpillars per tree) were placed across five branches with buds at stage 4 (bud elongated, swollen and green) to 6 (bud bursting, leaf shoots can be distinguished but are still intricately) (figure S2) (Du Merle and Mazet 1983). During infestation, the experimenter observed the caterpillars for a few minutes to ensure they entered the buds. In addition, when a tree was removed from an aviary, it was examined for caterpillar damage on leaves to ensure that HIPVs had been released. First trees were introduced in the aviaries between 16th and 20th April. In total, seven tree changes were done for each experimental aviary until 24th May. When no more caterpillars and trees were available for new infestation, we stopped changing trees and let them progress in the aviaries until the final blood samples were taken (see details below).

Reproduction monitoring

Nesting material (moss and dog hair) was provided from early March. Reproduction was monitored as described in a previous study (Delaitre et al. 2023). Briefly, from mid-March, we monitored nest building and egg laying activities. We began to check the nest boxes once and then twice a week as birds were closer to lay (Dufva 1996), and then daily until the last egg of the clutch had been laid. The pair laying date is defined as the date that the first egg was found. We weighed (to the nearest 0.01 mg with an electronic balance, Eidyer), and measured all eggs (to the nearest 0.05 mm using a calliper, Ecotone Measy DG) and calculated their volume (egg volume = $0.4673 \times \text{length} \times \text{breadth}^2 + 0.042$ (Dufva 1996)). The clutch size was determined after birds laid their last egg. Nests and eggs were removed after at least five days of incubation, so that birds could start a new clutch. Since six females started laying before the first trees and caterpillars were introduced in the aviaries, we excluded these females from the analyses. Analyses were thus realized on 30 pairs of birds. As only three females produced a second clutch, we only analysed the first clutch laid.

Physiological measures

Blood samples

We followed the procedures described in Delaitre et al (2023). Briefly, birds were caught in their aviary and transported to a laboratory room, where they were weighed and bled in the jugular vein using an insulin syringe (max. 150 μ l). Blood was transferred to heparinised tubes, plasma was separated by centrifugation and stored in freezers (-80°C) until assayed for

hormones. Birds were returned to their aviary after the sampling. Each bird was sampled every two weeks (18 aviaries per week (36 birds)) between 18th March (before first laying) and 17th June (seven blood samples per bird). Mean time between catching and blood sampling was 3 min 22 s \pm 46 s for females and 6 min 01 s \pm 1 min 10 s for males.

17 β -estradiol assay

The assay procedure used for 17 β -estradiol assay is described in detail in Delaitre et al (2023) and validated for blue and great tits (Caro et al. 2019). Concisely, plasma 17 β -estradiol (E₂) was measured using a commercially available double-antibody ¹²⁵I-E₂ radioimmunoassay (DSL-4800, Ultra-sensitive Estradiol RIA, Beckman Coulter, Brea, CA, USA) modified to increase the sensitivity of the assay (Charlier et al. 2010; Caro et al. 2019). Steroids were extracted from 30 μ L of plasma, 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). Recovery value after extraction was 91.5%. Samples were then assayed in duplicate and counted on a gamma counter. Seven assays were needed to estimate the concentration of the 250 samples. The intra- and inter-assay coefficients of variation were 3.73% and 4.10%, respectively. Assay sensitivity was 0.65 pg/mL.

Testosterone assay

Plasma testosterone was measured using a commercially available double-antibody ¹²⁵I-testosterone radioimmunoassay (IM1087, RIA Testosterone, Beckman Coulter, Brea, CA, USA). Steroids were extracted from 50 μ L of plasma using diethyl ether (DEE). First, 3 mL of DEE were added to the plasma. The tubes were dipped in a methanol and dry ice bath for 15 seconds and organic phase was poured in another tube. This step was repeated a second time with the aqueous phase and with 2 mL of DEE. The organic phase was then dried under nitrogen gas at 40°C. Finally, dried samples were resuspended in 120 μ L of extraction assay buffer and shaken overnight at 4°C. Recovery value after extraction was estimated at 91%. Concentrations were adjusted for samples that did not have 50 μ L of plasma available ($n = 32$ out of 237). Resuspended samples were then assayed in duplicate and tubes were counted on a gamma counter (Automatic Gamma Counter, Perkin Elmer, Waltham, MA, USA). Concentrations of testosterone were obtained using a linear regression with the log-transformed concentrations of the standards provided in the assay kit. The 237 plasma samples were run across six assays, and the intra- and inter-assay coefficients of variation, as estimated by assaying one high and one low concentration testosterone standard in duplicate, were 2.80% and 7.48%, respectively.

Assay sensitivity was 0.075 ng/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. We validated the assay following the methods of Caro et al. (2019). Briefly, pooled plasma samples were spiked with exogenous testosterone and serially diluted. Percentages of tracer bound (B/B0) from the serial dilutions were parallel to the standard curve (figure S3).

Statistical Analyses

Analyses were performed in R version 1.3.1093 (R CoreTeam). The lme4 package (Bates et al. 2015) was used for linear mixed-effects and generalized linear mixed-effect models. Generalized additive models were conducted using the mgcv package (Wood 2017). P-values of mixed-model analyses were obtained with the lmerTest package (Kuznetsova et al. 2017). Plots were created with the ggplot2 package (Wickham 2016).

Reproduction

We compared reproductive parameters between HIPV and control pairs by performing (i) a linear model on lay date, (ii) a generalized linear model with negative binomial distribution on clutch size; and (iii) a linear mixed-effect model on egg volume. For clutch size, we also tried a Poisson distribution, which is regularly used for analysing clutch sizes (e.g. Marini et al. 2017; Thornton et al. 2017; Eyck et al. 2020; Martay et al. 2023). Analysing clutch size with a Poisson distribution revealed that females in the odour treatment tended to lay larger clutches than in the control treatment ($z = 1.89$, $p = 0.057$; details of this statistical model not shown). However, because the variance of clutch size (27.98) was larger than the mean (10.24), and because our data were slightly overdispersed when using a Poisson distribution (ratio residual deviance / ddl = $87.425 / 27 = 3.23$), we pursued with a negative binomial distribution, which had a lower overdispersion (ratio residual deviance / ddl = $32.303 / 27 = 1.19$). In the linear mixed effect model for egg volume, female identification was added as a random intercept to account for the fact that each female laid several eggs. All models included treatment as explanatory variable.

Hormone level analyses

We tested whether the HIPV treatment played a role on plasma steroid levels in both sexes. Because we measured a different hormone in each sex, we ran separate models for males and females. None of the females were removed from the dataset but four males were: one male

with only three blood samplings, two males that showed a testosterone increase far after the laying period, and one male without any increase in testosterone during the whole breeding season. We used generalized additive models (GAMs) because we did not want to impose any prior assumption on the shape of the relationship between hormone levels and time. We tested whether there was variation in E_2 and T concentrations over the course of the breeding season, whether steroids levels were impacted by the time spent between the catching and the bleeding of the birds, and whether E_2 and T levels differed between HIPV and control birds over the course of the breeding season, by fitting GAMs with date (continuous variable, centred on first egg date, and ranging from -71 to 66) in a spline function, the time between catching and bleeding (continuous variable, in seconds, ranging from 143 to 564 seconds for females, and from 270 to 890 seconds for males), the treatment (HIPVs vs. Control) and the interaction between time (days from laying date) and treatment in a spline function as explanatory variables. The bird identification was added as a random intercept to take into account that each bird had several hormone measures during the course of the breeding season. To identify if at some point, the hormone levels fitted by the GAM models were significantly different in the HIPV compared to the control groups, we used the “get_smooths_difference” function from the “tidymv” package (Coretta 2022). This function calculates the difference between the mean control and the mean HIPV curves for each day and the corresponding 95% confidence interval. When the confidence interval does not overlap zero, the hormone concentration between the control and the HIPV curves are considered significantly different.

Results

All the 30 pairs included in the analyses built a nest and only one female did not lay. We found no significant difference in laying date ($F_{1,27} = 1.26$, $p = 0.271$, figure 2A) or clutch size ($z = 1.055$, $p = 0.291$, figure 2B) between HIPV and control birds. Out of the 208 eggs measured, we did not find evidence for a significant effect of the odour treatment on egg volume ($F_{1,26.67} = 1.13$, $p = 0.299$, figure 2C). Analyses of the other data relating to egg size (length, breadth, weight) also showed no effect of the treatment (data not shown).

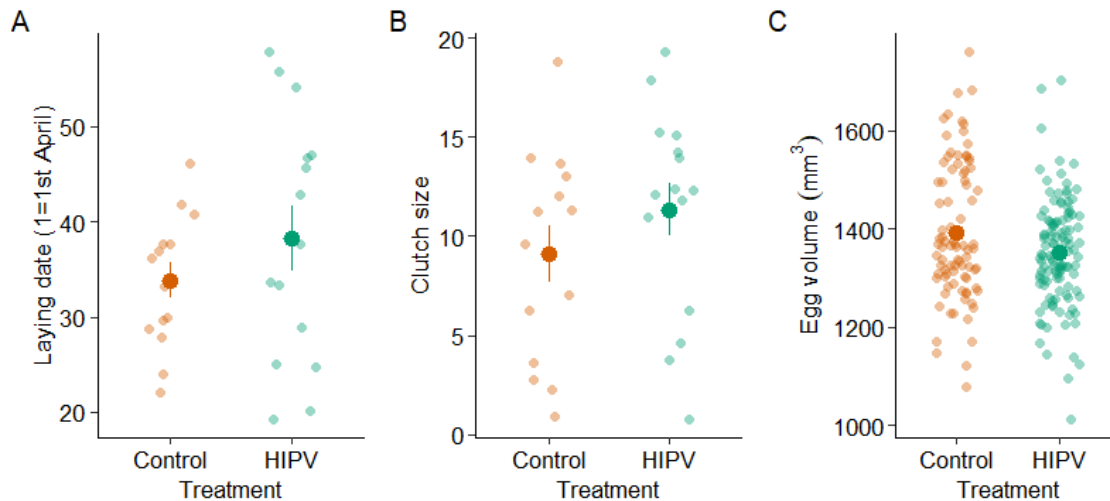


Figure 2. Effect of HIPVs on reproductive traits in great tits.

Females did not significantly (A) advance their laying date ($n = 29$), (B) lay larger clutches ($n = 29$) or (C) produce larger eggs ($n = 208$) when exposed to HIPVs. Large dots and error bars represent mean \pm SE, smaller dots depict raw data.

Individual variation in 17β -estradiol and testosterone over time are depicted at figures 3A and 3B, respectively. Both hormones varied significantly over time (E_2 : $F = 6.01$, $p < 0.001$, figure 3A, T: $F = 2.80$, $p = 0.03$, figure 3B) and were more elevated around laying. Time between catching and bleeding did not influence E_2 levels of females ($F = 0.37$, $p = 0.55$) but tended to have an effect on male T levels ($F = 3.32$, $p = 0.07$), with T levels decreasing when the time interval increased.

The “get_smooths_difference” function, from which the curves in figures 3E and 3F originate, calculated the daily difference between the control and the HIPV curves of figures 3C and 3D, and the 95% confidence interval. The confidence interval of these curves overlaps zero over the entire breeding period for E_2 , meaning that E_2 concentrations in females were not significantly different between the control and HIPV groups over the course of the breeding season (figure 3E, Table S1A). In males, T concentrations were significantly different between control and HIPV birds around the laying period, from 12 days before laying until eight days after (figure 3F, Table S1B). An additional linear model including treatment and time between catching and bleeding, run on the period 12 days before laying to eight days after, confirmed that testosterone levels were higher in the HIPV group than in the control group for this time period ($F_{1,31} = 4.74$, $p = 0.03$, figure 3G).

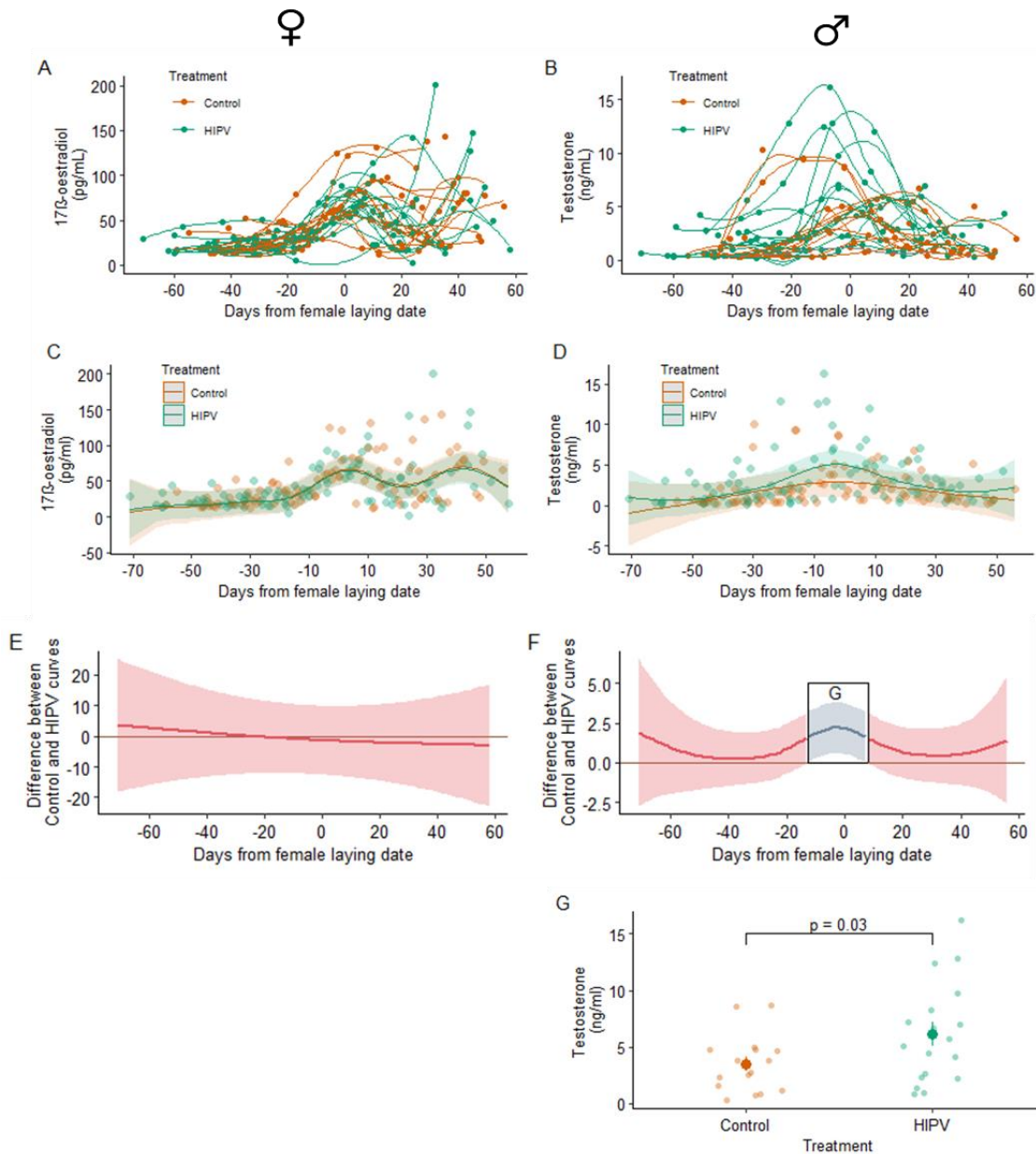


Figure 3. Hormonal profiles for 17β -estradiol (E_2) in females and testosterone (T) in male great tits.

A-B: E_2 /T profiles of females/males relative to the female egg laying date. Each line depicts one individual.

C-D: E_2 /T profiles of females/males in the HIPV and control groups. Predicted values of the GAM model and 95% confidence interval are depicted.

E-F: The line represents the differences between the mean control and the mean HIPV curves from figures C-D for E_2 and T levels, respectively. The area around the line represent the 95% confidence interval for the estimation of this difference. Line and area are red when the confidence interval overlap zero, i.e. that hormone concentration between the control and the HIPV curves are not significantly different, otherwise they are blue. T concentrations is significantly different around the laying period, from 12 days before laying to 8 days after.

G: Males exposed to HIPVs had higher T levels around the laying period. Large dots and error bars represent mean \pm SE. Smaller dots represent raw data ($n = 34$ samples from 25 males: 12 in control, 13 in HIPV).

Discussion

We found no evidence that great tits exposed to HIPVs, an olfactory cue released by trees in response to caterpillar infestation, advanced their laying date, laid larger clutches or produced larger eggs in captivity. We however found that males in the HIPV group had higher concentrations of testosterone than control males just before and during the female laying period. These results suggest that males, more than females, adjust their sexual behaviours in response to odorous alarm signals emitted by trees in spring.

One interesting finding of our study is that males exposed to HIPVs had higher testosterone (T) levels during the laying period than control males. Graham and colleagues (2021) found a similar relationship between HIPVs detection and T levels in male blue tits. After having trained birds to associate the presence of HIPVs with that of food, they found a positive correlation between the time spent close to the HIPV-emitting trees and male T levels (Graham et al. 2021). Males could use HIPVs as an environmental signal indicating higher food abundance (Horiuchi et al. 2003; Girling et al. 2011; McCormick et al. 2012; Miresmailli et al. 2012) and upregulate their T levels accordingly, as it has been observed with others stimuli like the presence of a nest box in starlings (*Sturnus vulgaris*) (Gwinner et al. 2002) or food availability in zebra finches (*Taeniopygia guttata*) (Lynn et al. 2015). Since T concentrations in males are associated with courtship and mating behaviour (Silverin 1980; Ketterson et al. 1992; Enstrom et al. 1997; Hill 1999), males could have upregulated their hormonal status in response to HIPVs to stimulate their female to invest in reproduction.

Males reacted to HIPVs, but we did not find that females adjust their timing of laying, clutch size or egg size when exposed to the odorous treatment. This goes against our initial prediction of a higher investment of females following detection of HIPVs, but is in line with a recent study in which adding artificial HIPVs around nest boxes in the wild did not advance laying, nor increase individual clutch sizes (Delaitre et al., in prep). Another recent study with blue tits found that HIPVs increased female ovarian follicle size, but that was restricted to fast exploring females, a proxy for personality (Caro et al. 2023). The absence of a clearer effect of HIPVs on female reproduction remains surprising given that HIPVs represent a seemingly good predictor of caterpillar phenology and abundance in spring, when insectivorous birds like great tits start breeding. HIPVs are indeed already emitted when oak tree buds are growing and young caterpillars just hatched from their eggs (Graham et al. 2021, Graham et al. in prep), and high concentrations of HIPVs in the environment indicate higher herbivorous insect availability

(Horiuchi et al. 2003; Girling et al. 2011; Miresmailli et al. 2012). The present results are also surprising knowing that female tits are able to detect HIPVs and to use them in a foraging context (Amo et al. 2013; Rubene et al. 2019; Graham et al. 2021; Sam et al. 2021; Rubene et al. 2023). We presently see three potential explanations for the lack of an effect of HIPVs on reproductive timing and investment in our captive great tits.

First, the influence of supplementary cues on reproductive traits is generally subtle and often requires long-term experiments with large sample sizes to be able to detect an effect at the individual level. For example, while several correlative studies conducted in the field have repeatedly shown a link between temperature and seasonal timing of reproduction in birds (Charmantier et al., 2008; Dunn, 2004; Visser et al., 2003), it took almost ten years of experiments in controlled conditions to understand the direct effect of temperatures on timing of reproduction in great tits (Visser et al. 2009; Schaper et al. 2012; Verhagen et al. 2020). In the present study, it is important to stress that we only conducted this experiment for one breeding season, and with only 30 females analyzed. Advancing laying dates experimentally using supplementary cues has generally proven difficult, very few studies using natural photoperiodic profiles managed to induce laying earlier than what female would normally do (Nilsson and Svensson 1993; Gienapp and Visser 2006; Visser et al. 2009; Schaper et al. 2012).

Second, the sole presence of HIPVs might not be sufficient to trigger laying and increase investment in reproduction. Contrary to males that already exhibit robust gonadal response to the change in day length (Farner and Wilson 1957), female reproduction results from a complex association of several environmental cues (Wingfield and Kenagy 1991; Ball and Ketterson 2008; Nakazawa et al. 2023), which could explain why it is difficult to show an effect of each cue tested in isolation of the others. This mainly owes to the supplementary nature of those cues, which only modulate the powerful effect of photoperiod (Wingfield et al. 1992). Temperature, food and water availability, social cues from conspecifics or heterospecifics are all cues that birds are likely to use to decide when to breed and how much invest in it (Chmura et al. 2019), and females, more than males, might integrate those cues together for their annual reproductive decisions (Caro et al. 2009). In that context, future studies should consider testing the interactive action of multiple cues, like HIPVs and temperature for example.

Third, for an unknown reason, great tits in climate aviaries started laying particularly early in 2021. It was in fact one of the first times that captive females at the NIOO laid earlier than great tits in the wild (first egg date in our experiment : 7th April; first egg date in the wild: 17th April), which on average lay three weeks earlier than in captivity (Visser et al. 2009). Inducing an even earlier lay date in our experiment was thus probably even more difficult than

usual. Our captive birds also lay surprisingly large or small broods compared to the wild. We have observed some clutches with only 1 ($n = 2$), 2 ($n = 1$) or 3 ($n = 1$) eggs and others with 18 ($n = 1$) and 19 ($n = 2$) eggs, whereas the usual average clutch size in great tit varies from 7 to 12 eggs (Perrins and McCleery 1989). Such a large, unexplained, variance in clutch size renders the detection of a HIPV treatment effect quite challenging.

We found no evidence for an effect of our HIPV treatment on 17β -estradiol (E_2) levels in females. Given that E_2 is tightly correlated with rapid yolk development (Williams et al. 2004 Caro et al. 2009), and that we found no effect of HIPVs on laying dates or clutch sizes, finding no link between E_2 and HIPVs might therefore not be surprising. E_2 is also under control of the gonadotropin hormone LH, which in female great tits was found not to be influenced by the visual presentation of leafing branches and caterpillars (Schaper et al. 2011). On the other hand, in a recent study we found an effect of HIPVs on the growth of ovarian follicles, which produce E_2 , but that was restricted to a subset of females that were more exploratory when exposed to a novel environment (a proxy for personality) (Caro et al. 2023). We have here checked for an effect of personality in preliminary analyses, but found no reliable effects (data not shown).

In conclusion, our results do not provide evidence that odours released by developing tree buds in response to caterpillar infestation trigger laying or increase reproductive investment in female great tits, even though they have been shown to attract females as well as males in a foraging context (Amo et al. 2013; Rubene et al. 2019; Graham et al. 2021, Delaitre et al. in prep), and to modulate female gonadal responses in some cases (Caro et al. 2023). The picture in males is clearer, with a higher plasma testosterone in the presence of HIPVs (Graham et al. 2021, this study). The fact that males are generally less picky than females in terms of environmental signals eliciting a reproductive activation suggests that HIPVs might well be a player in the reproduction of insectivorous birds, but that it is challenging to detect and request longer, or more complex experimental designs. Future studies should now focus on how detection of HIPVs could affect reproductive decisions, offspring condition and reproductive success in the wild, and on how females weigh the different environmental signals shaping their reproductive decisions.

Declaration of interests

The authors have no competing interests to declare.

Data availability statement

The datasets needed to reproduce the analyses will be available on DRYAD.

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Chapter 4

Influence of artificial Herbivore-Induced Plant Volatiles on blue and great tit reproductive decisions



In **Chapter 3**, I investigated the effect of HIPVs emitted by caterpillar-infested trees on the reproductive decisions and physiology of captive birds. If I found interesting results, the sample sizes that we could afford in such settings may be a bit too restrictive for the subtle roles that such kind of supplementary cues play. Furthermore, the responses of birds in captivity can be a bit different from that observed under natural conditions ³⁷², even if I used hand-reared birds that are well adapted to captivity and reproduce well in these conditions. Combining captive studies with field studies thus constitutes complementary approaches to the study of populations adaptations to environmental fluctuations, in particular when one has the possibility to manipulate the environment in both settings in parallel. I consequently also investigated the response to HIPVs in free-living birds under natural conditions. Studying environmental cues in the wild is challenging. Since it was not feasible to modulate HIPV emissions by manually infesting tree buds with caterpillars in the forest, I opted for an artificial blend of HIPVs that was diffused around the birds' nest-boxes. In a previous experiment carried out at the CEFÉ, Graham et al. (in prep.) identified the volatile organic compounds emitted by caterpillar-infested oak tree buds. They identified eight compounds that differed in concentration between caterpillar-infested and control trees, and assembled those compounds in an artificial blend that I used in my experiments.

I first tested the attractiveness of blue tits to this artificial blend in a Y-shaped aviary. As birds were collected as chicks and hand-reared indoor until their independence, they had never foraged in trees and never smelled bud HIPVs. They were therefore naïve to these odours. I found that the birds were significantly more attracted to the HIPVs side of the aviary than to the control side. This showed that blue tits are naturally attracted to HIPVs, even without having had the chance to learn to associate their presence with that of caterpillars, and it also showed that the artificial HIPV blends that we had created worked. The next question was whether the birds would use these odours in a reproductive context in the field. In different free-living populations, I investigated whether blue and great tits would adjust their reproductive timing and investment in response to the presence and abundance of HIPVs in their breeding territory. Blue and great tit reproduction was monitored in deciduous downy oak forests, both on the mainland near Montpellier and on the island of Corsica. From 2019 to 2022, before the onset of bud burst and nest building, odour diffusion flasks containing artificial HIPVs were placed around the nest-boxes studied. As HIPVs reflect the timing of the caterpillar emergence and abundance, I expected birds to lay earlier and larger clutches where the odour cue was diffused.

I did not find that individual birds that built their nest in HIPV nest-boxes laid earlier or more eggs. Interestingly though, overall, there were much more eggs, nestlings and fledglings produced in the areas that had been equipped with HIPVs than in the control areas. There were thus more new birds that had grown surrounded by our artificial blend that fledged and populated the sites I studied. By adding HIPVs around the nest-boxes, we expected an increase in the number of eggs, and potentially chicks, produced, but we did not expect more fledglings as we only increased the signal and not the quantity of caterpillars in the environment. These results suggest that the perception of HIPVs stimulated parents to increase their foraging effort during the chick-rearing phase. This chapter highlights the possibility that birds adapt their reproductive investment to the amount of food they predict from an olfactory cue.

Contribution: For the behavioural experiment in a Y-shaped aviary with captive blue tits, Samuel Caro and I designed the protocol, I conducted the experiment and analysed the data. This was mostly done during my Master's thesis project. For the field experiment, I installed half of the HIPVs flasks in the field in 2020, 2021 and 2022, and participated to the monitoring of the bird reproduction. I contributed to the development of the new emission method in 2021-2022, together with Bruno Buatois. I wrote the first draft of the manuscript with Jessica Graham.

Article 6: The manuscript entitled “Olfactory communication from trees to birds: Effects of herbivore-induced plant defences on a songbird behaviours and reproductive decisions” is in revision for *The American Naturalist*.

Article 6

Olfactory communication from trees to birds: Effects of herbivore-induced plant defences on a songbird behaviours and reproductive decisions.

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Abstract

In insectivorous birds the reproductive period often coincides with the spring development of vegetation, but whether there is a direct relationship between the two, and through which mechanisms this link could come about, is not clear. Trees release Herbivore-Induced Plant Volatiles (HIPVs) when they are attacked by insects, and we test here whether birds use those alarm odours to modulate their reproductive behaviours. Using a two-step experimental approach, we first show in controlled conditions that blue tits are attracted to HIPVs mimicking a caterpillar attack on oak buds in early spring, without having to learn them. We then equipped nest-boxes in the field with the same artificial HIPVs. We did not find that birds that built their nest in HIPV nest-boxes advance lay date or produced larger clutch. However, although this result is not significant, slightly more nest-boxes equipped with HIPVs were occupied compared to control ones, resulting in more eggs, nestlings, and fledglings introduced in the population from these treated nest-boxes. Our study thus reveals a potential for new multitrophic interactions across plants, insects, and birds by linking plant alarm odours with reproductive decisions in a common insectivorous songbird. The use of olfaction in birds thus represents an encouraging area of study for understanding adaptations to a constantly changing environment.

Keywords: olfaction; herbivore-induced plant volatiles; reproductive investment; reproductive timing

Introduction

In seasonal environments, many species raise offspring at a specific time of the year that coincides with the local surge in resources (Rutberg 1987; Boutin et al. 2006; van Asch et al. 2010; Peláez et al. 2020). Phenological synchronization of peak food abundance and offspring needs is a crucial determinant of fitness (Plard et al., 2014; Reed et al., 2013; Thomas et al., 2001; Van Asch & Visser, 2007). However, there could be large annual variation in the optimal timing for breeding that must be predicted and anticipated (Williams 2012; Kriegsfeld et al. 2015; Tissier et al. 2020). Organisms thus need to use cues present in their environment to predict the future peak in resources (McGrath et al. 2009; Ogutu et al. 2010) and decide when to initiate reproduction (Tyler et al. 1982; Wingfield and Moore 1987).

In the well-studied oak-caterpillar-insectivorous bird food chain, the caterpillar peak timing is a major source of selection pressure on the timing of breeding in tits. A mismatch between maximum caterpillar abundance, the main food of nestling tits, and the chick feeding period results in lower nestling condition, higher nestling mortality, or decreased local recruitment into the following generations (Naef-Daenzer and Keller 1999; Thomas et al. 2001; Gienapp and Visser 2006; Visser and Gienapp 2019). Thus, birds must anticipate and use cues to synchronize the chick feeding period with the period of maximal biomass of caterpillars.

The initial predictive cue is photoperiod (i.e. daylength) (Lambrechts et al. 1997; Dawson et al. 2001; Rani and Kumar 2014) but photoperiodic conditions are the same every year so it cannot explain how individuals adapt to year-to-year variation in environmental conditions. Among the wide array of other possible signals of optimal breeding conditions, temperature has by far been the most studied (Caro et al., 2013; Parmesan & Yohe, 2003; Woodroffe et al., 2017) but the influence of this cue on seasonal timing of reproduction seems relatively subtle (Schaper et al. 2012; Verhagen et al. 2020) as several studies have failed to show a causal link between mean temperatures and first-egg dates, gonadal growth, or reproductive hormone levels (Caro et al., 2013; Dawson, 2005; Visser et al., 2011). Other environmental cues may have more decisive influences on breeding phenology.

The most favorable period for leaf-feeding moths to hatch is just after the buds of their host plant open, as this is at this time that trees provide the best food for them (van Asch and Visser 2007). In our studied system, freshly hatched caterpillars immediately bore into developing oak (Du Merle and Mazet 1983) and this is generally at this time that birds should initiate reproduction to be able to synchronize chick feeding period with caterpillar abundance peak. When they just hatch, caterpillars are too small and cryptic to represent a food source so

they are unlikely to serve as a visual cue to make reproductive timing decisions. Instead, olfaction may be one sense that organisms could use to infer caterpillar phenology. Although birds were considered anosmic for a long time, they have an olfactory system that can allow them to detect odours, at low concentrations (Caro and Balthazart 2010) and in different contexts (e.g., burrow recognition (Bonadonna et al. 2003); partner recognition (Bonadonna and Nevitt 2004; Grieves et al. 2022), foraging behaviour (Nevitt et al. 1995), predator detection (Roth et al. 2008; Amo et al. 2011)).

In response to damage by herbivorous insects, many tree species release herbivore-induced plant volatiles (HIPVs) (Arimura et al. 2005). Carnivorous arthropods and parasitoids are known to use HIPVs as a cue to locate herbivorous insects (Turlings et al. 1990; Giunti et al. 2016). A large number of studies have shown that HIPV blends are used by birds to locate insect prey (Mäntylä et al. 2004, 2008, 2014, 2017; Mrazova and Sam 2017; Hiltbold and Shriver 2018; Sam et al. 2021; Nguyen et al. 2022), although this effect may be specific to certain plant species (Amo et al. 2013; Koski et al. 2015). Recent studies also show that the ability to discriminate between caterpillar infested and uninfested trees only by smell is acquired through learning (Amo et al. 2013; Mrazova et al. 2019; Graham et al. 2021; Rubene et al. 2022). Whether birds use their olfactory abilities in other situations, like determining the future peak in food biomass to decide when to initiate reproduction, is currently unknown. Only two studies in blue tits (*Cyanistes caeruleus*) investigated this possibility (Graham et al. 2021; Caro et al. 2023). The authors found that more exploratory (i.e. a proxy of personality) females had larger ovarian follicle when exposed to HIPVs (Caro et al. 2023) and that males with higher testosterone level (i.e. a hormone controlling sexual behaviour in males) spent more time around caterpillar-infested trees (Graham et al. 2021). Birds could use this olfactory signal to settle in high food density territories (Graham et al. 2021), adjust their laying date and synchronize the feeding period of their nestlings with the timing of maximal biomass of caterpillars (Blondel et al. 1991; Thorley and Lord 2015) and adjust their clutch size to match predicted food availability of the breeding territory (Hussel and Quinney 1985; Marciniak et al. 2007). As a result, better detection and correct interpretation of HIPV cues could result in higher reproductive success (Mägi et al. 2009; Harriman et al. 2017) compared to birds that would not rely on this odour cue to make their reproductive decisions.

The purpose of this study was two-fold. First, we tested in captivity, using Y-mazes, whether blue tits detect and orient to an artificial HIPVs odour bouquet mimicking HIPVs released by caterpillar infested oak buds, without having had the possibility to learn them. Studies so far have focused on HIPVs emitted by mature leaves, which qualitatively and

quantitatively differ from HIPVs emitted by buds and young leaves (Fischbach et al. 2002; Röse and Tumlinson 2004). Many studies have indeed demonstrated that HIPV emissions, particularly emissions of monoterpenes (ocimene), terpenes (isoprene) or sesquiterpene (caryophyllene) differ widely during the course of the growing season for different tree species in different genera (*Quercus*, *Cistus*, *Prunus*, *Pinus*) (Staudt and Bertin 1998; Hakola et al. 2001; Röse and Tumlinson 2004; Pio et al. 2005; Holopainen and Gershenson 2010; Bracho-Nunez et al. 2011). Experimental approaches investigating the effect of HIPVs on bird behaviour also always have trained birds to associate HIPVs with food presence (Amo et al. 2013), because it was found that great tits at least do not seem to innately detect HIPVs (Amo et al. 2016; Sam et al. 2021; Rubene et al. 2022).

In a second step, we tested in two free-living populations whether blue and great tits use their perceived timing and abundance of HIPVs emitted by oak buds to adjust reproductive timing and investment. We hypothesized that adding HIPVs on birds' territories prior to bud burst and caterpillar hatching would mislead the birds, causing them to advance first egg laying date and increase their reproductive effort (i.e. more eggs).

Methods

Ethics

Blue tits were trapped and maintained under licenses 2018-s-11 issued by the Direction Régionale de l'Environnement, de l'Aménagement et du Logement Languedoc-Rousillon; and 15-XIX-116 issued by the Direction Départementale de la Protection des Populations de l'Hérault. 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 numbered 036 with reference APAFIS#8608-2017012011062214 v4.

Y-maze experiment

Subjects

The experiment took place at the CEFÉ-CNRS in Montpellier, France, from 4th to 20th February 2020. Twenty-nine blue tits were captured in 2019 from the long-term study population of La Rouvière at Montarnaud near Montpellier. They were born in the wild, collected as nestlings (10 days old), and hand-raised in captivity until independence (35 days old), following the procedure described in previous studies (Reparaz et al. 2014; Caro et al. 2021) and then maintained in outdoor aviaries (27 m³) following the methods of Graham et al.

(2021). Since birds had been hand-reared indoors and were not released into outdoor aviaries until late June 2019 (i.e., after the period when caterpillars forage on oak buds) we considered that these birds were naïve to HIPVs emitted by oaks in early spring. Birds were moved from aviaries to individual cages (0.8 x 0.4 x 0.35 m), distributed across 3 indoor rooms in mixed sex groups on 3rd February 2020.

Exploration score

We scored exploration by releasing each bird separately in a novel environment, which here consisted in an exploration cage equipped with two perches (0.6 × 0.4 × 0.4 m). Once the bird entered the cage, its movements were observed for five minutes. Birds were given an exploration score on a continuous scale based on the total number of movements between the perches, as well as hops up and down and flights. Exploration score ranged from 88 to 290, with higher scores indicating faster exploration, and lower scores indicating slower exploration. This kind of measurement has been used to measure exploration behaviour in great tits (Stuber et al. 2013) and blue tits (Mutzel et al. 2013; Charmantier et al. 2017; Dubuc-Messier et al. 2017), and provides exploratory scores that are repeatable and well correlated to those performed in the laboratory (Dingemanse et al. 2002; Stuber et al. 2013).

Artificial HIPVs used in the experiment

Green oak leafroller (*Tortrix viridana*) and winter moth (*Operophtera brumata*) caterpillars are oak specialists and hatching needs to be closely timed with elongation and development of oak buds, so caterpillars can perforate the bud and eat young developing leaves inside the bud (Du Merle 1999; Ivashov et al. 2002). To create an artificial HIPVs cocktail, HIPVs emitted by downy oak buds (*Quercus pubescens* Willd.) infested with freshly hatched green oak leafroller and winter moth caterpillars were compared with volatile organic compounds emitted by uninfested buds (Graham et al., in prep). Graham and colleagues (in prep) identified the compounds emitted in significantly higher concentrations by caterpillar infested buds to constitute the odour bouquet used in the present experiment (figure 1). Based on these results we then made flasks to diffuse the artificial HIPVs (see figure S1A-B-C for details on flask design). Briefly, HIPVs flasks consist in 150 mL opaque plastic containers containing eight 1.5 mL amber vials each filled with 10 µL of the selected compounds while control flasks were empty.

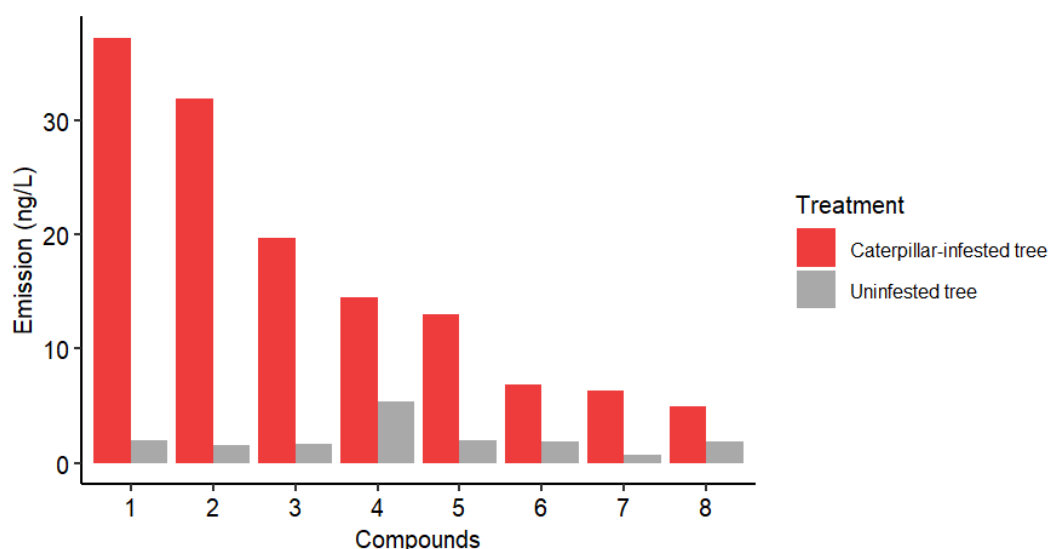


Figure 1. The eight compounds emitted in higher concentration in caterpillar infested oak buds compared to control trees.

The eight compounds selected to create the artificial HIPV flasks were emitted in higher concentration in caterpillar infested oak tree compared to uninfested oak tree.

List of the compounds: (1) Aromadendrene, (2) (E)- β -ocimene, (3) Farnesene, (4) (Z)-hex-3-enyl benzoate, (5) DMNT, (6) α -humulene, (7) (E)-caryophyllene, (8) Methyl salicylate.

Experimental setting

Two outdoor Y-mazes were used to test for naïve preference for artificial HIPVs (2.5 x 2 m arms, figure 2). The dimensions of the Y-mazes are similar to previous studies (Amo et al. 2013; Graham et al. 2021) showing that blue and great tits can discriminate between caterpillar-infested and uninfested trees. Additionally, the flasks were mildly heated with an electric heat cable (Waldbeck Greenwire, Germany) to increase their emission rates (Tingey et al. 1979) and it has been shown that insect responses to HIPVs are generally consistent out to a range of 8 m (Braasch and Kaplan 2012), we can therefore expect at least the same detection sensitivity in birds. Thus, our device allowed the birds to detect the odours from the entry box. The frames of the Y-mazes were made of wood and wire mesh. Three perches were placed in each maze; one in the neutral zone (zone containing no stimuli in which the bird could orient itself before making a choice), one in the left arm, and one in the right arm. Two flasks containing HIPVs or two empty control flasks were placed on the right and left perches according to the treatment (treatment and control side of the maze was randomized). Two GoPro cameras (GoPro, Inc., USA) were fixed on the perch in the neutral zone to simultaneously record the movements of the birds in the right and left arms. To control for the effect of the wind on the orientation of the birds in the Y-maze, wind strength and direction were measured using the weather station on site. Bird behaviours started to be recorded when the tested bird entered the maze.

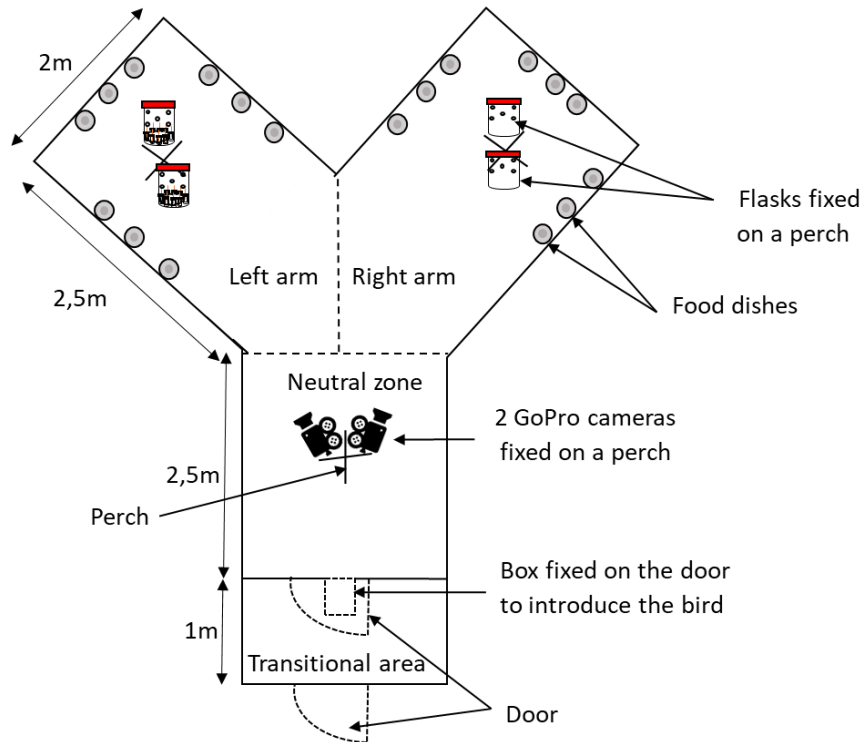


Figure 2. Diagram of the two Y-mazes used in the behavioural experiment.

In each maze, nine empty food dishes were placed inside the left and right arm to evaluate food searching activity of birds during testing. A small box fixed on the entrance door of the maze was used to introduce the birds into the maze. A wind break was placed around each maze to reduce visibility on the outside of the maze and to reduce strength of the wind.

Testing

Birds were placed during one day by group of five before testing to habituate to the maze, at this time containing only control flasks in each arm. Then, birds were individually tested with two flasks containing artificial HIPVs placed in one arm of the maze and two control flasks in the other arm. Approximately half of the birds were tested with HIPVs in the right arm ($n = 14$) and the left arm of the maze ($n = 15$). At that time, birds were considered naïve to the odour bouquet. Each bird was recorded for 30 min.

Behavioural observations

Recorded videos were analyzed with the freely available Behavioural Observation Research Interactive Software (BORIS) (Friard and Gamba 2016) version 7.9.7–14/01/2020 (<https://www.boris.unito.it/>). Behavioural events were time stamped as they were observed. We recorded: (i) the first arm chosen when entering the maze, (ii) the number of visits to empty food dishes in each arm, and (iii) the time spent in each arm of the maze.

Field experiment

Study areas

Blue and great tit reproduction was monitored in a deciduous downy oak (*Quercus humilis*) forest (Charmantier et al. 2016) on the mainland near Montpellier (La Rouvière wood, Montarnaud, 43.621°, 3.733°, hereafter called mainland) across four successive years (2019-2022). From 2020, we also ran the experiment in another deciduous downy oak forest on the Mediterranean island of Corsica (Avapessa, 42.557°, 8.887°, Feliceto, 42.538°, 8.937°, hereafter called Corsica). Caterpillar density is typically higher in Corsica than on the mainland (Blondel et al. 1999; Tremblay et al. 2003), we thus hypothesized that HIPVs could constitute a more prominent environmental cue for reproductive timing and investment of tits in this population than on the mainland.

Experimental setting

Sixty-eight and forty (thirty-eight in 2021-2022) nest-boxes on the mainland and in Corsica, respectively, spaced ~50 m apart, were used in this study. Two experimental settings to diffuse artificial HIPVs have been used. In 2019-2020, three odour diffusion flasks containing artificial HIPVs (figure S1A-S1C) were placed in a triangle around half of the nest-boxes studied. The other half of the nest-boxes had three control flasks (figure S1B). Each flask was hung at the height of the nest-box, ~5 m away, at a distance that birds can smell HIPVs from the nest-box. In 2021-2022, we used 1.5 mL amber glass vials to diffuse HIPVs (figure S1D). Two different solutions were prepared before going on the field in the lab. A control solution containing 300 mL of paraffin oil, and a treatment solution containing 300 mL of paraffin oil and the appropriate quantities of the different compounds selected for treatment flasks (table S1). The quantities used in 2021 and 2022 for treatment flasks depend on the molecular mass, the relative density, and the boiling point of each compound. Mixing all compounds in paraffin oil (figure S1D) instead of releasing each compound independently in a vial through a capillary glass tube (figure S1A-C) enable to better control the concentration and duration of emission of each compound. In paraffin oil, emission rate of a compound only depends on its molar concentration and its boiling point. Contrary to the capillary glass tube, all the compounds had the same exchange surface air/paraffin with the setup used in 2021-2022. By adjusting the quantities of the different compounds according to their physical parameters (introducing a smaller quantity of heavy compound which hardly emits, and a bigger quantity of lighter compounds which emits easily), the emission is more stable over time. In 2021-2022, five flasks (figure S1D) were placed the nest-boxes at the height of the nest-box. Half of the

nest-boxes received flasks filled with 1 mL of the treatment solution, and the other half had 1 mL of the control solution. In 2020 on the mainland, and in 2022 in both population, nest-boxes had the opposite treatment compared to 2019, and 2021, respectively, so each nest-box was its own control. Flasks were always placed prior to the onset of bud burst and nest building. Nest-boxes were monitored weekly to collect nest-box occupation rate (i.e. at least one egg laid) and bird reproduction data: date of first egg, number of eggs, egg size in 2021-2022 in the mainland, number of hatchlings, number of 15-day old nestlings, nestling weight at day 15, and number of fledglings. We only included first nest attempts of blue and great tits in our study.

Statistical analysis

We analyzed the data in two steps, from (1) simple and straightforward count tests, to (2) more complex (mixed) models that investigate the effects of the different explanatory variables. All the analyses were performed in R version 1.3.1093 (R CoreTeam). Linear mixed-effects models and generalized linear mixed-effects models were conducted using the lme4 package (Bates et al. 2015). P-values were obtained with the lmerTest package in the case of mixed-model analyses (Kuznetsova et al. 2017). Plots were created with the ggplot2 package (Wickham 2016). Post-hoc tests were conducted using the lsmeans package (Lenth 2016). We set $\alpha = 0.05$ and report mean \pm standard errors (S.E.).

Y-maze experiment

Step 1: To determine if there was a preference for the HIPV side of the maze, we first used binomial tests comparing the number of birds that (i) visited the HIPV *vs.* the control side when they first entered the maze, (ii) visited more food dishes in the HIPV *vs.* the control side, and (iii) spent more time in the HIPV *vs.* the control side.

Step 2: We tested several variables that could affect the number of visits and the time spent on each side by running (i) a generalized linear model to explain the choice of first side (HIPV *vs.* control) using a binomial distribution; (ii) a generalized linear mixed-effects model using a Poisson distribution to explain the number of visits to the food dishes in each side of the maze and; (iii) a linear mixed effects model to explain the time (in seconds) spent in each arm of the maze. All three models included sex (M or F), exploration score, wind, and the interaction exploration score*sex, as explanatory variables. For the analyses on the number of visits to the food dishes and the time spent in each side of the maze, the treatment (control *vs.* HIPV) was added, as well as the interactions treatment*sex, treatment*wind, treatment*exploration score, and a 3-way interaction of treatment*sex*exploration score. We

considered interactions that included treatment only, as they represent the most biologically meaningful interactions. The identification of the bird was included as a random intercept in the mixed-models to account for the fact that there were two non-independent values per bird in the dataset (one for each side of the maze). When interactions were significant, we also ran a Tukey's post-hoc analysis to identify which pairs of factor levels differed.

Field experiment

We used binomial tests to compare (i) the number of HIPV *vs.* control nest-boxes occupied, the total number of (ii) eggs laid, (iii) 15 day-old nestlings produced, and (iv) fledglings produced in HIPVs *vs.* control nest-boxes, across populations and years. We then tested whether these counts varied across years or populations in interaction with the treatment, by running generalized linear models with Poisson distributions on the total numbers of (i) occupied nest-boxes, (ii) eggs, (iii) 15 day-old nestlings, and (iv) fledglings. These models included a year*treatment and a population*treatment interaction.

At the individual level, we first ran a PCA on egg size (data on 2021-2022 in the mainland) including length, breadth and volume of the eggs, as these variables were correlated. The PC1 axis explained 91.3% of the variability in the data (figure S2). We then compared reproductive parameters between HIPV and control nest-boxes by performing (i) linear mixed-effect models on lay date, nestling weight and egg size, (ii) generalized linear mixed-effects models with Poisson distribution on clutch size; and (iii) generalized linear mixed-effects models with binomial distribution on reproductive success. Models included year (2019, 2020, 2021, 2022), population (Mainland or Corsica), species (blue or great tit), the interaction of treatment*year, treatment*population as well as the interaction treatment*species as explanatory variables. Nest box identification was added as a random intercept to account for the fact that several nest-boxes had nestlings in different years. The model looking at nestling weight included female ID as a random intercept to account for females having multiple nestlings measured.

Results

Y-maze experiment

When birds were exposed to HIPVs in the maze, 21 out of 29 birds first went to the arm containing the HIPVs (binomial test, $p = 0.024$, figure 3A). A generalized linear model (GLM) revealed that this initial attraction to the HIPV side tended to be reinforced by a favorable wind (higher speed and blowing from the experimental arm towards the maze entrance), although the

effect is not statistically significant ($p = 0.066$, Table S2). This model also shows that this attraction to HIPVs was similar in males and females ($p = 0.579$, Table S2) and across the range of exploration scores ($p = 0.824$, Table S2).

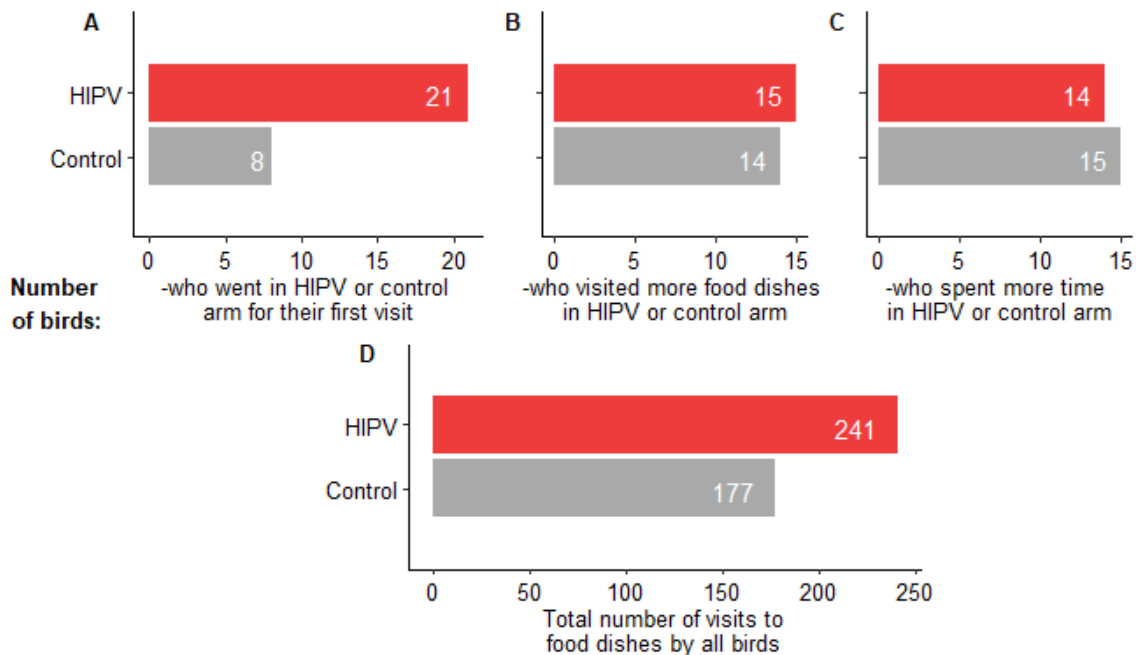


Figure 3. Blue tit preference for the HIPV side of the maze.

(A) More blue tits went to the HIPV side when entering the maze for the first time ($p = 0.024$) but (B) they were not more likely to visit food dishes in the HIPV arm ($p = 1$) (C) nor to spend more time in the HIPV side ($p = 1$). If we do the sum of all visits to the food dishes made by all the birds, they made more visits in the HIPV side (241 vs. 177, $p = 0.002$)

Regarding the visits to the food dishes, overall only 15 out of 29 birds visited dishes more frequently on the HIPV side than on the control side (binomial test, $p = 1$, figure 3B). However, if we compare the total number of times that dishes were visited on each side of the maze (summed for all the birds), dishes on the HIPV side were visited on 241 occasions, while dishes on the control side were visited on 177 occasions (binomial test, $p = 0.002$). Moreover, GLMMs run on the number of visits individuals made to the food dishes revealed that birds had a higher rate of food dishes visits in the HIPV arm when the wind was favorable ($p < 0.001$, figure 4A, Table S2). A significant treatment*sex interaction ($p = 0.012$, Table S2) revealed that males visited food dishes in the HIPV arm more often than females (post-hoc test; males: $p = 0.017$, females: $p = 0.846$). Adding exploration score to this interaction (3-way interaction treatment*sex*exploration score, $p = 0.002$, Table S2) showed that more exploratory males were driving this pattern (figure 4C).

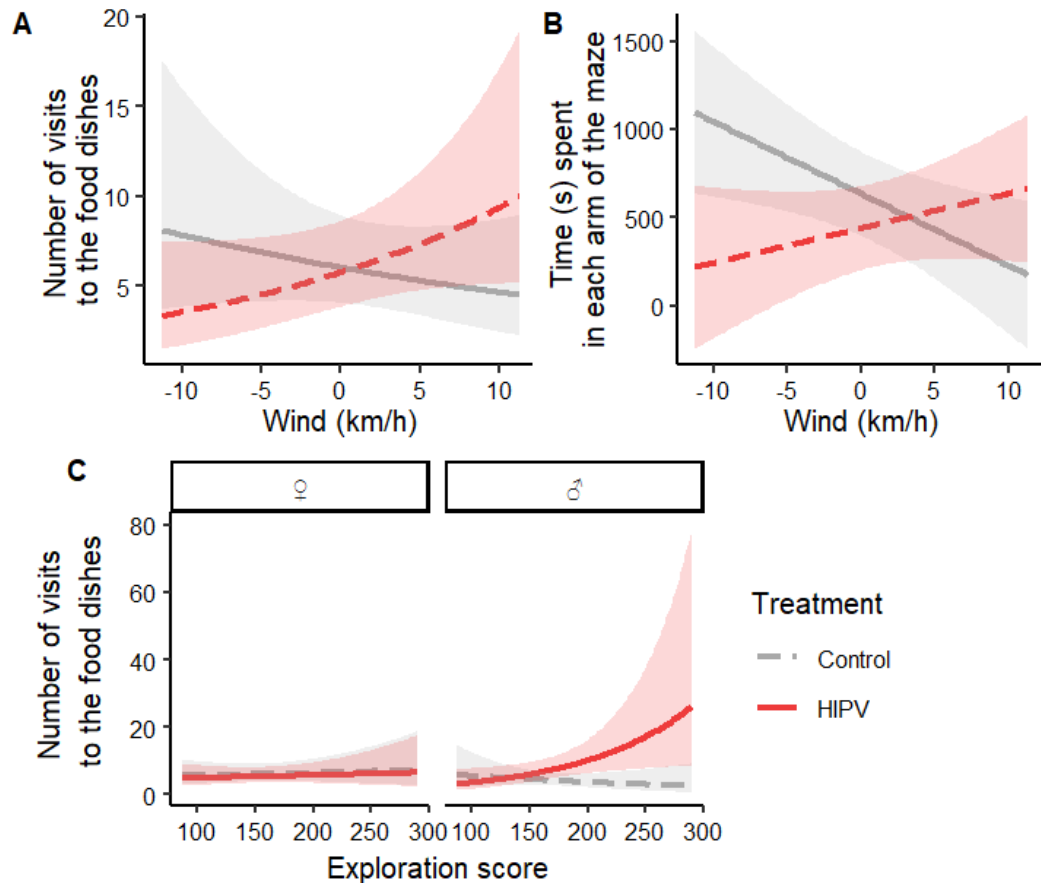


Figure 4. Food searching activity in the maze is driven by wind and exploratory behaviour.

Increased food searching activity and time spent in the HIPV side of the maze appears to be primarily driven by (A-B) a favorable wind (higher speed and facing the bird) and (C) more exploratory behaviours in males. Model estimates \pm SE are depicted.

Regarding the time spent in each side of the maze, only 14 out of 29 birds spent more time in the HIPV side than in the control side (binomial test, $p = 1$, figure 3C). Interestingly however, birds spent more time in the HIPV arm when the wind was favorable (treatment * wind interaction, $p = 0.011$, figure 4B, Table S2). Time spent in each arm did not depend on sex ($p = 0.845$) or exploration score ($p = 0.409$). Although not statistically significant, more exploratory males tended to spend more time in the HIPV arm, while more exploratory females spent more time in both arms of the maze (whether this is in HIPV or control arm) rather than in the neutral zone (treatment * exploration score * sex, $p = 0.064$, Table S2).

Field experiment

At the population level, for both study populations and across the four years, birds occupied slightly more nest-boxes equipped with HIPVs than control nest-boxes (135 vs. 118),

but this effect was far from significant (binomial test, $p = 0.315$, figure 5A). In total in both population, birds in HIPV nest-boxes laid significantly more eggs (binomial test, $p = 0.001$, figure 5B), raised more nestlings (binomial test, $p < 0.001$, figure 5C) and produced more fledglings (binomial test, $p < 0.001$, figure 5D), compared to control nest-boxes.

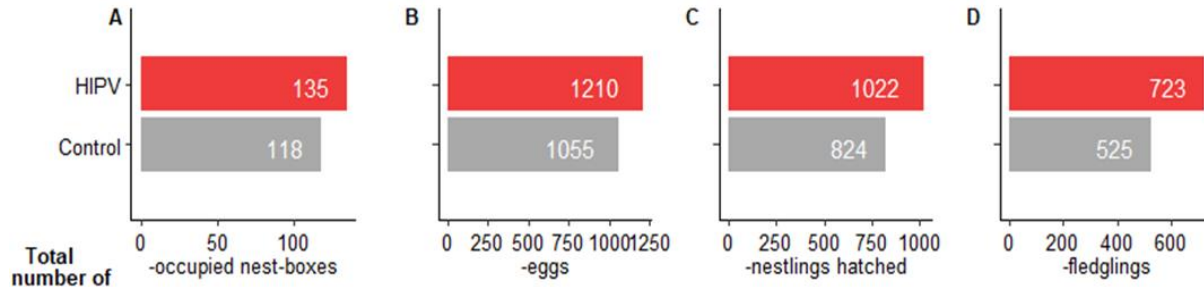


Figure 5. More reproductive outputs were produced in HIPV nest-boxes.

(A) Across the four years and in both populations (Mainland and Corsica), wild blue tits and great tits had a slightly higher occupation rate in HIPV vs. control nest-boxes, yet this result is not significant ($p = 0.315$). (B) They however laid more eggs ($p = 0.001$), (C) raised more nestlings ($p < 0.001$), and (D) produced more fledglings ($p < 0.001$) in HIPV nest-boxes.

We tested whether the treatment effects in the field experiment varied across populations or years. We found that the effect of treatment on the number of fledglings varied across years ($p = 0.002$) and populations ($p = 0.005$) (Table S3A). Post-hoc analyses showed that more fledglings were produced in HIPV nest-boxes in 2020 (185 vs. 99, $p < 0.001$) and 2022 (280 vs. 182, $p < 0.001$) (Table S3B, figure S3A), and in HIPV in the mainland (414 vs. 269, $p < 0.001$, Table S3B, figure S3B) compared to control nest-boxes. For all the other response variables, the effect of the odour treatment was similar across years and populations (see Table S3A).

At the individual level, linear model showed a significant interaction between the odour treatment and population ($p = 0.045$, Table S4A), but a post hoc test revealed that this significant result was only due to a tendency for earlier laying date in control nest-boxes in Corsica compared to control nest-boxes in the mainland ($p = 0.091$, Table S4B, figure 6A). GLMM for nestling weight showed a significant interaction between the odour treatment and the species ($p = 0.043$, Table S4A) but the post hoc test on this interaction showed no difference between the control group and the experiment group of nestlings when analyzing the effect of the odour treatment on each species independently (Control vs. HIPV Blue tit: $p = 0.999$, Control vs. HIPV Great tit: $p = 0.156$, Table S4B, figure 6B). Other (G)LMMs showed no evidence of an odour treatment effect on clutch size, egg size or reproductive success, either as a main factor, or in interaction with year or population (see Table S4A).

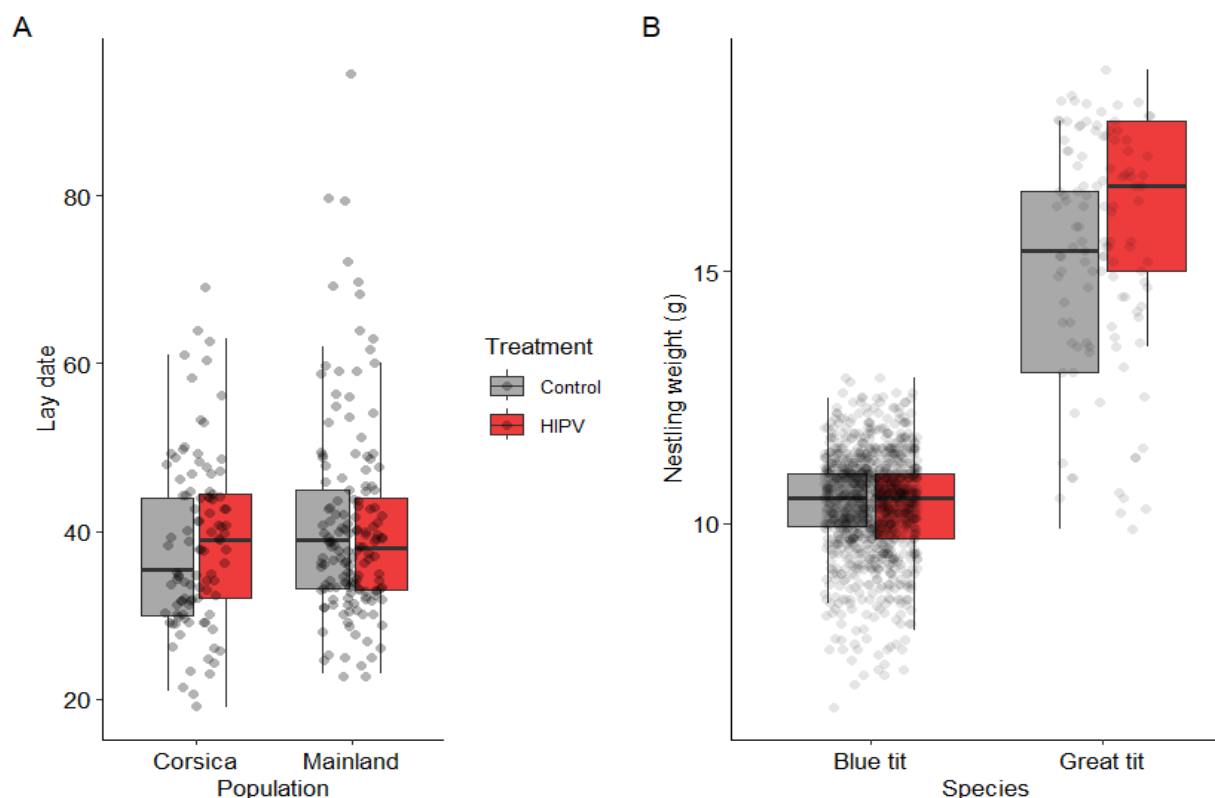


Figure 6. Odour treatment effect on laying dates and nestling weight.

A: Linear model showed a significant interaction between the odour treatment and population ($p = 0.045$), but a post hoc test revealed that this was only due to a tendency for earlier laying date in control nest-boxes in Corsica compared to control nest-boxes in the mainland ($p = 0.091$). Laying date: 1=1st March.

B: GLMM for nestling weight showed a significant interaction between the odour treatment and the species ($p = 0.043$) but the post hoc test showed no difference between the control group and the experiment group of nestlings when analyzing both species independently (Control vs. HIPV Blue tit: $p = 0.999$, Control vs. HIPV Great tit: $p = 0.156$).

Discussion

In captivity, we found that blue tits expressed a preference for artificial HIPVs. We also found that food dishes were visited more often when surrounded by HIPVs; visits that were mostly attributable to fast exploring males. This means that blue tits are readily attracted to an odour normally emitted by caterpillar-infested buds, potentially without having to learn it. A favourable wind reinforced the attractiveness of the HIPVs. Using the same bouquet of artificial HIPVs in the wild, we did not find that female blue and great tits use HIPVs to time reproduction or adjust individual clutch size. Although this result is not significant, we found that birds occupied slightly more nest-boxes equipped with HIPVs, indicating that birds potentially use them to identify territories that will have high caterpillar density. This higher occupancy of

HIPV nest-boxes then conducted to a higher overall production of eggs, nestlings, and fledglings in HIPV areas.

The main finding of the present study is that blue tits seem to be able to innately detect HIPVs. Up to now, however, it was believed that passerines needed to learn to associate HIPV presence with food (Amo et al. 2016; Sam et al. 2021; Rubene et al. 2022). For small insectivorous species like tits that are short-lived (average longevity in our population is only 2 years (Oliosio 2017)), it would seem adaptive not to have to learn on their first breeding attempt that HIPVs emitted by growing buds in early spring can signal food presence and seasonal timing of peak food biomass. Our results add to the recently growing body of literature showing that passerines, like blue and great tits, are in fact exquisite smellers, and that they can use their sense of smell in a large variety of contexts, which include foraging and potentially reproduction. The discovery that birds can use olfactory signals from primary producers (i.e., plants) to infer information about higher levels of the food chain they prey on, originally came from a few seabird species (Nevitt et al. 1995). It was not until recently that terrestrial birds, which have notably smaller olfactory bulbs than seabirds (Bang and Cobb 1968; Caro et al. 2015), were also shown to be able to use plant-released volatile compounds to find their food (Amo et al. 2013; Mrazova et al. 2019; Graham et al. 2021).

Even if female blue tits were initially as attracted to the HIPV side of the maze as males when they entered the maze for the first time, they did not visit more food dishes on this side. This is consistent with our hypothesis that oak bud HIPVs can be used more as a reproductive signal of future caterpillar abundance rather than a cue for current food availability. This is also consistent with the fact that timing for breeding and investment in egg production is determined by the females only (Caro et al. 2009; Williams 2012). Males might behave differently, as we have seen that more exploratory males were not only initially more attracted to HIPVs, but also visited more food dishes on that side of the maze, suggesting they were also looking for immediate presence of food, probably in order to obtain the energy necessary to defend a territory (Serrano-Davies et al. 2017). Blue tits are known to consume growing buds in early spring (Betts 1955) so HIPV presence may indicate presence of developing buds to consume with an extra source of protein inside due to the presence of young caterpillars (Shutt et al. 2019). Interestingly, in another recent study with blue tits, males with the highest levels of circulating steroids (i.e., testosterone) spent the most time around an oak tree infested with caterpillars (Graham et al. 2021). Some species exhibit the ability to respond to odour cues only

during the breeding season (Clark and Smeraski 1990) and experimentally elevating testosterone in males increases olfactory bulb volume (De Groof et al. 2010). Olfactory sensitivity in males could thus be upregulated by testosterone production in the olfactory epithelium (Lupo et al. 1986; Horie et al. 2017). Moreover, linkage between testosterone and exploratory behaviour have been suggested in some species, with testosterone potentially increasing foraging persistence (Andrew 1972; Daisley et al. 2005; Németh et al. 2015). This suggests that the interplay between oak bud HIPVs, bird reproduction and exploratory behaviour differs between males and females, and future studies will have to disentangle these complex interactions.

We cannot rule out that birds may have learned to associate HIPVs with food availability before leaving the nest. For example, in chicken chicks, exposure to strawberry odour 5 days before hatching influences the chick's preference for this odour afterwards (Sneddon et al. 1998). However, blue tits are born inside a closed nest chamber, and nestlings hatch several weeks after their parents made the decision to initiate egg laying. Thus, nestlings are in the nest at a time that is already far from caterpillar hatching and bud burst, and by extension, from the HIPVs emitted at that time. Many studies have indeed demonstrated that HIPV emissions, particularly emissions of monoterpenes and isoprene, differ widely during the course of the growing season for different tree species in different genera (*Quercus*, *Cistus*, *Prunus*, *Pinus*) (Hakola et al. 2001; Pio et al. 2005; Bracho-Nunez et al. 2011). Emission rate of caryophyllene and ocimene, two compounds we used in our HIPV flasks, differ during the developmental stages of oak leaves (Staudt and Bertin 1998). Additionally, in many species, the odour bouquet of buds, mature leaves, and fruits or flowers that have been damaged by insect herbivory have different compositions (Röse and Tumlinson 2004; Holopainen and Gershenson 2010). Consequently, when nestlings hatch, they are exposed to HIPVs of fully developed leaves and no longer to HIPVs from early developmental stages of buds. We can thus be quite confident that our blue tits were naïve with respect to the HIPV blend we created.

At the individual level, females did not adjust their timing of laying or clutch sizes when exposed to HIPVs. The influence of some cues on seasonal timing may be subtle and requires large sample sizes and long-term experiments to be detected at the individual level (Schaper et al., 2011; Visser et al., 2002). The manipulation of supplementary cues in controlled conditions has almost never triggered an advancement in laying date whereas clear correlations are highlighted in the wild. Temperature is a good example. While in the field, correlative studies have repeatedly shown a link between temperature and onset of reproduction (Charmantier et al., 2008; Dunn, 2004; Visser et al., 2003), it required nearly ten years of complex experiments

in controlled conditions to show a direct effect on seasonal timing (Schaper et al., 2012; Visser et al., 2009).

Our artificial blend was designed to mimic the HIPVs emitted early in spring by oak buds infested with caterpillars. We cannot exclude the possibility that our odour bouquet does not accurately reflect the real HIPVs emitted by oak trees. Indeed, we focused on selecting the compounds emitted in greater concentrations by caterpillar-infested trees compared to non-infested trees (Graham et al., in prep), and we used two methods to diffuse odours to optimise the accuracy of the signal. However, the relative proportions of the different compounds can be an important clue to the identification of HIPVs for birds (Webster et al. 2010; Koski et al. 2015). If the ratios of the different compounds in the blend have to be controlled at a very fine scale to be relevant for birds, our bouquet may have misled them in their interpretation of the odours. Future studies should investigate the importance of HIPVs nature for the recognition and use for reproduction of this olfactory cue by birds.

Although we did not find an effect of our HIPVs blend on individual reproductive investment or timing of insectivorous birds, our results suggest that they could use HIPVs in other contexts than foraging, specifically as a cue for territory selection for reproduction, as observed by the slightly higher number of nest-boxes equipped with HIPVs occupied compared to control nest-boxes. We used artificial HIPVs inspired by oak bud emissions in response to green oak leaf-roller and winter moth caterpillars that have been shown to act as a central selection factor on blue and great tit reproduction (Blondel et al. 1999; Thomas et al. 2001). Oak tree is the main tree species found in our studied populations and it is the main tree species on which the caterpillars feed. We thus think that oak trees are the primary key species for birds to use HIPVs. A recent study found that blue tit females have larger ovarian follicle sizes when exposed to HIPV odours emitted by caterpillar infested oak trees (Caro et al. 2023). and males spend more time near the infested trees (Graham et al. 2021). However, it is also possible that other HIPV blends from other dominant tree species in other population could modulate reproductive behaviour of birds. For example, HIPVs of apple trees (*Malus silvestris*) seemed to attract insectivorous birds (Amo et al. 2013), while HIPVs of mountain birches (*Betula pubescens* and *Betula pendula*) seemed to have no such effect (Koski et al. 2015) and do not appear to modulate reproductive timing (Visser et al. 2002; Schaper et al. 2011). Many studies investigated the effect of two general HIPVs found in any plant species (methyl salicylate and methyl jasmonate) on bird foraging behaviour (Mäntylä et al. 2014; Mrazova and Sam 2017; Mrazova et al. 2019) but their effect on reproductive decisions is unknown. Future studies

should investigate the ability of different HIPVs composition to modulate bird breeding phenology.

We cannot exclude the possibility that birds could also rely on caterpillar odours themselves to modulate reproductive behaviour. Songbirds have been shown to use sex pheromones of winter moths (*Operophtera brumata*) as a foraging cue (Saavedra and Amo 2018; Amo and Saavedra 2021) but when oaks are still in buds and freshly-hatched caterpillars start to infest the buds, caterpillars are very small (~1 mm). Consequently, they do not represent nutritious food for tits at this time and they do not seem to be used as a visual cue (Schaper et al. 2011). We ran preliminary odour emissions measurement from caterpillars previously to our experiment and did not find biologically relevant concentration of odour emitted by caterpillars that birds might use as a cue to infer caterpillar's phenology.

In conclusion, our results suggest that small birds can detect and use minute amounts of HIPVs released by primary producers as an odour cue to infer relevant information about future availability of primary consumers, which, in the case of blue and great tits, constitute their main selection factor (Dias and Blondel 1996; Blondel et al. 1999; Gienapp and Visser 2006). This is a stunning finding because Passeriformes possess the smallest relative olfactory bulb size in bird orders (Bang and Cobb 1968). Blue and great tits are furthermore generalist species so if HIPVs has an effect on those species, it is possible that birds more specialized on a type of food rely on HIPVs for their reproductive decisions, which provides many opportunities to study the use of olfactory cue for reproductive behaviour in the bird kingdom.

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Conflict of Interest

The authors have no conflict of interest to declare.

Data Accessibility Statement:

If accepted, data and code will be uploaded to Dryad

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General discussion



Reminder of the context

While warm weather and longer days in early spring evoke summer images, many seasonal animals are racing against time to raise a new generation. Deciding when to reproduce is a key factor in an individual's ability to raise offspring; it therefore impacts its reproductive success. The optimal timing to rear offspring is generally concomitant with a period of high food availability. Organisms thus need to use multiple sources of information present in their environment to predict the timing of the food peak and organise the different stages of their reproductive cycle²⁶. In the case of birds, they have to find a mate, develop their reproductive system, find a breeding territory, and build a nest even before they actually start laying and incubating the eggs²². Social interactions with the sexual partner are one of the first factors to modulate the reproductive timing and investment of birds³⁷³. Mate choice and the amount of time the partners spend together affect the capacity of the pair to coordinate their behaviours and improve their reproductive success^{66,67}. However, mate choice is the result of a complex series of events mixing mate preferences, competition with other females, or male availability³⁰⁰⁻³⁰³. Mate preference is therefore an important part of the mate choice process and can influence individual investment in reproduction⁶⁸⁻⁷⁰. Being mated to a non-preferred partner may lead to reduced motivation to reproduce, or cause physiological stress that alters reproductive investment^{70,89-92}.

Although breeding pairs must coordinate with each other, they also have to coordinate with their environment. In this prospect, organisms rely on environmental cues to try to predict the environmental conditions that will prevail at the time they will raise their offspring. Photoperiod is the main predictive cue that triggers the physiological mechanisms preparing birds for reproduction⁹⁸. However, the seasonal changes in day length are invariable across years. Photoperiod consequently does not allow individuals to predict inter-annual variations in the optimum reproductive period, and organisms need to rely on supplementary cues to regulate their reproductive timing²². The insects that many species of insectivorous birds use to feed their offspring are selected to emerge synchronously with tree bud burst^{174,185}. Vegetation phenology has therefore been suggested to be a cue that insectivorous birds could use to predict the phenology of their food source and, by extension, to adapt their reproductive timing^{186,188}. One particular cue that could be associated with the development of vegetation is the emission of Herbivore-Induced Plant Volatiles (HIPVs), which are odorous compounds emitted by plants when grazed by herbivorous insects^{374,375}. Birds are known to use these odours in a foraging context^{229,268}. Since HIPV emissions reflect the timing of emergence and the future abundance of insects in the environment^{211-213,322}, birds could use this olfactory cue in early spring to

predict the period and the amount of food that will be available later in spring, and consequently adjust their onset of breeding and their clutch size to the predicted food biomass.

This thesis aimed to examine how social and environmental cues influence the reproductive decisions of two insectivorous bird species: the blue tit and the great tit. More specifically, I investigated the role of female mate preference as well as that of vegetation development and HIPVs in bird reproductive timing and investment.

Main results of this thesis

In **Chapter 1**, I investigated whether the degree of female preference for her mate influenced her timing of reproduction and her reproductive investment. I tested female preferences, and then paired females with either their preferred or their avoided male. While great tit females confirmed my initial hypothesis by advancing their reproductive timing when they were paired with a male they preferred (**article 2**), blue tits did not seem to adapt their reproduction to the male with which they were paired (**article 1**). In addition, I found that female great tits selected mates based on their exploratory behaviour, whereas female blue tits selected mates based on their plumage colour (**figure 14**).

In **Chapter 2**, I used a descriptive approach to investigate the correlation between tree budburst and tit reproductive timing and investment, in two Mediterranean populations of blue and great tits. Using high-resolution satellite imagery, I quantified the Normalized Difference Vegetation Index (NDVI), a proxy for the development of leaves on trees, and I estimated the date of leaf emergence (“date of vegetation green-up”) around each nest-box. I then estimated whether bird laying dates and vegetation green-up dates were correlated at the level of each breeding pair, and whether pairs more synchronised with green-up had a better reproductive success. Contrary to my initial hypothesis, I found no clear evidence that the dynamics of vegetation development on each bird territory influenced the laying dates of the territory owners, and only moderate evidence that the synchrony with vegetation green-up influenced bird fitness. However, I did find some support for a relationship between bird reproductive timing and vegetation phenology, but that was only when using more classical, lower, spatial and temporal resolutions (**article 3**) (**figure 14**).

In **Chapter 3**, I used experimental approaches to explore the effect of HIPVs emitted by caterpillar-infested trees on the reproductive physiology of captive birds. In a first experiment (**article 4**), we kept pairs of blue tits in airtight compartments in which the air came

from enclosures containing oak trees infested with caterpillars. In a second experiment (**article 5**), pairs of great tits were housed in climate-controlled aviaries, together with caterpillar-infested trees, hidden behind an opaque screen. In both experiments, birds could not see the trees, they could only smell the HIPVs. We measured the gonadal growth of blue tits, and the sexual hormone levels of great tits, over the course of the breeding season. Blue and great tits did not lay earlier, or more eggs when exposed to HIPVs, which invalidated my hypothesis. Although I found no effect of the odour treatment on female great tit reproductive hormones (**article 5**), more exploratory female blue tits developed larger ovarian follicles when exposed to the odour of caterpillar-infested trees (**article 4**). In contrast, male blue tits showed no differences in gonadal development between the control and treatment groups (**article 4**), but male great tits exposed to HIPVs presented higher testosterone levels during the egg-laying period (**article 5**) (**figure 14**).

In **Chapter 4**, I performed a four-year field experiment where I examined the effect of an artificial blend of HIPVs on the reproductive decisions and the reproductive success of natural populations of birds. Based on previous experiments conducted in our laboratory, in which the authors characterised the volatile compounds emitted by caterpillar-infested trees (Graham et al., in prep.), we created an artificial bouquet of HIPVs mimicking those emissions. Using a Y-shaped aviary set-up, I found that naïve blue tits, i.e. hand-reared blue tits that had never foraged on trees and had never been exposed to bud HIPVs, could detect and orient to the artificial odour bouquet (**article 6**). I then placed those same artificial HIPVs around the nest-boxes of great and blue tits in two Mediterranean populations monitored for their reproduction. Similar to the results I found with captive birds in **Chapter 3**, birds in the field did not advance their laying dates, nor increase their clutch size in the presence of artificial HIPVs. However, more nest-boxes equipped with HIPVs were occupied compared to control ones, resulting in more eggs, nestlings, and fledglings introduced in the population from these treated nest-boxes (**article 6**) (**figure 14**).

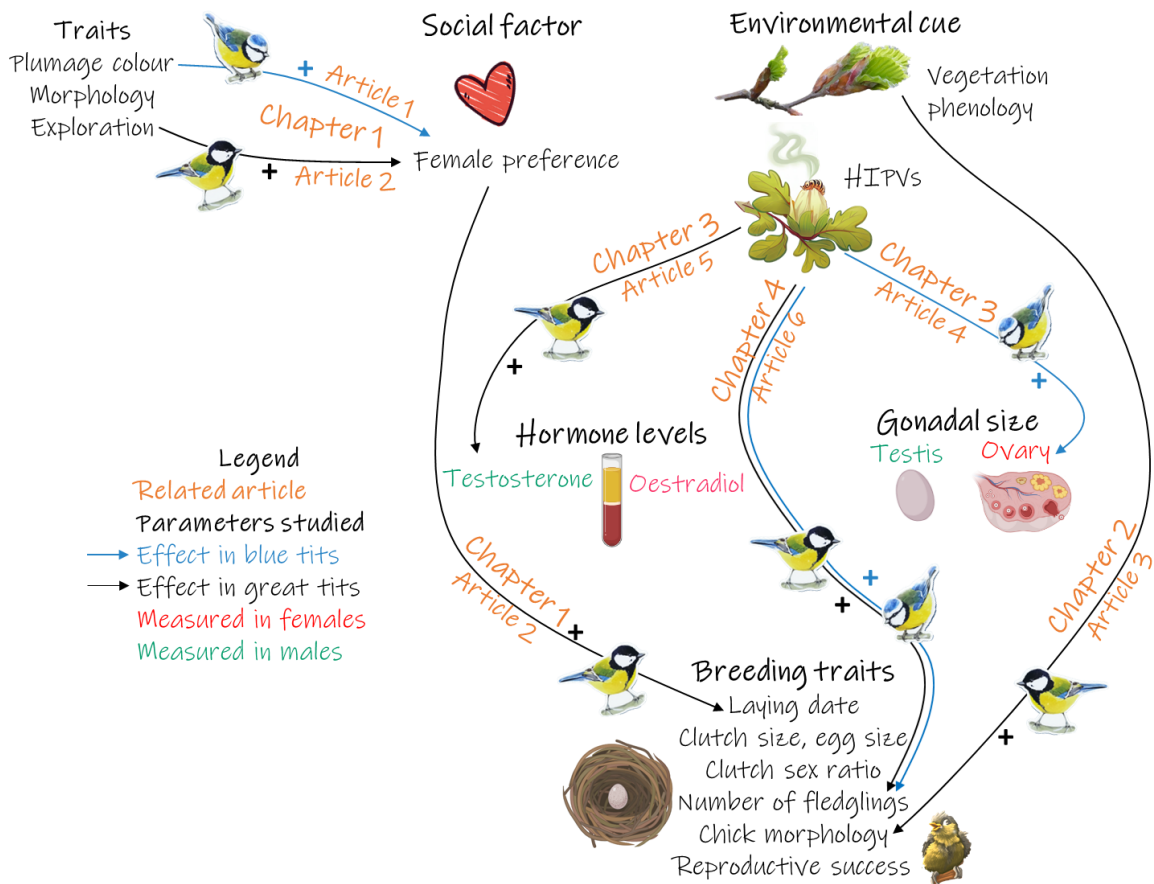


Figure 14: Diagram summarising the main results of this thesis.

Female blue tits preferred males based on their plumage colouration, while female great tits showed a higher preference for more exploratory males. Female great tits paired with their preferred male advanced their laying dates. Vegetation phenology advanced bird laying dates at the population level and synchronisation of bird laying date and vegetation phenology showed moderate effect on bird reproductive parameters. HIPVs increased testosterone levels of male great tits around the laying period, increased follicle size in fast-exploring female blue tits, and increased the number of chicks produced in the field.

The role of mate preferences, vegetation phenology and HIPVs in avian reproduction

In **Chapter 2**, using a vegetation index derived from high-resolution satellite images, I found no evidence for a synchronisation of individual bird laying dates with vegetation phenology, nor any clear evidence of an effect of vegetation phenology on bird reproductive performances. However, when using more classical method, at lower spatial and temporal resolutions, I found a correlation between the mean annual laying date of the bird population and the mean vegetation green-up date of the territory (**article 3**), which is in agreement with previous studies suggesting a link between vegetation bud burst dates and bird laying dates at the population level ^{186,187,195} but see ¹⁹⁷. Similarly, birds in captivity exposed to real HIPVs

emitted by caterpillar-infested trees (**Chapter 3**), or in the field exposed to artificial HIPVs (**Chapter 4**), did not advance their laying dates, nor increase their clutch size (**articles 4-5-6**). However, in **Chapter 4**, when examining the bird population in the field as a whole, rather than focusing on individual cases, more eggs, nestlings, and fledglings were introduced in the population from the treated nest-boxes than from the control areas (**article 6**). Thus, these chapters suggest that vegetation phenology may exert some influence on avian reproduction, but that this effect remains statistically imperceptible in terms of individual laying date or clutch size. Detecting an effect of vegetation development on bird reproductive timing or investment probably requires longer-term studies. It might be the accumulation of minute, statistically undetected, effects at the individual level that produces a noteworthy impact at the population level. Along those lines, the effect of temperatures on avian timing of breeding required long-term experiments with quite large sample sizes to demonstrate an effect at the individual level. While several correlative studies conducted in the field have repeatedly suggested a link between temperature and seasonal timing of reproduction in birds ^{134,376,377}, it took almost ten years of careful experiments in controlled conditions to prove the direct effect of temperatures on timing of reproduction in great tits ^{143,144,149}. And even then, the effect of temperature remained much smaller than what had been predicted by correlative field data ¹⁴⁷. The same may be happening here, with vegetation development. Similarly to the experiments with temperature, I might have had to repeat the same experiment for more than five consecutive years, using a large set of climate-controlled aviaries, to be able to show the effect of HIPVs on individual bird seasonal timing. Like temperature, vegetation signals are categorised among the well-named "supplementary cues" ²⁷, meaning that their effects on animals' seasonal timing are subtle and complementary to other cues, among which stands the powerful and initial effect of photoperiod.

Social factors are nothing else than supplementary cues, as well. If I found an effect of female partner preference on reproductive decisions in great tits (**article 2**), but not in blue tits (**article 1**), the reason could be that female blue tits are indifferent to their male partners, an explanation we put forward in our article and which has been supported by earlier studies on the same species ¹¹⁸. However, it could also be that in this species, social factors interact with other environmental factors, such as food availability for example, and that the interactions only become apparent when these other factors become limiting factors, leading to some sort of cocktail effect. In zebra finches for example, it has been shown that long day lengths only stimulated gonadal growth if birds were fed *ad libitum*, not if they were food-restricted ³⁷⁸. Food

may therefore stimulate gonadal growth more than photoperiod in this species, but these results may also indicate that a long photoperiod is required for birds to respond to the food signal. Such interactions between environmental cues are probably widespread in nature. For example, the development of vegetation and insects depends on temperature ^{144,174}, which has been extensively studied in birds, but mostly independently of other cues. Interactions with heterospecifics (predation, competition) or conspecifics (social information, competition, courtship), photoperiod, rainfall, temperature, food availability, nest-box location, and vegetation are all cues that could influence bird reproduction in a non-independent way ²⁹, and it may well be that one such cue was missing in my experiment manipulating social factors in blue tits. However, investigating them under controlled conditions is challenging due to the complex experimental designs and large sample sizes required.

In the articles included in this thesis, I did not directly consider the interplay that might exist between female preference and HIPVs on bird reproduction, the two environmental factors I studied. I have therefore carried out additional preliminary analyses here, to explore whether the social and environmental cues might have interacting effects on bird reproduction. I tested the potential interactive effects of the exposure to HIPVs and the social relationships within a pair on gonadal size. To recall the experiment in which we measured gonadal size (**Chapter 3, article 4**), blue tits were placed in opposite-sex pairs in terrariums and exposed to the odours of caterpillar-infested trees (HIPVs). In this experiment, pairs were formed at random. Here, I tested whether exposure to HIPVs could favour the physiological synchronisation of the pair, which I estimated by the correlation between the female and the male gonadal size. I studied the correlation between the volumes of the testes and the volumes of the ovarian follicles as a function of exposure to HIPVs, at each sampling point (one measure before the exposure to HIPVs, and two during exposure) (details of the statistical models in appendices). I found no effect of the odour treatment on the correlation between male and female gonadal sizes (10th March: $p = 0.71$, 10th April: $p = 0.24$, 10th May: $p = 0.91$). More surprisingly, I also found no overall correlation between male and female gonadal volumes, thus independently of the odour treatment (10th March: $p = 0.99$, 10th April: $p = 0.16$, 10th May: $p = 0.47$) (**figure 15**). Thus, I found no interacting effect between the synchronisation of the gonadal development between pair members and the exposure to olfactory compounds released by caterpillar-infested trees. However, we did not consider female preferences for males when forming the pairs in this experiment, which may explain the overall lack of synchronisation of partners, even

independently of the odour treatment. If we had taken female preferences into account, there might have had wider possibilities of interactions between social factors and HIPVs.

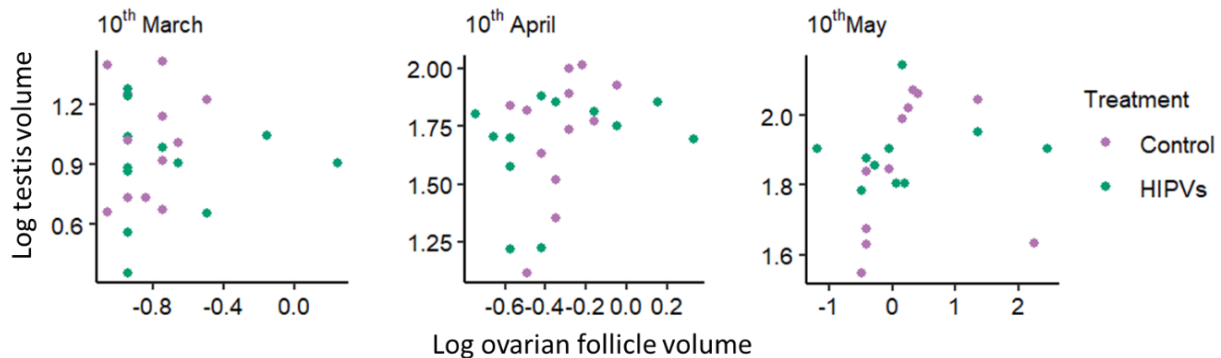


Figure 15: Correlation between male and female gonadal size as a function of exposure to Herbivore-Induced Plant Volatiles.

These data refer to the gonadal volumes measured in **Chapter 3, article 4**. Birds were exposed to the smell of caterpillar-infested trees. At all time points (before exposure to HIPVs (10th March) and during exposure (10th April and 10th May)), male and female gonadal sizes (log transformed) were not significantly correlated, even independently of the odour treatment. Dots depict individual data.

In **Chapter 3, article 5**, in which great tit pairs were housed in climate-controlled aviaries and either exposed to the odours of caterpillar-infested trees, or to a control, females varied in their degree of preference for the male with which they were paired (preference test realised at **Chapter 1, article 2**). This configuration enables me to investigate the interacting effect of the exposure to HIPVs and the female degree of preference for her male on her reproduction. Although I found no overall effect of the odour treatment on laying dates or hormone levels (**Chapter 3, article 5**), I found that females that expressed a stronger preference for their partner laid earlier (**Chapter 1, article 2**). Here, I wanted to investigate whether the interplay between the exposure to HIPVs and the preference for the partner could have an effect on female oestradiol levels, clutch sizes, and laying dates. I expected that females that were both exposed to HIPVs and paired with their preferred male would have higher levels of oestradiol, lay earlier and more eggs, compared to females not exposed to HIPVs and paired with a less-preferred male. I however found no significant interaction between the female preference and the odour treatment on any of female mean oestradiol levels ($p = 0.10$, **figure 16A**), laying dates ($p = 0.77$, **figure 16B**) or clutch sizes ($p = 0.89$, **figure 16C**) (details of the statistical models in appendices). These analyses remain preliminary and need to be further developed, but contrary to my hypothesis above, it seems that social and vegetation factors do not interact to shape bird reproductive physiology and behaviours.

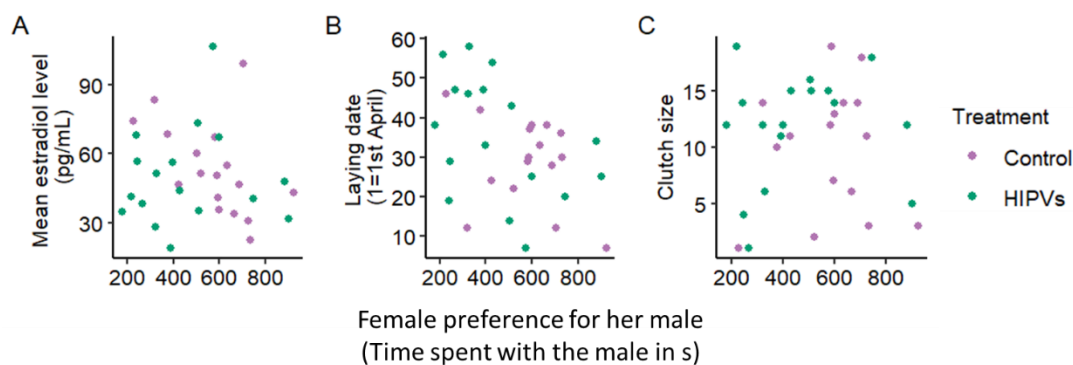


Figure 16: Interactive effect of female preference for a mate and exposure to HIPVs on female reproductive parameters.

Females showing stronger preference for their mate did not (A) present higher mean levels of oestradiol ($p = 0.10$), (B) lay earlier ($p = 0.77$) or (C) larger clutches ($p = 0.89$) when exposed to HIPVs. Dots depict individual data.

The effects of environmental factors like social stimulations and vegetation development may not be restricted to easily observable traits like laying dates and clutch sizes. In fact those behavioural decisions necessarily come about via cascades of neuroendocrine mechanisms^{22,379}. We could thus expect that any effect of the environmental cues on blue and great tit behaviours should find its origin in some underlying physiological control mechanisms. If this should theoretically always be the case, in practice, many studies have failed to show mirroring effects of environmental factors at both the phenotypic and the physiological levels, especially when dealing with subtle supplementary cues. Several studies in great tits for example, have shown that effects of temperatures on reproductive timing and performance were not visible at the endocrine level, and vice versa^{147,380}. This happened also in my studies, and it further highlights the fact that effects of the environment are not always straightforward to detect overall. For example, in **Chapter 1**, I described a stimulating effect of female preference for their male on their laying dates, but without detecting a concomitant effect of their preference on their plasma oestradiol concentrations (see **figure S5** of **article 2**), even though laying dates and oestradiol concentrations are supposed to be intrinsically linked³⁸¹. In **Chapter 3**, I showed that males exposed to HIPVs had higher testosterone levels during the egg laying period, but I failed to show an effect of HIPVs on their females' laying dates (**article 5**).

Regarding testosterone, it is not the first time that a link between HIPVs and the main sexual steroid hormone in males is shown. Recently, Graham and colleagues indeed found that males with higher testosterone levels spent more time around caterpillar-infested trees releasing HIPVs in a Y-shaped aviary²⁶⁹. In this earlier study however, the authors did not know whether the increase in male testosterone followed exposure to HIPVs (i.e. HIPVs trigger an increase in

testosterone), or whether males with already high testosterone levels spent more time near HIPVs. Olfactory sensitivity in rats appears to be upregulated by local sex steroid production in the olfactory epithelium^{382,383}, suggesting that elevated testosterone concentrations during the reproductive season could promote HIPV detection. In **article 5**, I however found that males had higher testosterone concentrations in the experimental group exposed to HIPVs, which suggests that it is the exposure to HIPVs that triggers the increase in testosterone, and not testosterone promoting olfactory sensitivity. Since HIPVs are an indicator of the presence of food in the environment, and therefore of environment quality, the increase in testosterone could enable males to defend their territories, or their female, against other males. Further research is needed to understand the complex interplays that may exist in male birds between the detection of HIPVs, olfactory sensitivity, testosterone levels, and their potential effects on male foraging and sexual behaviours.

Contrary to males that already exhibit robust gonadal response to the change in day length³⁸⁴, female reproduction results from a complex association of several environmental cues^{27,385}. This could explain why it is difficult to show an effect of each cue tested separately. Mechanisms underlying the final stages of follicle growth, determining the key life-history traits of clutch size, egg size and potentially laying date²², last only three to four days in small passerines. Since I could only take blood samples every two weeks to measure hormonal levels, or measure gonadal size three times over the whole breeding season, it is consequently likely that I missed the short crucial period in which female endocrine system responds differently in the HIPV than in the control group. Having found effects, even small and only in some individuals, at the level of the gonads for HIPVs (**Chapter 3, article 4**) and on laying dates for partner preference (**Chapter 1, article 2**) suggests that these factors have subtle influence on female reproduction, although we do not yet precisely understand the underlying mechanisms. It is also possible that we were not tracking the right hormone, or the right time in the female reproductive cycle to detect the effect of these two cues on female reproductive decisions. For example, a potential effect could be observed on prolactin (PRL) levels, which regulate many aspects of the reproductive events³⁸⁶, such as clutch size^{387,388} or incubation^{22,389}, and that have been suggested to be modulated by environmental cues such as temperature³⁹⁰⁻³⁹².

The regulatory mechanisms used to transmit information from environmental cues to the reproductive system could somewhat differ between the sexes. Nakazawa and colleagues³⁸⁵ have recently proposed a “sexual match/mismatch hypothesis”, which claims that the reproductive phenology of individuals may respond to environmental cues in a sex-specific

manner. If supplementary cues are integrated differently between the sexes and modify reproductive phenology depending on sex, the use of these cues could disrupt the synchronisation of the reproductive cycle of male and female and, consequently, complicate the ability of individuals to mate. This mismatch of cue processing between sexes could ultimately affect the timing of emergence and the success of juvenile recruitment through a lack of synchrony with the food source dynamics. Although it is quite likely that I did not detect all the potential effects of HIPVs on the physiology of males and females, the results I obtained in **Chapter 3** suggest that HIPVs may be integrated in different ways between males and females (at the gonadal level in some females (**article 4**) and at the hormonal level in males (**article 5**)). While further investigations are necessary to fully unravel the intricacies of the effect of environmental factors on bird reproductive physiology, the first evidence from my thesis suggests that these cues might have different impact between the two sexes.

This thesis highlights that we still have limited knowledge about the physiological mechanisms involved in the use of environmental and social cues to regulate reproduction. The best evidence to support the impact of environmental and social signals on reproduction is to detect the specific mechanisms that females use to identify and combine these signals with the HPG axis to regulate the final stages of egg-laying. I managed to identify some of these processes in my thesis, even if many grey areas persist. Some limitations could partly explain the subtle and variable effects found. In **Chapters 3** and **4** in particular, birds were exposed to HIPVs released from only two caterpillar-infested trees (**Chapter 3**) or artificial compounds (**Chapter 4**). These conditions may have not recreated a reliable cue for birds. It has been suggested that birds could be sensitive to the ratio of different compounds emitted by trees ²⁶³. HIPVs being complex assemblies of several chemical compounds, the concentration of each compound could be detected by birds and thus influence their reproductive behaviours. As the compositions of HIPVs vary depending on the plant species ²⁰⁵, the level of infestation that the plant undergoes ^{211–213}, and sometimes even the herbivore species attacking them ^{214,215}, birds could detect the differences in HIPV compositions, analyse the relevance of the signal, and adjust their reproduction according to the information carried by the olfactory signal. Furthermore, in **Chapter 4**, the addition of HIPVs in the field might have been done at an inappropriate time. The artificial odours were added prior to the birds' breeding period and in large quantities. The aim was to make the birds think that caterpillars would be present earlier and in greater abundance in the environment, and thus to induce them to lay earlier and more eggs. I expected that adding HIPVs on birds' territories prior to bud burst and caterpillar

hatching would mislead the birds, causing them to advance their laying dates and increase their reproductive efforts (i.e. more eggs). However, we were unable to advance the birds' egg-laying date. Experimentally advancing the dates on which birds lay their eggs seems to be a complex task, as very few studies using natural photoperiodic profiles managed to induce earlier laying than what females normally do^{143,144,393,394}. This highlights the complexity of the female's decision-making process concerning her reproductive timing. A reason why we did not succeed to advance the bird's laying date could be that we may have added the artificial odours too early in the breeding territory compared to when the birds make the decision to reproduce. Consequently, by the time the birds chose to breed, the bottles of artificial HIPVs would no longer have emitted enough compounds, or they may have undergone chemical modifications over time, making the signal unusable for birds. However, in **Chapter 3**, tree budding and caterpillar emergence were synchronised before exposing the birds to the tree odours. Thus, the odours were not included too early compared to caterpillar emergence in the bird environment, but we still found no effect of the treatment on reproductive timing and investment. Therefore, the protocols used do not appear to be a potential source of bias that could have prevented us to detect an effect of volatile compounds on bird reproduction, but it rather seems that the effects of HIPVs on bird reproduction are too fine to be detected using our methods.

From an evolutionary point of view, several points emerge from my thesis which support the idea that it could be adaptive for birds to use plant development as a cue for reproduction. Although **Chapter 2** first appears to contradict this idea, the case studied might represent a highly specific scenario. In this chapter, I failed to highlight any evidence indicating that the synchronisation of breeding with vegetation development could be beneficial for birds. However, our study area, the Rouvière forest, appears to be relatively deprived of caterpillars. In comparison, blue tit pairs that breed in deciduous downy oak forests (*Quercus pubescens*) in Corsica rear their young on a caterpillar peak that is nearly 25 times higher than that available to pairs breeding in the Rouvière forest¹⁷³. During the satellite image collection campaigns conducted to realise the study of **Chapter 2**, satellite images in areas of Corsica with high densities of caterpillars were also collected, specifically in the Muro region situated in the northwest of Corsica. Due to time constraints, I was not able to analyse the data from these images, but it would be interesting to investigate whether the birds in these caterpillar-rich regions have a greater synchronisation of their reproduction with the surrounding plant development compared to La Rouvière forest. Birds living in caterpillar-poor forests, such as La Rouvière, could diversify their diet to fulfil the needs of their young³⁹⁵⁻³⁹⁸ and consequently

would not need to rely on vegetation development for reproduction, while birds in caterpillar-rich regions, as the Muro region, could completely depend on caterpillar to raise their young and so completely rely on vegetation development to synchronise the offspring rearing with the caterpillar abundance peak. On the one hand, selection for synchronising breeding with the caterpillar phenology, and so vegetation development, might be relaxed for birds rearing their offspring with various other insect species. On the other hand, the innate recognition of HIPVs that I established in **Chapter 4**, contrary to previously found^{270,271,278}, could enable populations dependent on caterpillars to raise their chicks, such as the population in Muro, to anticipate and predict caterpillar phenology from their first breeding attempt. Finally, as mentioned above, in **Chapter 4**, adding artificial HIPVs on birds' territories prior to bud burst and caterpillar hatching was supposed to mislead the birds, causing them to advance their laying date and increase their reproductive investment. As in reality, neither the phenology of the caterpillars was advanced nor their abundance increased, we expected that birds would not have been able to raise all their chicks. However, I found that more fledglings were introduced in the population from nest-boxes surrounded by HIPVs (**article 6**). These results suggest that the perception of HIPVs stimulated parents to increase their foraging effort during the chick-rearing phase. Birds could consequently adapt their reproductive investment to the amount of food they predict from an olfactory cue emitted by growing buds in early spring.

Perspectives

Body odours and MHC alleles in blue and great tit mate preferences

In **Chapter 1**, I investigated whether the degree of female preference for her mate influenced her reproductive investment. Although I found a preference of female great tits for more exploratory males (**article 2**) and that female blue tits relied on blue and white plumage characteristics of the male to designate their preferred one (**article 1**), these effects were subtle when considering the effect sizes of these parameters (effect sizes of : the interaction blue UV chroma male and female in **article 1**: $\eta^2 = 0.04$; of blue brightness male in **article 1**: $\eta^2 = 0.06$; of exploration score in **article 2**: $\eta^2 = 0.02$) and most of the traits studied were found to be non-significant in our statistical models. Therefore, the criteria on which females express a preference and choose their mate remain relatively obscure. In my experiments, I focused on the male physical appearance (colour and morphology) and behaviour (exploration) in order to explain the female preference. However, at the beginning of my thesis project, I had initially intended to explore whether the preference could be influenced by the olfactory signature of the males. The idea was to investigate the possibility that birds could select a mate based on the

Major Histocompatibility Complex (MHC) of individuals. The MHC is a large locus on vertebrate DNA containing a set of genes coding for cell surface proteins essential for the immune system³⁹⁹. Due to its association with microbial diversity⁴⁰⁰, MHC is also implicated in body odour²⁴⁴. Female of some bird species have been shown to select a mate based on the characteristics of his MHC^{250,296,401,402}. Consequently, I wondered whether tits could also use body odours to select their partner and to what extent these odours were linked to the MHC characteristics of individuals. Studying the body odours of birds could have created an interesting link between the use of olfaction both in mate choice and in the use of environmental information from HIPVs. Unfortunately, these investigations could not be conducted due to time constraints, but they could constitute a future research project. Moreover, if mate choice is indeed based on MHC and/or body odours, the reproductive performance of pairs formed according to the compatibility of their MHC/odours could be investigated. Females may invest more in reproduction when mated with a male of a specific MHC or body odour type. This research project could offer new insights into the mechanisms of mate choice and the potential interplay between reproductive performance and MHC/odour profiles of bird breeding pairs.

Investigating the role of bud ingestion in bird reproduction

Animals combine information from multiple cues of different types to interpret their environment. For example, birds use photoperiod, temperature, rainfall and/or social factors as cues for reproduction. Among these cues, a single cue can be perceived and used in different ways. For example, the perception and interpretation of temperature can be based on either its means or fluctuations within a specific period. Finally, a cue can also be interpreted and used differently depending on the sensory channel through which it is perceived. In this thesis, I have studied only one sensory channel through which birds can perceive plant phenology, namely olfaction (**Chapters 3 and 4**). However, they could also perceive vegetation development through a sensory channel other than vision or olfaction. Birds have indeed been shown to ingest buds in spring^{337,403–406} and recent studies also showed that leaf chemical composition is affected by the density of caterpillars infesting young oak trees in early spring⁴⁰⁷. Consequently, birds could use the chemical composition of the buds when ingesting them to gather information on the phenology and abundance of their prey. I am only aware of one example of a mammalian species where researchers have identified a correlation between the chemical composition of the ingested vegetation and the reproductive parameters of the animal. This species is the montane vole (*Microtus montanus*). The molecules present in the plant they consume before the breeding season influence their investment in reproduction^{408,409}. Indeed,

montane voles receiving 6-methoxybenzoxazolinone (6-MBOA), a plant derivative particularly abundant in growing seedlings, produce more and larger litters than individuals in control groups⁴⁰⁹. It would be interesting to study whether chemical compounds in buds or leaves have an effect on the physiology of birds, and whether they use this cue to predict the future caterpillar availability. A project investigating these questions would provide a better understanding of how birds use chemical signals from developing buds to regulate their breeding timing and reproductive investment, thereby providing essential information for the ecology and evolution of avian populations.

Conclusion

Responding to multiple cues is necessary for organisms to adjust their phenotype in complex environments. In this thesis, I combined descriptive and experimental approaches, both under controlled conditions and in the field, to explore the effect of a social factor, the female mate preference, and an environmental cue, the vegetation development and its associated olfactory alarm signals, on the reproductive decisions and the underlying mechanisms of avian seasonal reproduction. These complementary strategies allowed me to highlight an advancement of egg-laying linked to partner preference, an increased reproductive investment and physiological adjustments of sexual hormones and gonadal development following exposure to olfactory cues emitted from tree buds. The different results of this thesis suggest that we are dealing with mechanisms whose effects are relatively subtle, and whose overall functioning is complicated to grasp. However, the complexity of real-world conditions is hardly accurately captured by experiments. As suggested in a previous PhD thesis⁴¹⁰, the decision to initiate reproduction can be conceptualised as a series of traffic lights. Experiments that we conduct on a single cue in isolation from the others may turn the light to green for that particular cue, but not the others, so reproduction cannot begin. The multitude of potentially influencing environmental factors, the complexity of their integration, the multiple interactions occurring between them and the many “unknowns” make reproductive behaviour a difficult mystery to unravel.

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Appendices

Text and image for the regional final of the competition “My thesis in 180 seconds”.



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Maître Corbeau, sur un arbre perché, tenait en son bec un fromage. Maître Renard, par l'odeur alléché, lui tint à peu près ce langage. Plutôt intuitif de se dire que le renard sent le fromage n'est-ce pas ? Mais le corbeau, un oiseau, est-ce qu'il le sent si c'est un munster qui fouette ou un comté fruité son fromage ? Et bien, aussi surprenant que ça puisse paraître, les oiseaux sont bien capables de sentir. C'est principalement chez les charognards, humant le doux parfum des animaux morts, que l'odorat est le plus développé. Quant à la mésange bleue, qui est au cœur de ma thèse, les scientifiques ont découvert qu'elle ne rentrait pas dans son nichoir si elle y flairait l'odeur d'un de ses prédateurs.

Dans ce nichoir justement, la femelle peut pondre au printemps une dizaine d'œufs. Alors c'est bien beau de pondre autant, mais après il faut nourrir tout ce petit monde ! Et pour cela, il faut du flair, car les chenilles, essentielles à la survie des poussins, ne sont présentes qu'à un moment spécifique au printemps, il y a un seul pic, et si les mésanges le loupent, les poussins seront tous cuit-cuit... Et c'est là que la mésange doit avoir du nez, enfin du bec, pour anticiper.

Et oui, lorsque les minuscules chenilles éclosent au début du printemps, elles s'infiltrent à l'intérieur des bourgeons des arbres dont elles se nourrissent. Les bourgeons, étant attaqués de la sorte, émettent un cri d'alerte pour se défendre et attirer les prédateurs des chenilles, mais n'étant pas doté de la parole, ils émettent des odeurs qu'on appelle des composés organiques volatiles, les COVs. Et figurez-vous que les oiseaux sont capables d'utiliser ces COVs pour trouver de la nourriture. Par contre ce que l'on sait moins, et c'est ce que je cherche pendant ma thèse, c'est si les oiseaux sont aussi capables d'utiliser ces COVs pour adapter leur reproduction à la production de chenilles.

J'ai donc ajouté des COVs autour des nidoirs des mésanges un peu plus tôt dans la saison et en plus grandes quantités que ce qu'elles trouvent naturellement. Suite à cela, les mésanges n'ont pas pondu plus tôt comme je m'y attendais, mais elles ont pondu plus d'œufs, ce qui montre que les mésanges pourraient adapter leur nombre d'œufs pondus à la quantité de chenilles qu'elles prédisent grâce aux COVs.

On pourrait donc se servir des incroyables capacités olfactives des oiseaux dans la lutte biologique. Les agriculteurs pourraient attirer plus d'oiseaux dans leurs cultures infestées de chenilles en y ajoutant des COVs et ceux-ci se feraient alors un malin plaisir de s'occuper du problème ! Ces composés olfactifs représenteraient ainsi une solution écologique et économique de protéger les cultures tout en préservant les populations d'oiseaux de plus en plus menacées. Alors finalement, quelle est la morale de cette thèse ? Et bien pour les oiseaux, rien ne sert de courir, il faut sentir à point.

Supplementary material of Article 1 (Chapter 1)

Whoever their partner, female blue tits breed the same.

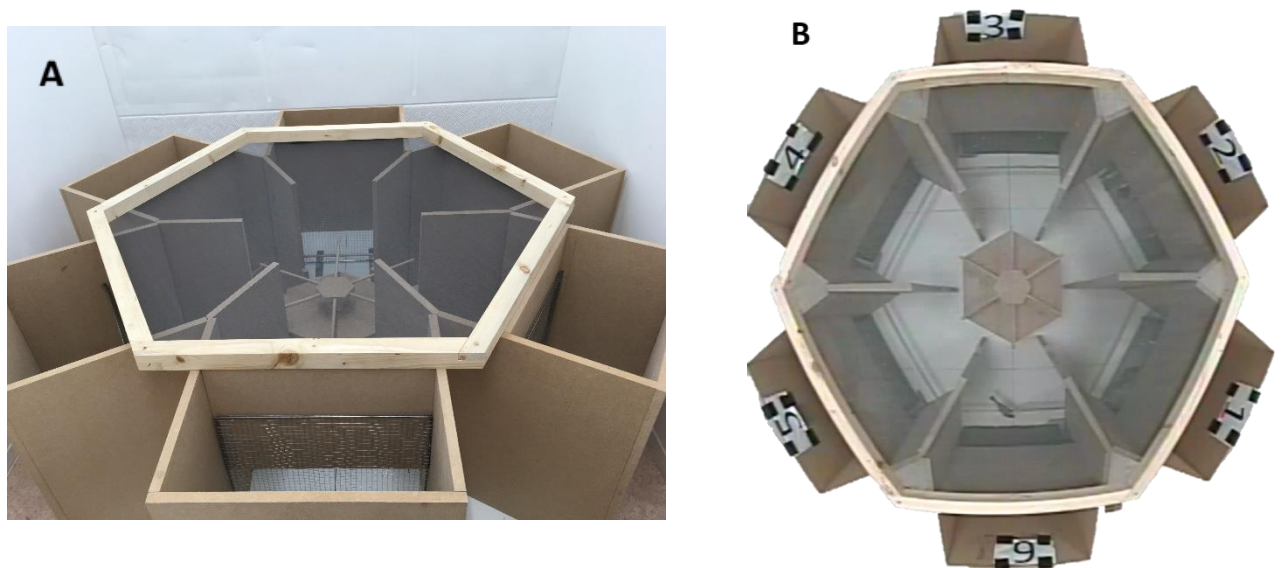


Figure S1: Pictures of the experimental setup for the mate preference test.

A: Picture of the carousel-shaped six-choice chamber. The roof can be moved to introduce the female into the setup.

B: Screenshot of the video of a test, the female is in the choice zone of the male 6. The males are not visible on the video.



Figure S2: Fertile germinal disc (GD) showing an early stage developed embryo (circle).

Embryos that have died at early stages of development cannot be identified by simply candling the egg. This can be done by opening the egg and analysing the germinal disc. For more information on the procedure, see: <https://www.zsl.org/science/bringing-threatened-species-back-from-the-brink-of-extinction/practical-resources-for>.

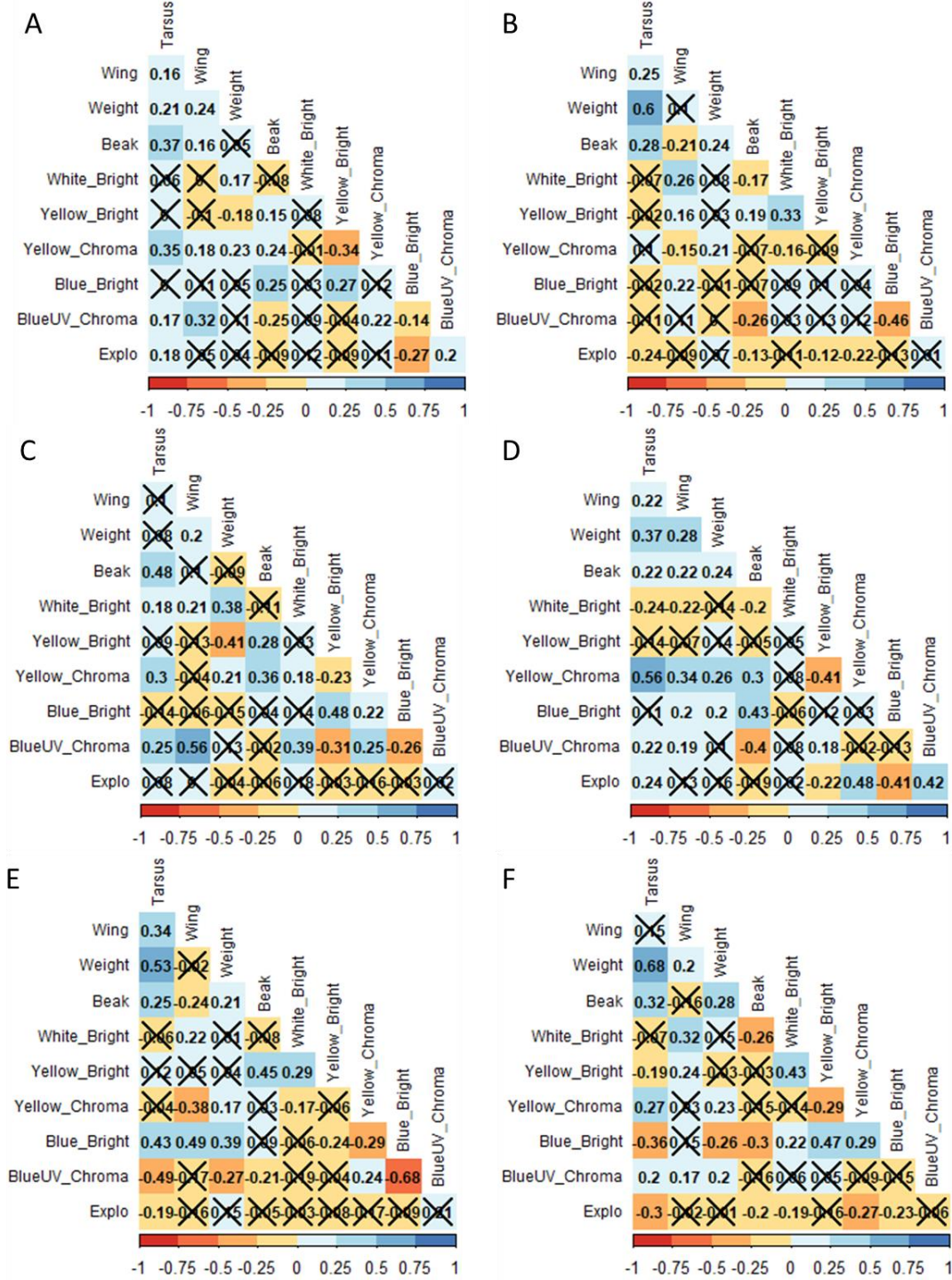


Figure S3: Correlation matrix of the traits of male and females.

A: Correlation of male traits for 2021 and 2022 together

B: Correlation of female traits for 2021 and 2022 together

C: Correlation of male traits for 2021

D: Correlation of male traits for 2022

E: Correlation of female traits for 2021

F: Correlation of female traits for 2022

Number in the coloured square are the Pearson correlation coefficients. Non-significant correlation ($p>0.05$) are crossed out.

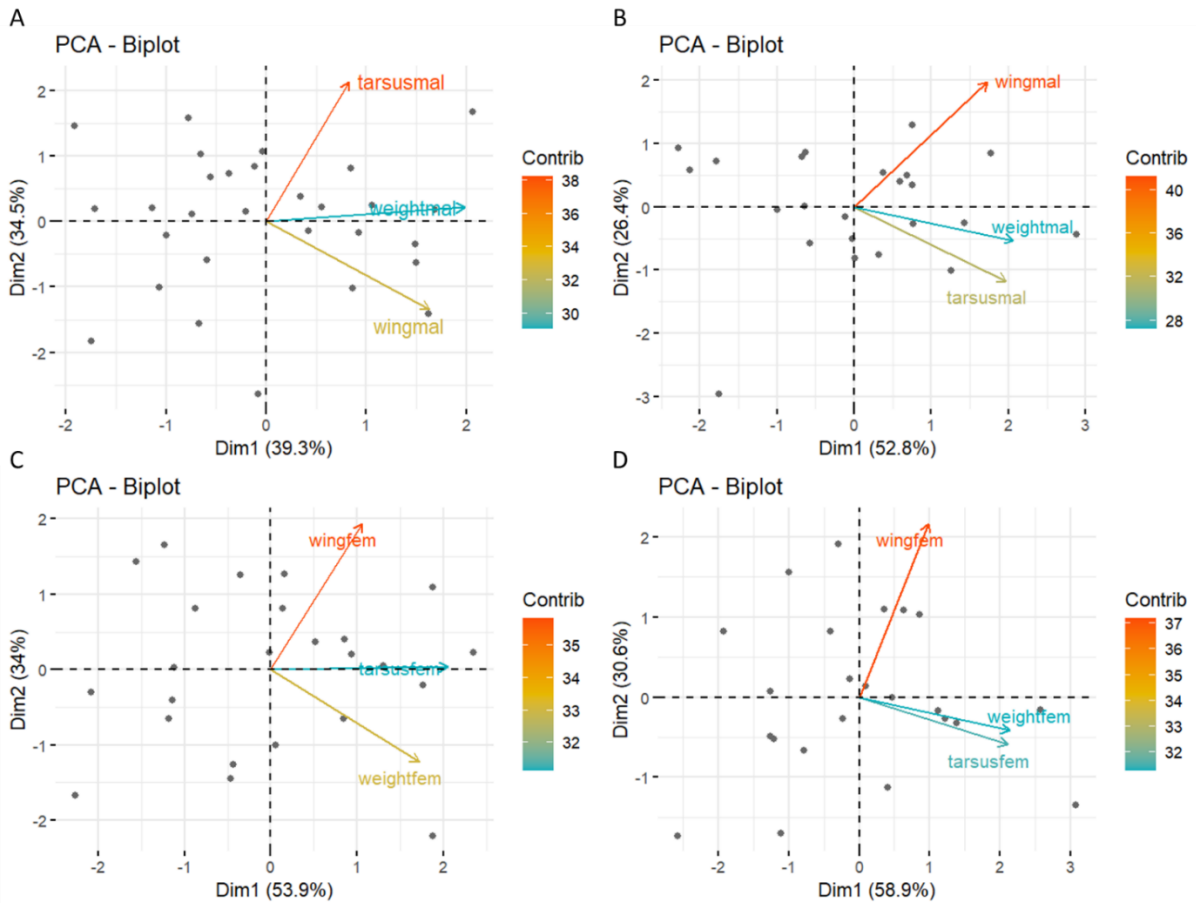


Figure S4: Biplot of the PCA ran for body size.

A: 2021 for males

B: 2022 for males

C: 2021 for females

D: 2022 for females

Each point depicts an individual.



Figure S5: Biplot of the PCA ran for egg size. (n = 320)

High value of the first axis represents bigger eggs.

Supplementary material of Article 2 (Chapter 1)

Female great tits (*Parus major*) reproduce earlier when paired with a male they prefer.

Table S1: Results from linear mixed effect models, exploring the variables that influence the time spent by the female in the choice zone of the males during the 90 minutes of the test ($n = 36$ females).

Variables in bold had a significant effect on the response variable ($p \leq 0.05$), italicized variables depict trends ($p \leq 0.10$). Variables in light grey were removed during the reduction of the model, and are presented in the reverse order in which they were removed.

Response variable	Explanatory variable	Estimate	S.E	df	F value	p-value
<i>Time spent by the female with the males (s)</i>	Intercept	315.68	21.29			
	Exploration score male	3.02	1.53	206.71	3.87	0.050
	<i>Axis 1 PCA Body size male</i>	-15.99	9.26	204.69	2.99	0.085
	Tie size male	-20.29	19.39	205.70	1.09	0.297
	Axis 2 PCA Coloration male	-10.18	11.44	206.08	0.79	0.375
	Axis 1 PCA Coloration male	-4.72	9.35	204.06	0.25	0.614
	Axis 1 PCA Beak size male	-2.59	14.27	195.16	0.03	0.856
	Axis 1 PCA Beak size female	0.24	13.71	34.26	0.01	0.986
	<i>Axis 1 PCA Beak size male*Axis 1 PCA Beak size female</i>	21.16	11.63	176.20	3.31	0.071
	Exploration score female	-1.17	2.19	82.42	0.29	0.593
	Exploration score male*Exploration score female	0.12	0.15	167.76	0.57	0.451
	Axis 1 PCA Coloration female	-2.87	11.04	29.78	0.07	0.796
	Axis 1 PCA Coloration male*Axis 1 PCA Coloration female	2.75	6.04	163.91	0.21	0.649
	Axis 1 PCA Body size female	17.19	16.78	27.35	1.05	0.314
	Axis 1 PCA Body size male*Axis 1 PCA Body size female	2.29	8.39	155.32	0.07	0.786
	Axis 2 PCA Coloration female	-26.51	16.33	31.19	2.63	0.115
	Axis 2 PCA Coloration male*Axis 2 PCA Coloration female	2.58	7.59	150.55	0.12	0.735
	Tie size female	12.12	167.95	163.49	0.01	0.943
	Tie size male*Tie size female	-7.96	39.07	155.96	0.04	0.839

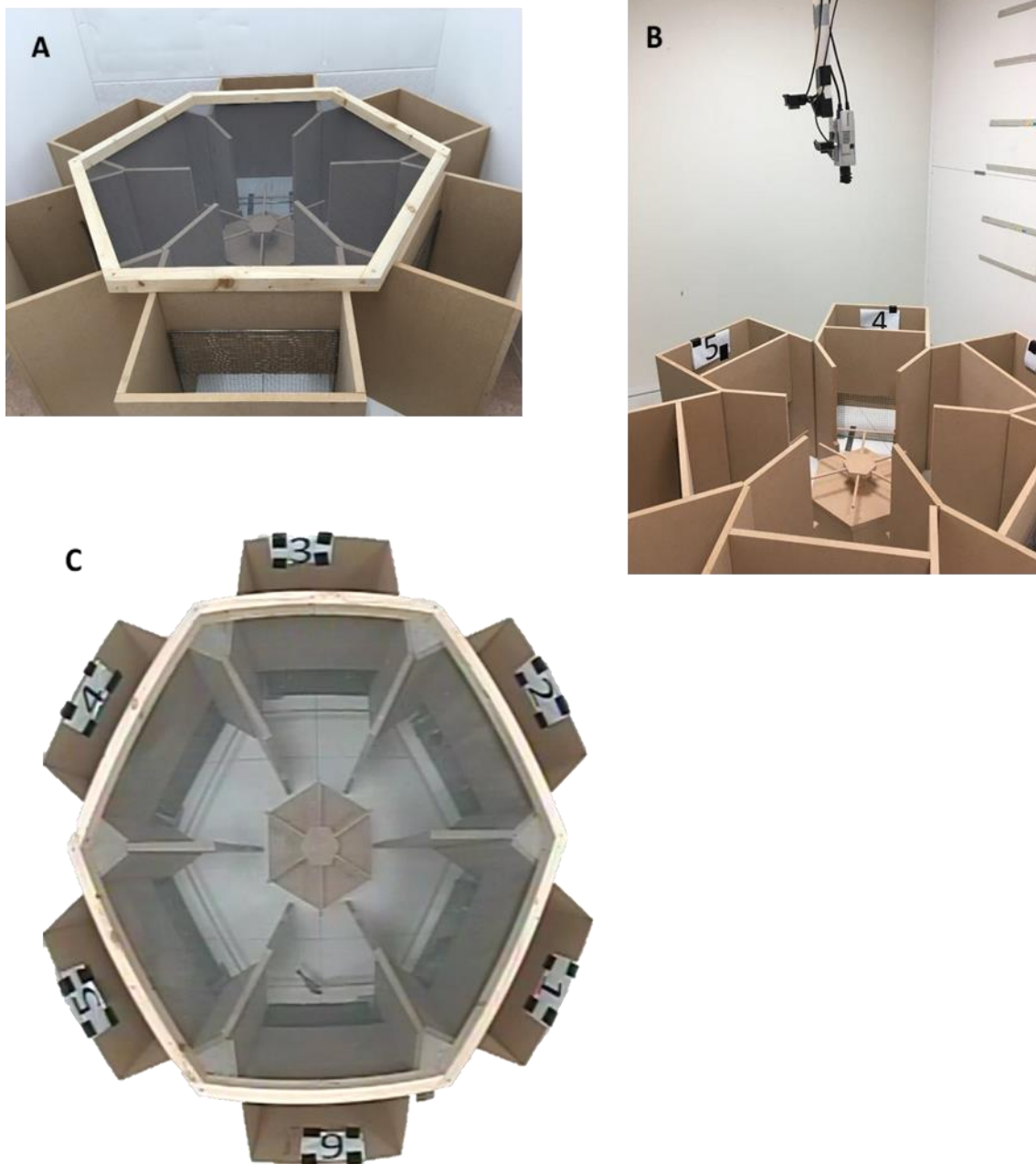
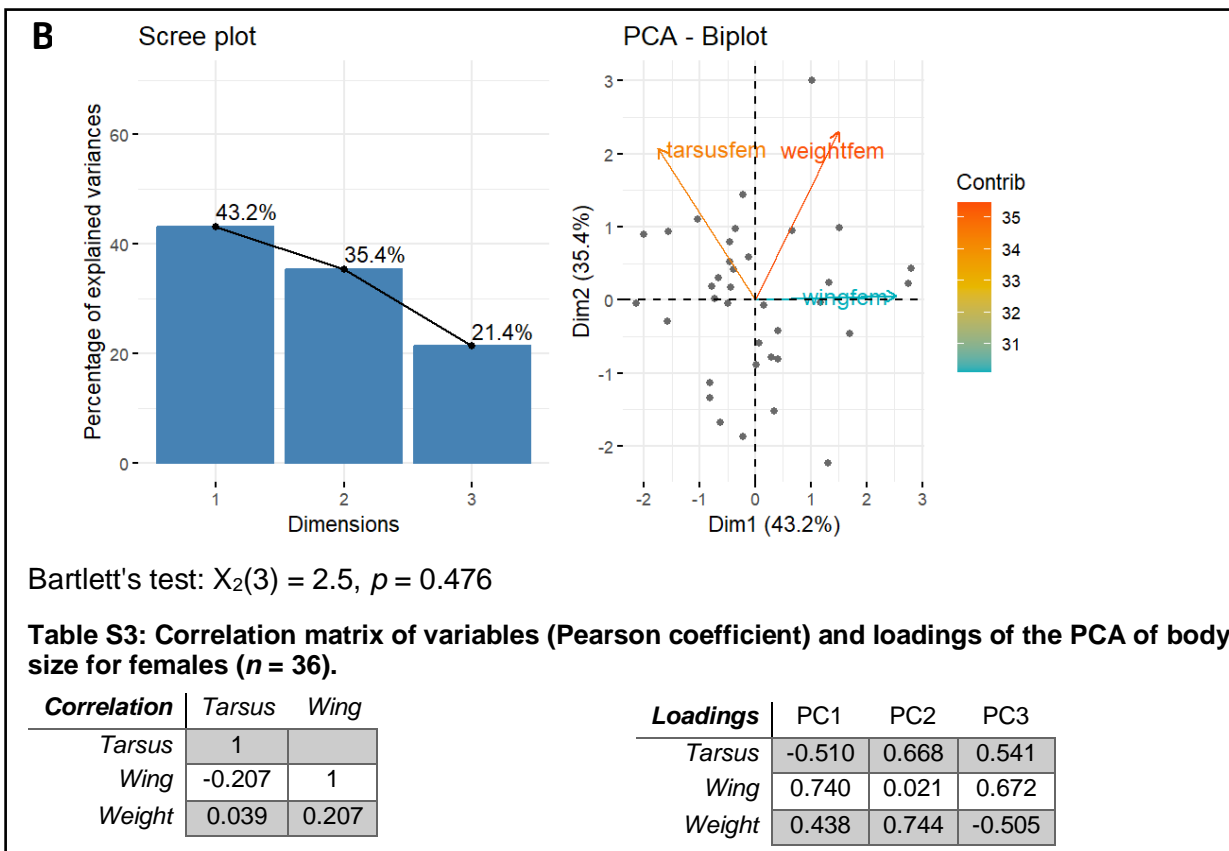
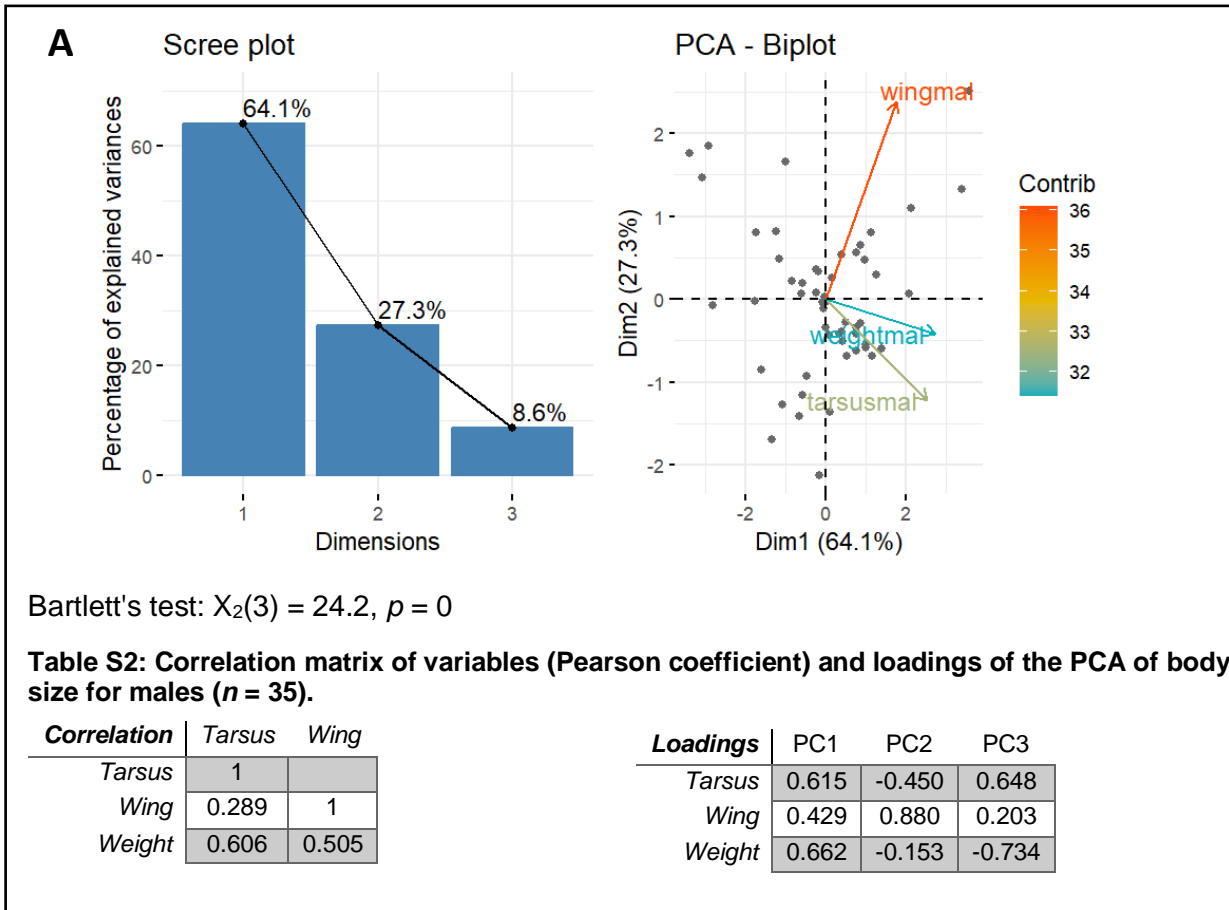


Figure S1: Pictures of the experimental setup for the mate preference test.

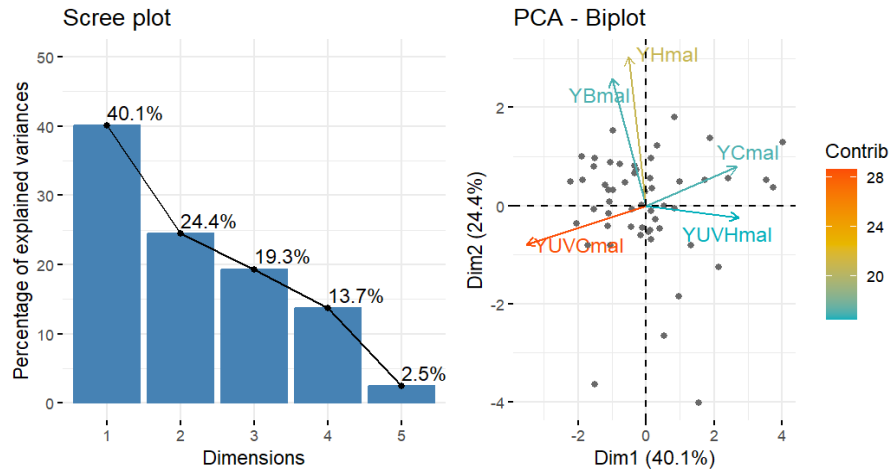
A: Picture of the carousel-shaped six-choice chamber. The roof can be moved to introduce the female into the setup.

B: Picture of the camera pointing down from the ceiling. The camera was centred to point in the middle of the setup.

C: Screenshot of the video of a test, the female is in the choice zone of the male 6. The males are not visible on the video.



C

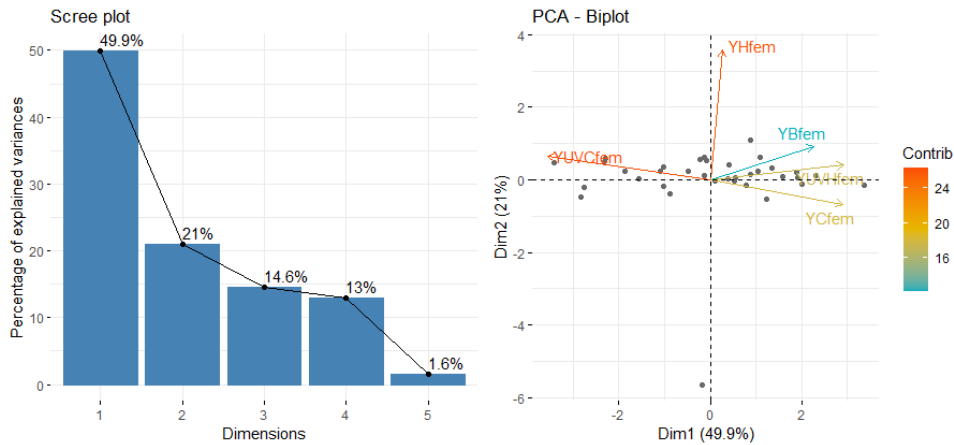


YB: achromatic yellow; YC: chromatic yellow; YUVC: chromatic UV yellow; YH: yellow hue (maximal slope in yellow part of the spectrum); YUVH: UV yellow hue (maximal reflectance in UV)
 Bartlett's test: $X_2(10) = 81.3, p = 0$

Table S4: Correlation matrix of variables (Pearson coefficient) and loadings of the PCA of colouration for males ($n = 54$).

Correlation	YBmal	YBmal	YBmal	YBmal	Loadings	PC1	PC2	PC3	PC4	PC5
YBmal	1				YBmal	-0.189	0.625	0.487	-0.567	0.121
YUVCmal	0.0422	1			YUVCmal	-0.663	-0.189	-0.089	0.089	0.714
YCmal	-0.176	-0.639	1		YCmal	0.501	0.190	-0.587	-0.359	0.488
YUVHmal	-0.099	-0.653	0.103	1	YUVHmal	0.513	-0.059	0.594	0.377	0.488
YHmal	0.238	0.020	0.051	-0.1285	YHmal	-0.099	0.731	-0.239	0.632	-0.006

D



YB: achromatic yellow; YC: chromatic yellow; YUVC: chromatic UV yellow; YH: yellow hue (maximal slope in yellow part of the spectrum); YUVH: UV yellow hue (maximal reflectance in UV)
 Bartlett's test: $X_2(10) = 72.7, p = 0$

Table S5: Correlation matrix of variables (Pearson coefficient) and loadings of the PCA of colouration for females ($n = 35$).

Correlation	YBfem	YBfem	YBfem	YBfem	Loadings	PC1	PC2	PC3	PC4	PC5
YBfem	1				YBmal	0.385	0.242	0.847	0.269	0.058
YUVCfem	-0.425	1			YUVCmal	-0.602	0.169	0.155	0.057	0.763
YCfem	0.291	-0.765	1		YCmal	0.493	-0.175	0.395	0.596	0.464
YUVHfem	0.344	-0.729	0.329	1	YUVHmal	0.495	0.108	-0.048	-0.745	0.432
YHfem	0.105	0.059	0.016	0.111	YHmal	0.045	0.933	-0.317	0.118	-0.116

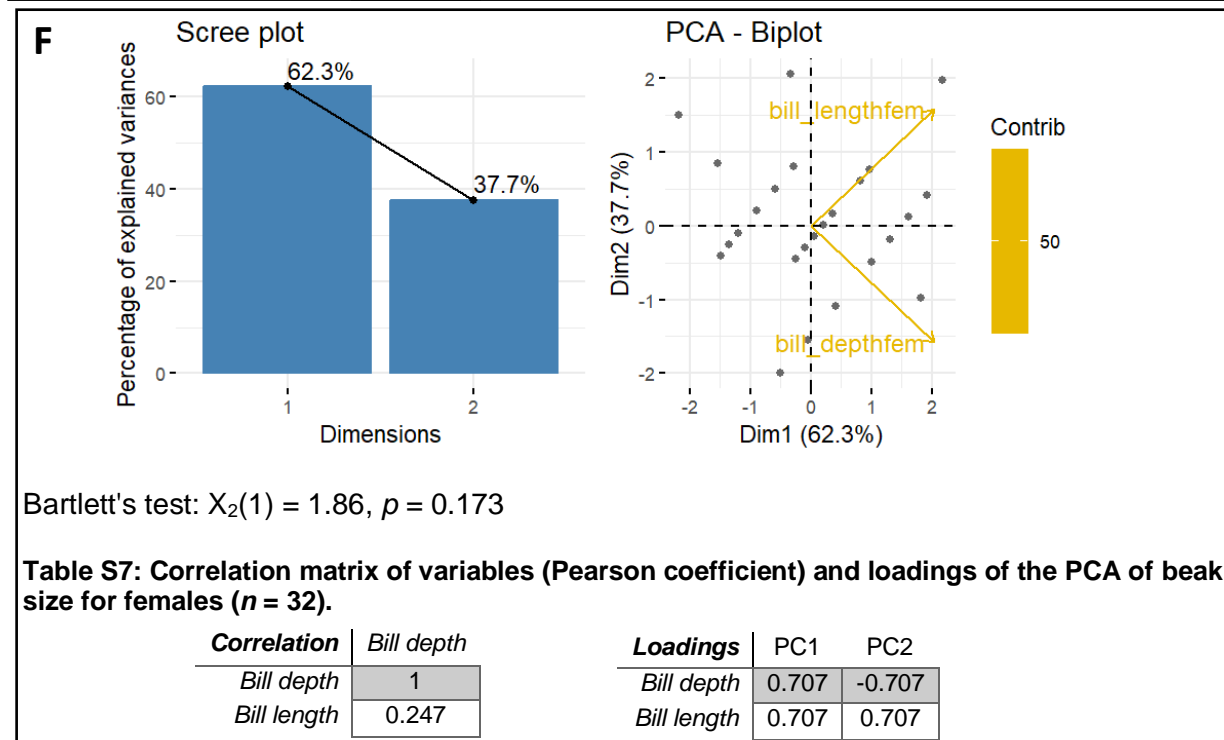
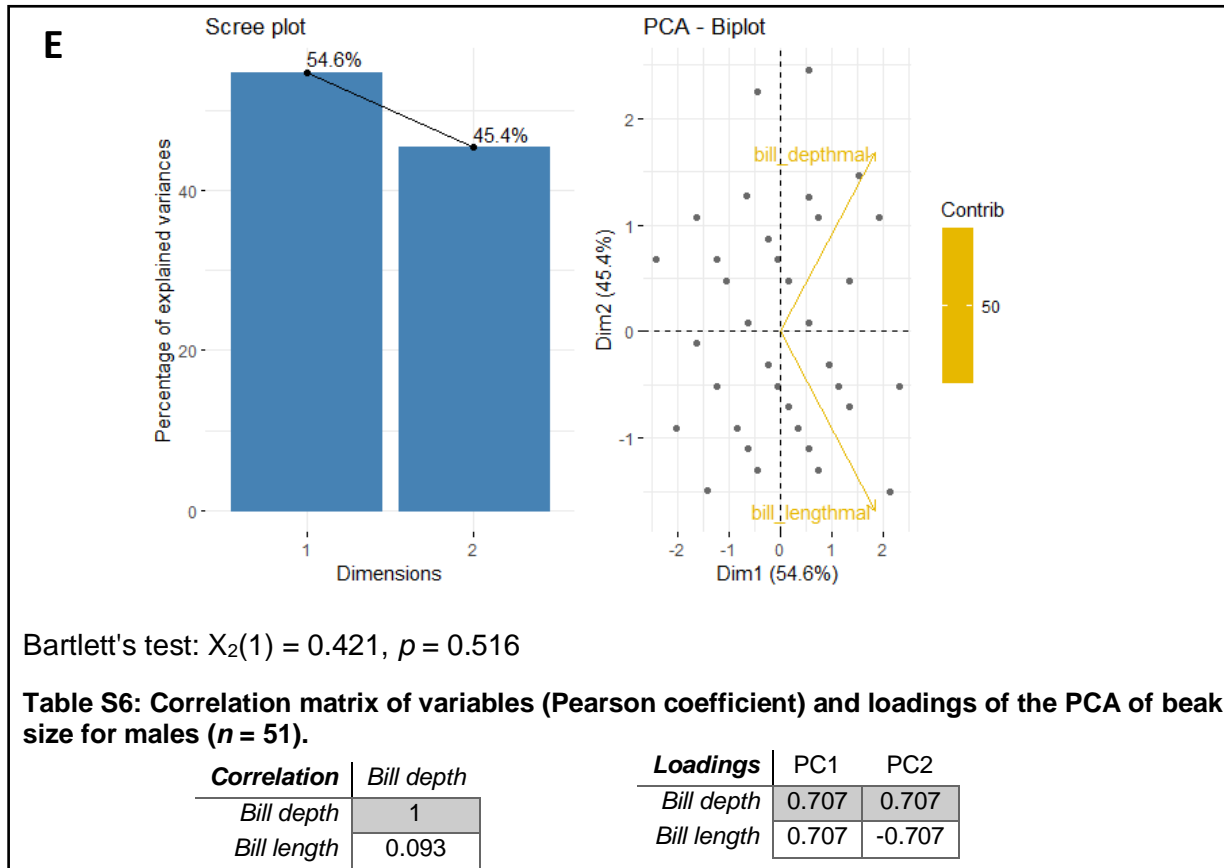


Figure S2: Results of the PCA ran for body size, colouration, and beak size of male and female great tits.

Correlation matrix was used for all PCAs.

A: Male body size; B: Female body size; C: Male colouration; D: Female colouration; E: Male beak size; F: Female beak size

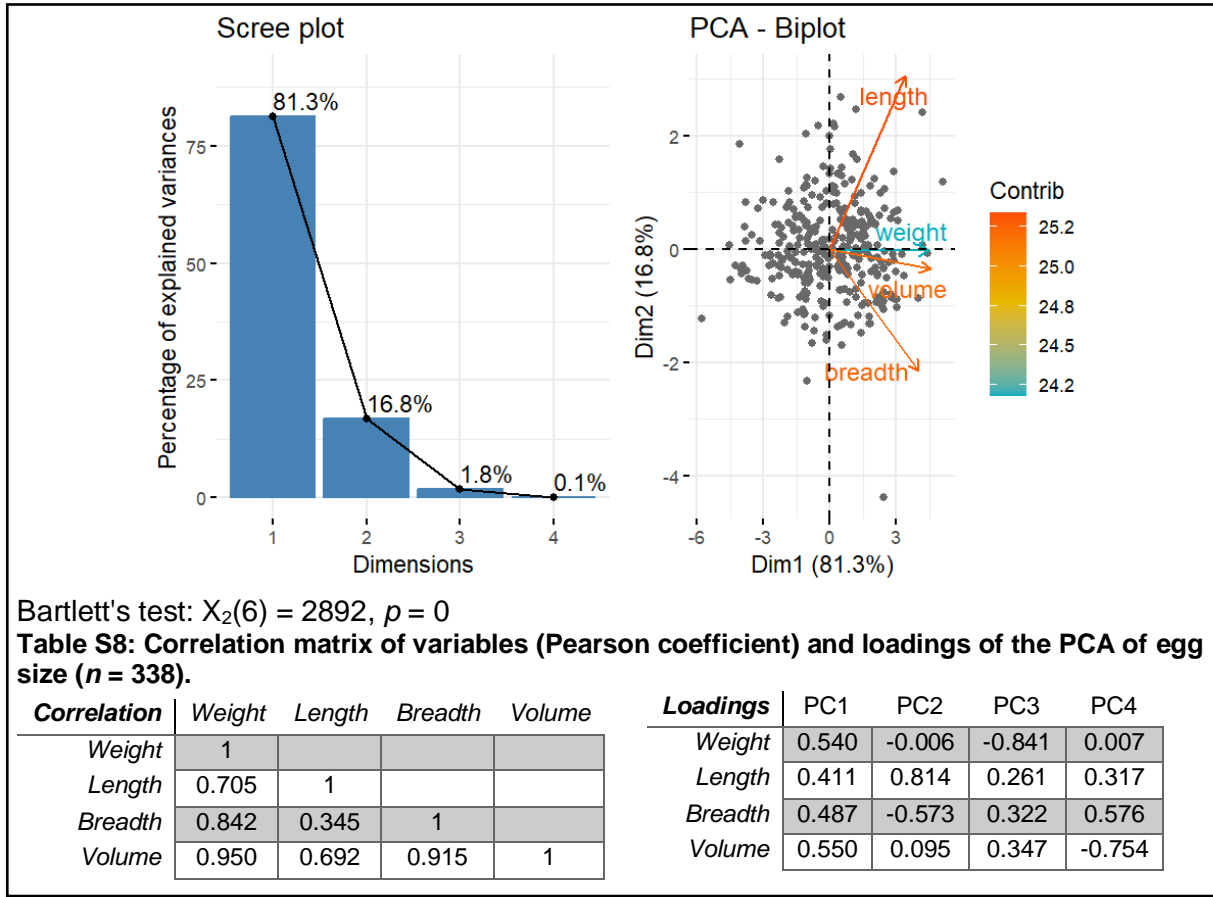


Figure S3: Results of the PCA run for egg size.
High value of the first axis represents bigger eggs.

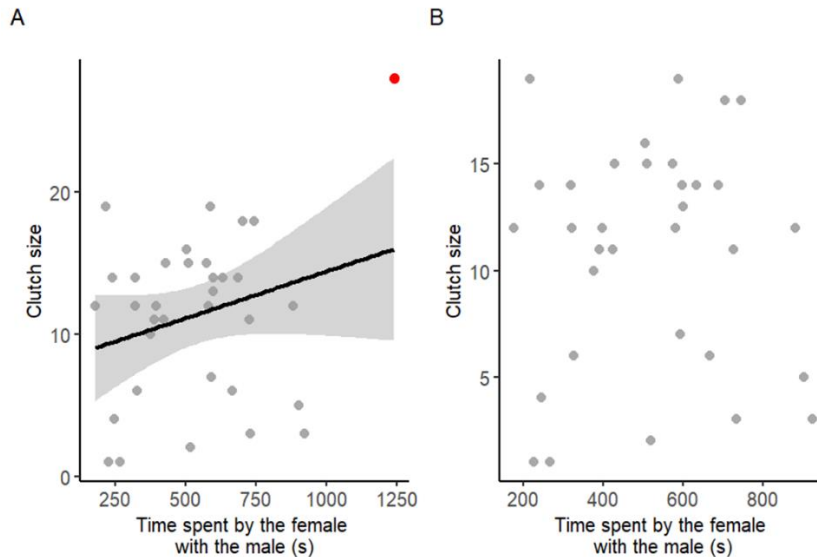


Figure S4: The strength of the preference of the female for the male did not affect clutch size, when excluding an outlier.

(A) Females showing stronger preference for their male laid larger clutches ($z_{1,32} = 2.69, p = 0.007$). However, one female, depicted by the red point on the graph, was an outlier in our data with 28 eggs laid. (B) When this female was removed from the data, the effect of preference intensity on clutch size was no longer significant ($z_{1,31} = 0.14, p = 0.891$).

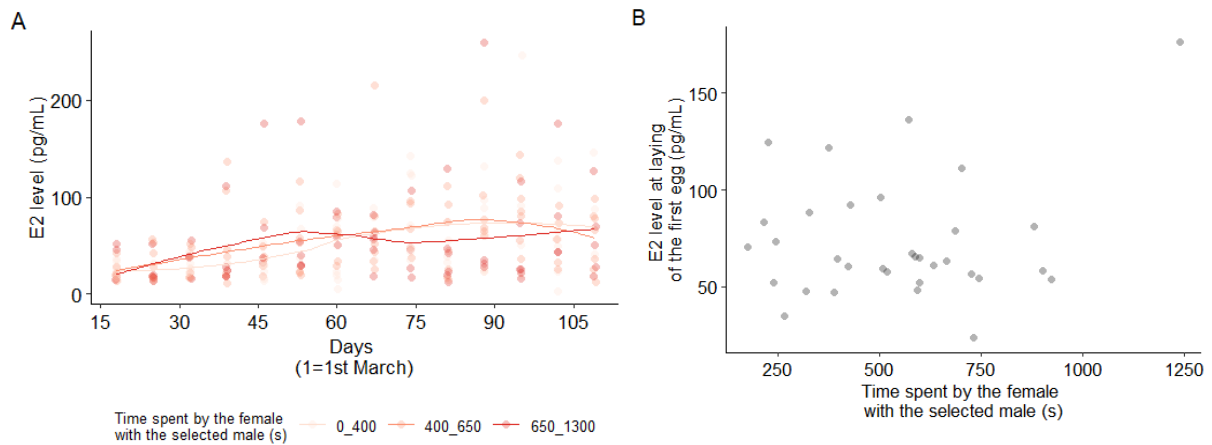


Figure S5: The strength of the preference of the female for the male did not affect E₂ level.

(A) E₂ concentrations during the breeding season did not increase sooner in females paired with a more preferred male (date*time spent with the male interaction: $F_{1,15.12} = 0.51$, $p = 0.484$). (B) Concentrations closest to the laying date of the first egg ($F_{1,31} = 0.29$, $p = 0.589$) were not significantly influenced by the strength of the preference of the female for her male.

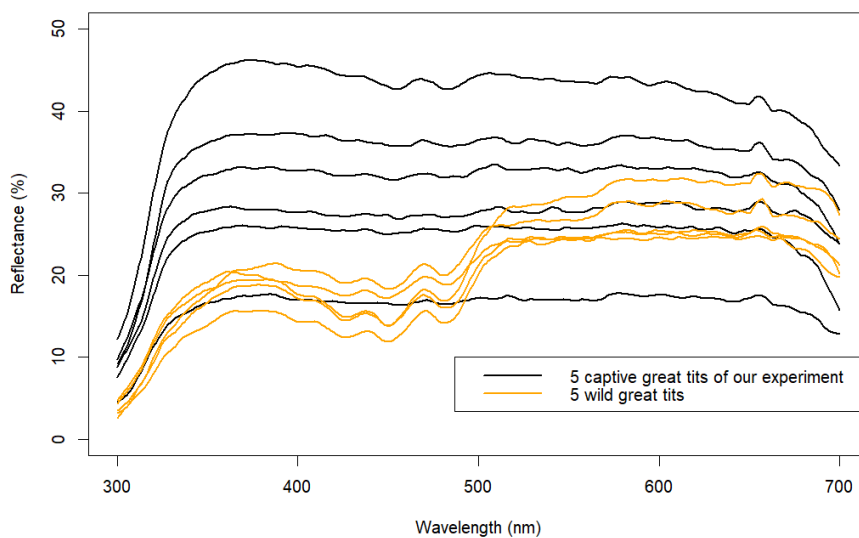


Figure S6: Reflectance spectra of male great tit breast feathers.

Reflectance spectra of breast feathers of ten great tit males revealing variability between individuals. Each line represents the mean of three measures of one individual. Black lines represent the data of five captive males that we used in our mate preference test. Yellow lines represent the data of five wild great tits. Wild great tits have a yellow breast, whereas captive great tits have a pale grey breast.

Supplementary material of Article 3 (Chapter 2)

Predicting individual reproductive decisions of passerine birds using a satellite-derived vegetation index.

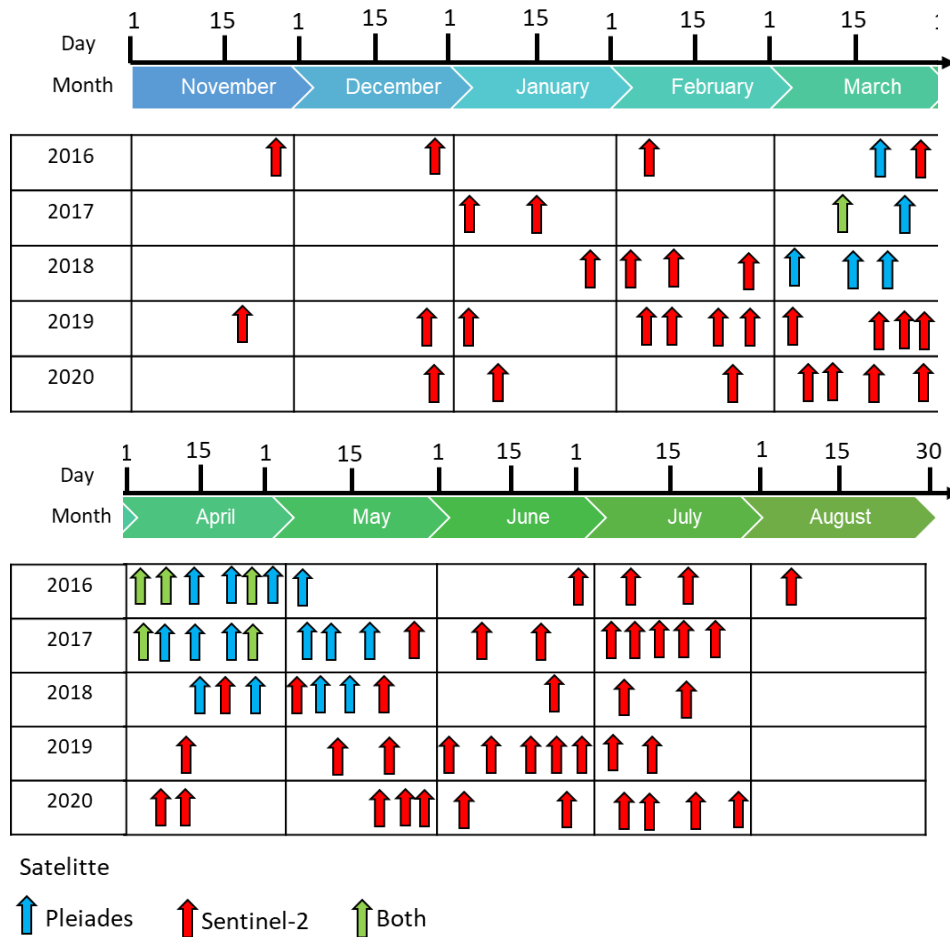


Figure S1: Timeline of the image collection of Sentinel-2 and Pléiade satellites.

In 2016, 2017 and 2018, we collected images from both satellites to minimise the time-gap between images during the bird breeding season. In 2019 and 2020, Pléiades images were no longer available for reasons beyond our control.

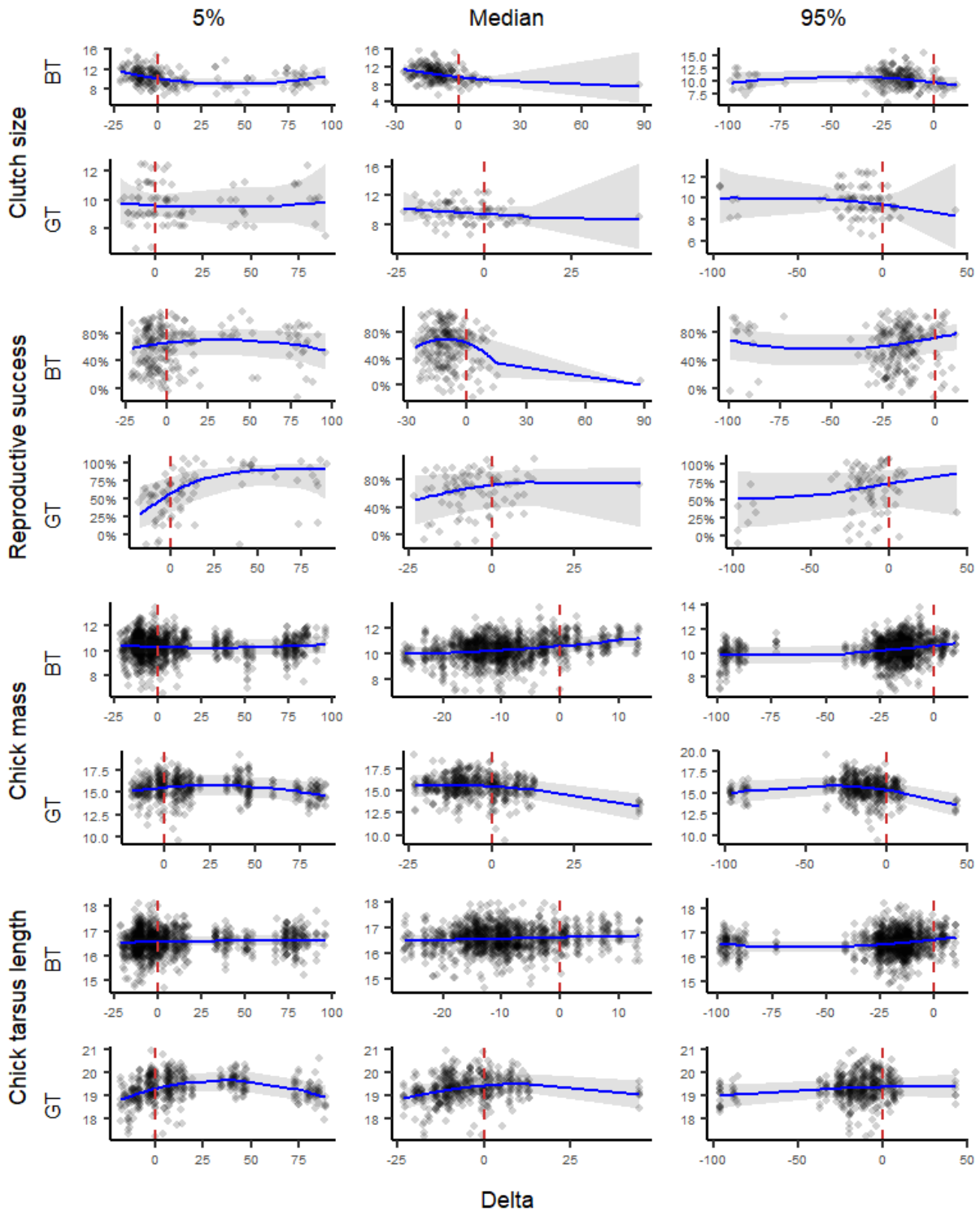
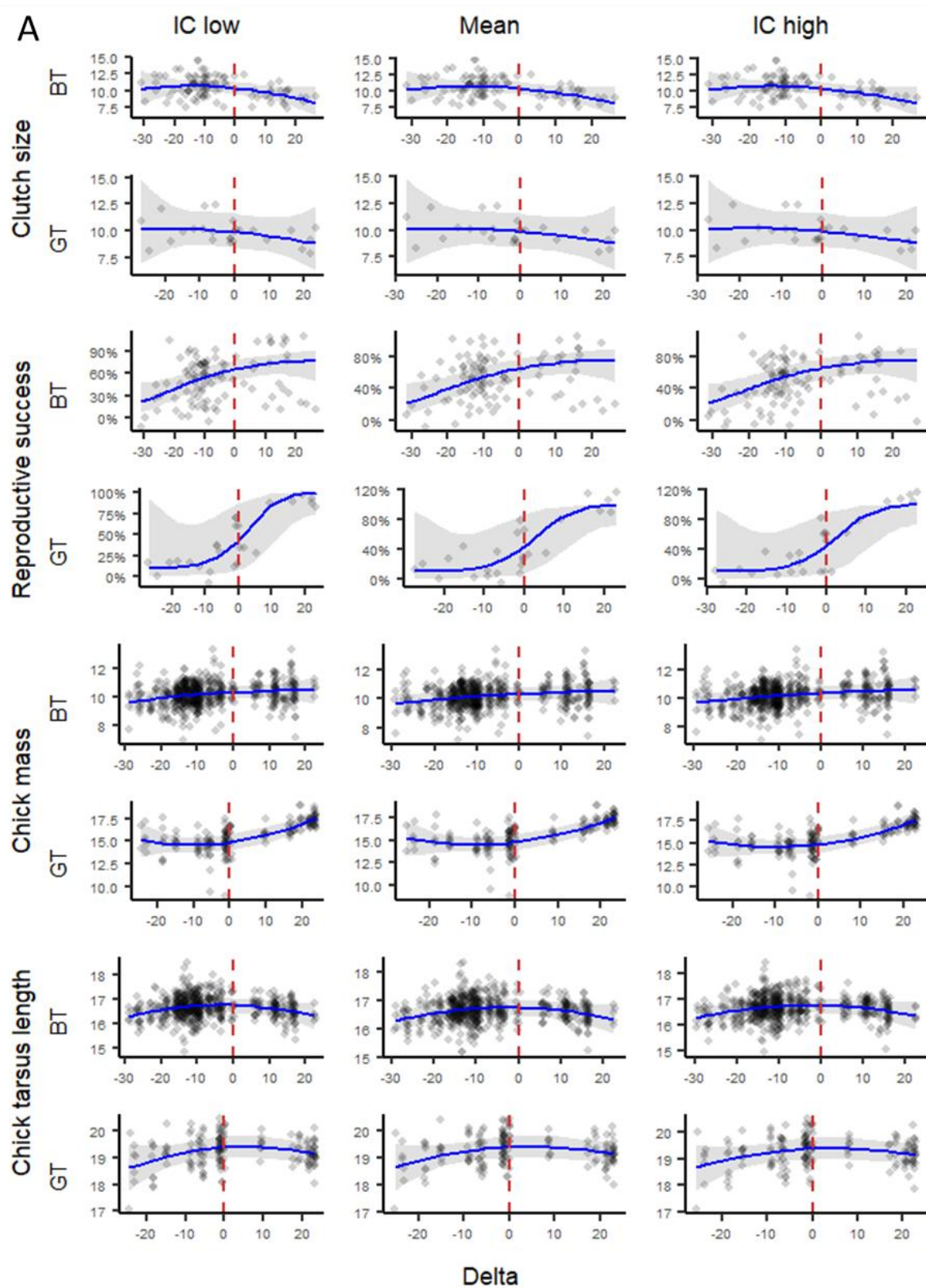


Figure S2: Sensitivity analysis with median and quantiles. Effect of the synchrony of bird laying date with vegetation green-up date on their reproductive parameters.

We used three other values of delta to estimate the effect of the synchrony of bird with vegetation green-up on their reproductive outputs: the median, quantile 5%, and quantile 95% delta. Points represent residuals and blue line depict the prediction of the model with a 95% confidence interval. Delta is defined as bird laying date – vegetation green-up date. Delta < 0 thus represent a bird that laid before vegetation green-up. Red dotted lines indicate a delta equal to 0, i.e. a bird that laid at the vegetation green-up date. GT: great tit, BT: Blue tit.



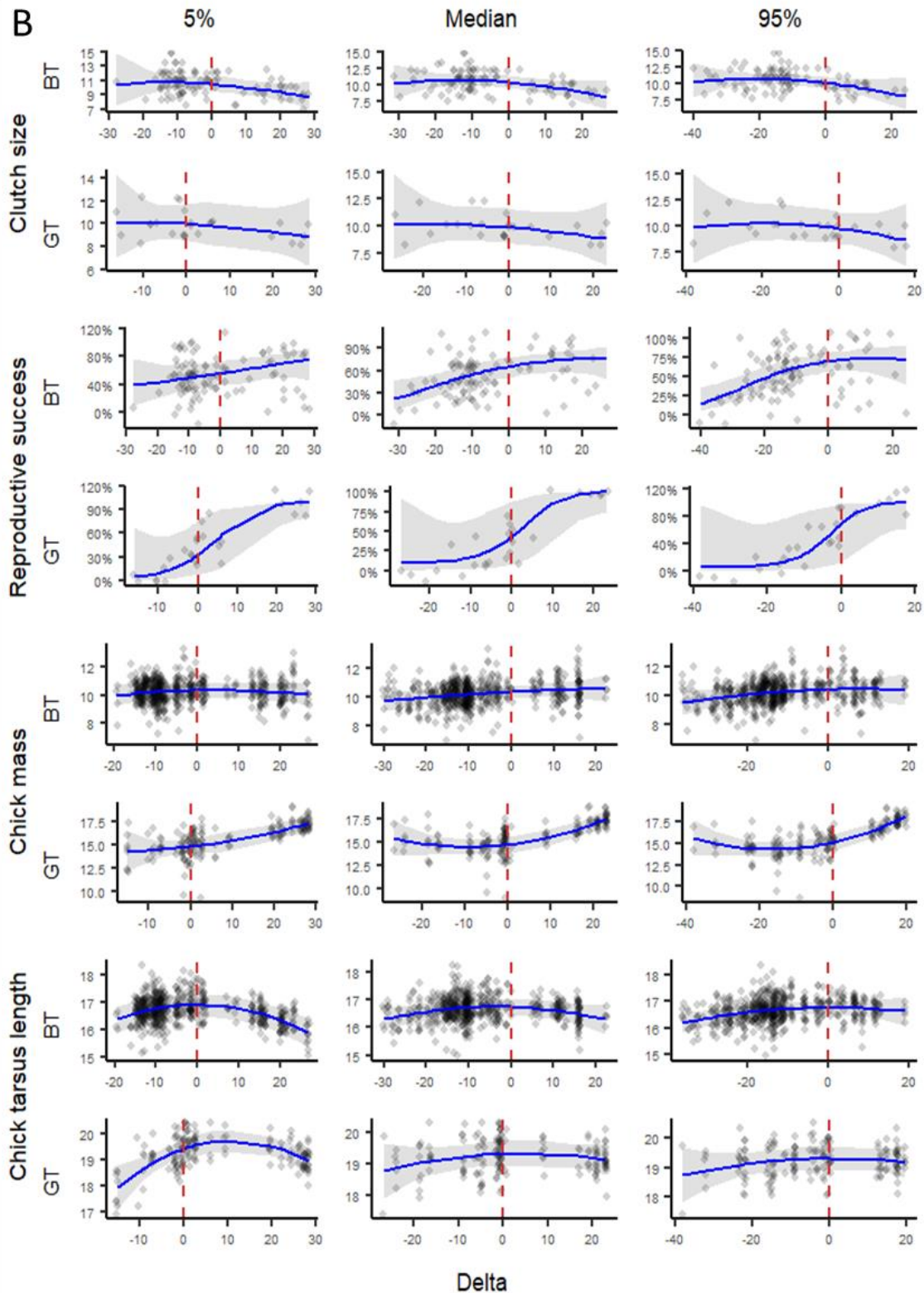


Figure S3: Sensitivity analysis with Sentinel-2 images only. Effect of the synchrony of bird laying date with vegetation green-up date on their reproductive parameters for 2019 and 2020 data only.

A: We used three values of delta to estimate the effect of the synchrony of bird with vegetation green-up on their reproductive outputs: the mean delta and the two delta of the high and low bound of its 95% confidence interval.

B: We used three other values of delta to estimate the effect of the synchrony of bird with vegetation green-up on their reproductive outputs: the median, quantile 5%, and quantile 95% delta.

Points represent residuals and blue line depict the prediction of the model with a 95% confidence interval. Delta is defined as bird laying date – vegetation green-up date. Delta < 0 thus represent a bird that laid before vegetation green-up. Red dotted lines indicate a delta equal to 0, i.e. a bird that laid at the vegetation green-up. GT: great tit, BT: Blue tit.

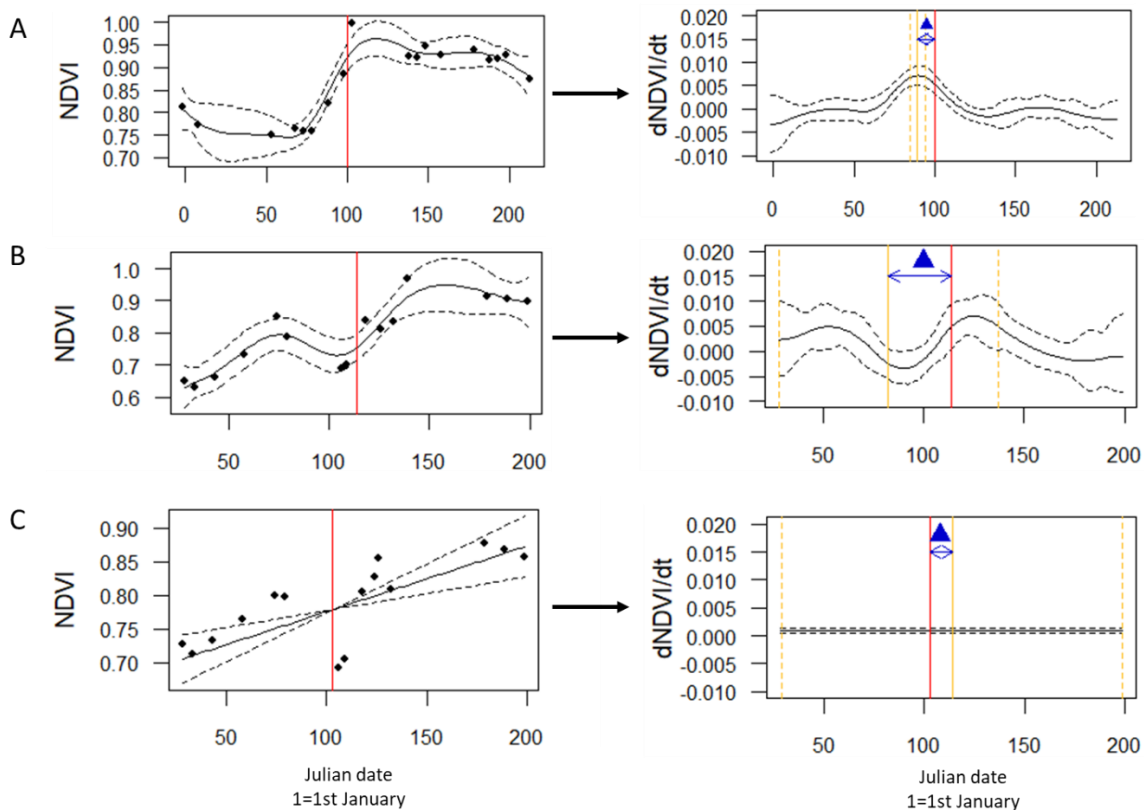


Figure S4: Annual NDVI time series and their derivatives.

Left panel: NDVI times series modelled by a Generalized Additive Model. The red line represents the laying date of the bird for this specific nest-box example.

Right panel: Derivatives of the GAM used to model the NDVI time series. The red line represents the laying date of the bird for this specific nest-box. The yellow line depicts the date of the inflexion point, estimated by the maximum of the derivative curve, and that we defined as the date of the vegetation green-up. The two dotted yellow lines represent the 95% confidence interval around this estimated date. The blue delta (Δ) and arrows represent the degree of synchrony between the bird laying date and the vegetation green-up date. Delta was calculated as the bird laying date minus the vegetation-green up date.

3 examples are depicted:

A: NDVI times series showing a sigmoid curve typical of deciduous habitat, and good estimation of the vegetation green-up date from the derivative.

B: NDVI time series with an increase before budburst, and thus the derivative gives a wide confidence interval of the vegetation green-up date.

C: NDVI time series not well modelled, and thus the derivative gives a wide confidence interval of the vegetation green-up date.

Supplementary material of Article 4 (Chapter 3)

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

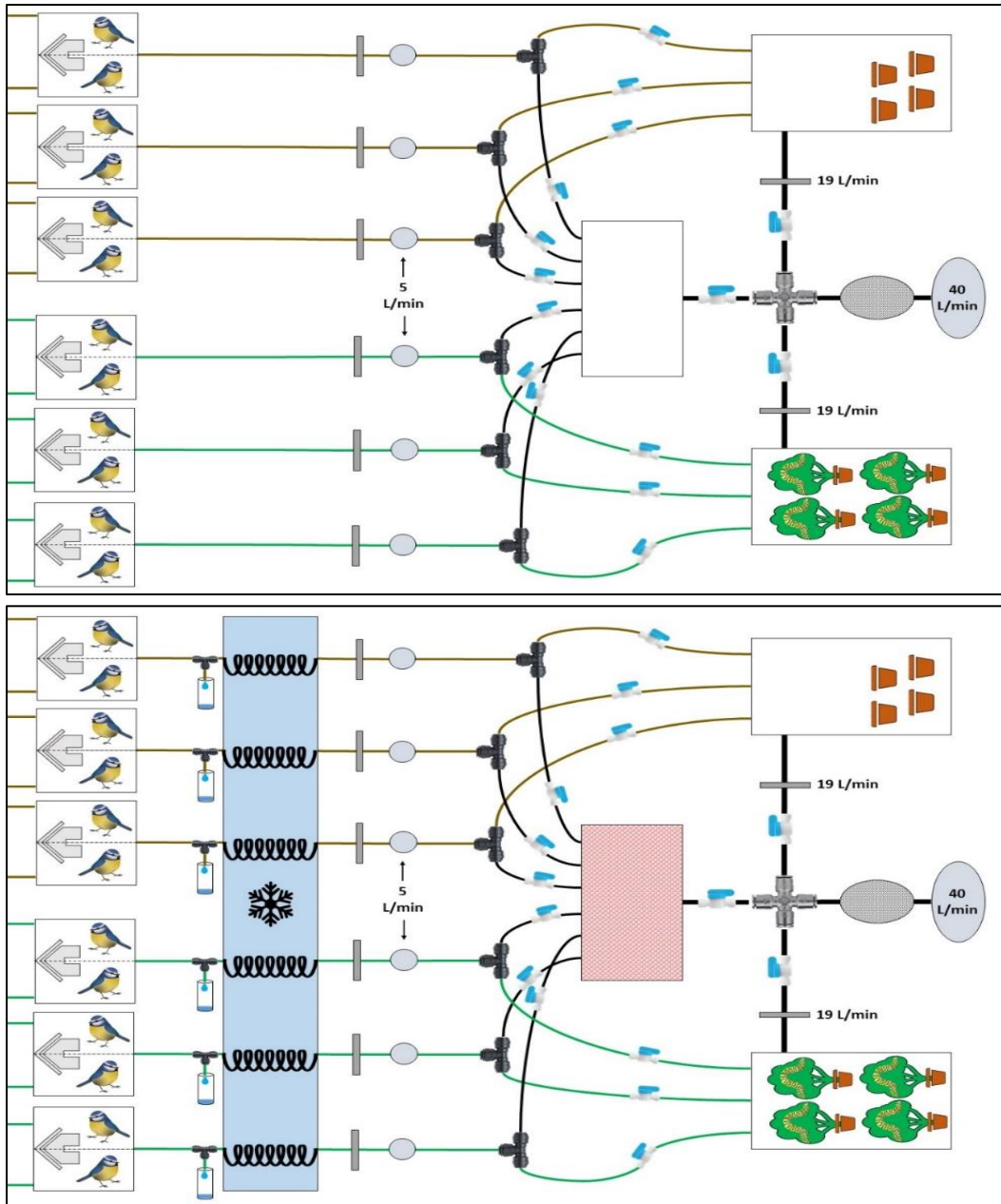


Figure 1. Diagram of the experimental setup in 2019 (A) and 2020 (B).

Air was purified through a charcoal filter before being split to one experimental and one control enclosure. Smaller pumps pulled air from the enclosure at 5 L/min and to the terrarium. In 2020, silica gel was used to remove moisture from the air when flow was not through the enclosures, and a cooling system was used to condense and remove water from the system. Diagram represents only half of the experimental setup, but both halves were set up identically.

Supplementary material of Article 5 (Chapter 3)

Odours of caterpillar-infested trees increase testosterone concentrations in male great tits.

Table S1: Comparison of the mean control and HIPV curves.

“Difference” represents the difference between the control and the HIPV curves. SE: Standard Error, CI upper: Upper bound of the 95% Confidence Interval, CI lower: Lower bound of the 95% Confidence Interval. The last column indicates if the 95% Confidence Interval around the difference overlap zero; if yes, it returns “FALSE”, meaning that the control and HIPV curves are not significantly different at this time point; if no, it returns “TRUE”, meaning that the curves are significantly different at this time point.

A: 17 β -estradiol (E₂)

<i>Day</i>	<i>Difference</i>	<i>SE</i>	<i>CI upper</i>	<i>CI lower</i>	<i>CI does not overlap zero</i>
-71	3,506389709	11,11146008	25,28485146	-18,27207205	FALSE
-69,6969697	3,402531597	10,91346215	24,79291741	-17,98785422	FALSE
-68,39393939	3,298679936	10,71814354	24,30624127	-17,7088814	FALSE
-67,09090909	3,194841178	10,52568019	23,82517436	-17,435492	FALSE
-65,78787879	3,091021775	10,3362179	23,35000886	-17,16796531	FALSE
-64,48484848	2,987228179	10,14987214	22,88097758	-16,90652122	FALSE
-63,18181818	2,883466839	9,966729341	22,41825635	-16,65132267	FALSE
-61,87878788	2,77974421	9,786849671	21,96196956	-16,40248114	FALSE
-60,57575758	2,676067606	9,610275283	21,51220716	-16,16007195	FALSE
-59,27272727	2,572447072	9,43704362	21,06905257	-15,92415842	FALSE
-57,96969697	2,46889543	9,267193186	20,63259408	-15,69480322	FALSE
-56,66666667	2,365429599	9,100774997	20,20294859	-15,47208939	FALSE
-55,36363636	2,262068937	8,937848796	19,78025258	-15,2561147	FALSE
-54,06060606	2,158835521	8,778484491	19,36466512	-15,04699408	FALSE
-52,75757576	2,055754319	8,622759655	18,95636324	-14,84485461	FALSE
-51,45454545	1,952851758	8,470751187	18,55552408	-14,64982057	FALSE
-50,15151515	1,850154517	8,322528107	18,16230961	-14,46200057	FALSE
-48,84848485	1,747692843	8,178154994	17,77687663	-14,28149094	FALSE
-47,54545455	1,645503767	8,037693351	17,39938274	-14,1083752	FALSE
-46,24242424	1,543631429	7,90119754	17,02997861	-13,94271575	FALSE
-44,93939394	1,442127058	7,768712391	16,66880334	-13,78454923	FALSE
-43,63636364	1,341048798	7,640273534	16,31598492	-13,63388733	FALSE
-42,33333333	1,240461326	7,515910023	15,97164497	-13,49072232	FALSE
-41,03030303	1,140435166	7,395648069	15,63590538	-13,35503505	FALSE
-39,72727273	1,041045814	7,279514485	15,3088942	-13,22680258	FALSE
-38,42424242	0,942372646	7,167538564	14,99074823	-13,10600294	FALSE
-37,12121212	0,844497584	7,059751502	14,68161053	-12,99261536	FALSE
-35,81818182	0,747503643	6,956183644	14,38162359	-12,8866163	FALSE
-34,51515152	0,651473363	6,856860475	14,09091989	-12,78797317	FALSE

APPENDICES

-33,21212121	0,556487212	6,761798647	13,80961256	-12,69663814	FALSE
-31,90909091	0,462622035	6,671003424	13,53778875	-12,61254467	FALSE
-30,60606061	0,369949597	6,58446857	13,27550799	-12,5356088	FALSE
-29,3030303	0,278535278	6,502178732	13,02280559	-12,46573504	FALSE
-28	0,188437068	6,424113556	12,77969964	-12,4028255	FALSE
-26,6969697	0,099704794	6,350252382	12,54619946	-12,34678987	FALSE
-25,39393939	0,01237963	6,28057805	12,32231261	-12,29755335	FALSE
-24,09090909	-0,073505982	6,215078488	12,10804785	-12,25505982	FALSE
-22,78787879	-0,157928122	6,1537458	11,90341365	-12,21926989	FALSE
-21,48484848	-0,240870918	6,096573285	11,70841272	-12,19015456	FALSE
-20,18181818	-0,322325741	6,043551331	11,52303487	-12,16768635	FALSE
-18,87878788	-0,402290191	5,994663761	11,34725078	-12,15183116	FALSE
-17,57575758	-0,480766961	5,949886066	11,18100973	-12,14254365	FALSE
-16,27272727	-0,557762593	5,909186156	11,02424227	-12,13976746	FALSE
-14,96969697	-0,633286206	5,872527494	10,87686768	-12,14344009	FALSE
-13,66666667	-0,707348298	5,839873865	10,73880448	-12,15350107	FALSE
-12,36363636	-0,779959663	5,811194279	10,60998112	-12,16990045	FALSE
-11,06060606	-0,851130471	5,786466356	10,49034359	-12,19260453	FALSE
-9,757575758	-0,92086957	5,765677319	10,37985798	-12,22159712	FALSE
-8,454545455	-0,989184017	5,748822392	10,27850787	-12,2568759	FALSE
-7,151515152	-1,056078863	5,735900988	10,18628707	-12,2984448	FALSE
-5,848484848	-1,121557189	5,726912187	10,1031907	-12,34630508	FALSE
-4,545454545	-1,185620337	5,72185112	10,02920786	-12,40044853	FALSE
-3,242424242	-1,248268357	5,720707415	9,964318176	-12,46085489	FALSE
-1,939393939	-1,309500586	5,723466275	9,908493313	-12,52749449	FALSE
-0,636363636	-1,369316326	5,730112024	9,86170324	-12,60033589	FALSE
0,666666667	-1,427715573	5,740632895	9,823924902	-12,67935605	FALSE
1,96969697	-1,484699739	5,755025344	9,795149935	-12,76454941	FALSE
3,272727273	-1,54027232	5,773296581	9,775388979	-12,85593362	FALSE
4,575757576	-1,594439504	5,79546444	9,764670799	-12,95354981	FALSE
5,878787879	-1,647210645	5,821554312	9,763035807	-13,0574571	FALSE
7,181818182	-1,698598601	5,851594255	9,770526139	-13,16772334	FALSE
8,484848485	-1,748619982	5,885609851	9,787175326	-13,28441529	FALSE
9,787878788	-1,797295239	5,923620277	9,813000505	-13,40759098	FALSE
11,09090909	-1,844648635	5,965636896	9,847999682	-13,53729695	FALSE
12,39393939	-1,890708158	6,011664873	9,892154992	-13,67357131	FALSE
13,6969697	-1,93550537	6,06170714	9,945440626	-13,81645137	FALSE
15	-1,979075185	6,115769238	10,00783252	-13,96598289	FALSE
16,3030303	-2,021455679	6,173863452	10,07931669	-14,12222804	FALSE
17,60606061	-2,062687914	6,236010717	10,15989309	-14,28526892	FALSE
18,90909091	-2,102815766	6,30223927	10,2495732	-14,45520474	FALSE
20,21212121	-2,141885804	6,372580465	10,34837191	-14,63214352	FALSE
21,51515152	-2,179947218	6,447062916	10,4562961	-14,81619053	FALSE
22,81818182	-2,217051753	6,525706589	10,57333316	-15,00743667	FALSE
24,12121212	-2,253253651	6,608518738	10,69944308	-15,20595038	FALSE
25,42424242	-2,288609587	6,69549306	10,83455681	-15,41177598	FALSE

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26,72727273	-2,323178553	6,786612249	10,97858146	-15,62493856	FALSE
28,03030303	-2,357021654	6,88185318	11,13141058	-15,84545389	FALSE
29,33333333	-2,390201815	6,981193162	11,29293678	-16,07334041	FALSE
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31,93939394	-2,454831482	7,192110806	11,6417057	-16,55136866	FALSE
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38,45454545	-2,609314203	7,790580762	12,66022409	-17,8788525	FALSE
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42,36363636	-2,69952058	8,197492173	13,36756408	-18,76660524	FALSE
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44,96969697	-2,75943534	8,487715994	13,87648801	-19,39535869	FALSE
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47,57575758	-2,81944445	8,792623971	14,41409853	-20,05298743	FALSE
48,87878788	-2,849516995	8,95051936	14,69350095	-20,39253494	FALSE
50,18181818	-2,87963725	9,112013608	14,97990942	-20,73918392	FALSE
51,48484848	-2,909800046	9,277069625	15,27325642	-21,09285651	FALSE
52,78787879	-2,939999094	9,445620069	15,57341624	-21,45341443	FALSE
54,09090909	-2,97022699	9,617566996	15,88020432	-21,8206583	FALSE
55,39393939	-3,000475483	9,792780089	16,19337349	-22,19432446	FALSE
56,6969697	-3,030736371	9,971092141	16,51260423	-22,57407697	FALSE
58	-3,061002828	10,15231886	16,83754214	-22,9595478	FALSE

B: Testosterone

Day	Difference	SE	CI upper	CI lower	CI does not overlap zero
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-69,71717172	1,778777771	2,248464275	6,185767749	-2,628212207	FALSE
-68,43434343	1,664101158	2,124544098	5,82820759	-2,500005274	FALSE
-67,15151515	1,550616998	2,007682796	5,485675278	-2,384441282	FALSE
-65,86868687	1,438921518	1,898728728	5,160429825	-2,282586789	FALSE
-64,58585859	1,329610944	1,798189901	4,854063151	-2,194841263	FALSE
-63,3030303	1,223281502	1,706174948	4,5673844	-2,120821396	FALSE
-62,02020202	1,12052942	1,622363146	4,300361187	-2,059302347	FALSE
-60,73737374	1,021945959	1,546039936	4,052184234	-2,008292317	FALSE
-59,45454545	0,928100137	1,476254074	3,821558123	-1,965357849	FALSE
-58,17171717	0,839501198	1,411991161	3,607003875	-1,928001478	FALSE
-56,88888889	0,75651992	1,352436588	3,407295633	-1,894255793	FALSE
-55,60606061	0,679468854	1,296973304	3,221536529	-1,862598822	FALSE
-54,32323232	0,608654417	1,245126714	3,049102777	-1,831793942	FALSE
-53,04040404	0,544280085	1,196653058	2,889720078	-1,801159909	FALSE
-51,75757576	0,486453527	1,15149117	2,743376221	-1,770469166	FALSE
-50,47474747	0,435276727	1,109606723	2,610105903	-1,73955245	FALSE

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-49,19191919	0,390769846	1,070988204	2,489906727	-1,708367035	FALSE
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-46,62626263	0,320885619	1,003577184	2,2878969	-1,646125663	FALSE
-45,34343434	0,29467996	0,974605486	2,204906712	-1,615546792	FALSE
-44,06060606	0,273593251	0,948498768	2,132650835	-1,585464334	FALSE
-42,77777778	0,257070203	0,924986165	2,070043087	-1,555902681	FALSE
-41,49494949	0,244608728	0,903861312	2,0161769	-1,526959444	FALSE
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-37,64646465	0,228536955	0,854699817	1,903748597	-1,446674687	FALSE
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-32,51515152	0,2595729	0,822578311	1,87182639	-1,35268059	FALSE
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-24,81818182	0,51180466	0,805834095	2,091239486	-1,067630166	FALSE
-23,53535354	0,58985134	0,804183601	2,166051198	-0,986348518	FALSE
-22,25252525	0,679539377	0,803226364	2,25386305	-0,894784296	FALSE
-20,96969697	0,780637908	0,803006752	2,354531142	-0,793255325	FALSE
-19,68686869	0,892376603	0,803439589	2,467118199	-0,682364992	FALSE
-18,4040404	1,013423159	0,804316449	2,5898834	-0,563037082	FALSE
-17,12121212	1,141895282	0,805358604	2,720398146	-0,436607583	FALSE
-15,83838384	1,275401449	0,806300685	2,855750792	-0,304947894	FALSE
-14,55555556	1,411110315	0,806976106	2,992783484	-0,170562853	FALSE
-13,27272727	1,545851366	0,807372001	3,128300489	-0,036597757	FALSE
-11,98989899	1,67623965	0,807632116	3,259198597	0,093280702	TRUE
-10,70707071	1,798816565	0,808003211	3,382502859	0,215130272	TRUE
-9,424242424	1,910156764	0,808702611	3,495213881	0,325099646	TRUE
-8,141414141	2,006973911	0,809793266	3,594168711	0,41977911	TRUE
-6,858585859	2,086355024	0,811210409	3,676327426	0,496382622	TRUE
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0,838383838	2,110995026	0,813859088	3,706158838	0,515831214	TRUE
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3,404040404	1,954587329	0,810981557	3,544111181	0,365063476	TRUE
4,686868687	1,854648003	0,80995144	3,442152825	0,267143181	TRUE
5,96969697	1,744341249	0,809490212	3,330942066	0,157740433	TRUE
7,252525253	1,626968986	0,809627228	3,213838352	0,04009962	TRUE
8,535353535	1,505859375	0,810237088	3,093924069	-0,082205318	FALSE

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9,818181818	1,384212361	0,811077551	2,973924361	-0,205499639	FALSE
11,1010101	1,264960566	0,811869575	2,856224933	-0,326303801	FALSE
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14,94949495	0,944574903	0,812588151	2,537247679	-0,648097874	FALSE
16,23232323	0,855303958	0,812690341	2,448177025	-0,73756911	FALSE
17,51515152	0,775942327	0,813314029	2,370037823	-0,81815317	FALSE
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22,64646465	0,554467014	0,825722148	2,172882424	-1,063948396	FALSE
23,92929293	0,519912745	0,831116096	2,148900293	-1,109074803	FALSE
25,21212121	0,491823755	0,836975938	2,132296594	-1,148649083	FALSE
26,49494949	0,469273646	0,843113906	2,121776901	-1,183229609	FALSE
27,77777778	0,451490295	0,849518665	2,116546879	-1,213566289	FALSE
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31,62626263	0,422587079	0,87342184	2,134493886	-1,289319728	FALSE
32,90909091	0,420990789	0,884625775	2,154857307	-1,31287573	FALSE
34,19191919	0,42381811	0,898213096	2,184315777	-1,336679558	FALSE
35,47474747	0,431570133	0,914515797	2,224021095	-1,360880828	FALSE
36,75757576	0,444864214	0,933801848	2,275115835	-1,385387408	FALSE
38,04040404	0,464351709	0,95629803	2,338695848	-1,409992429	FALSE
39,32323232	0,490630442	0,982334181	2,416005436	-1,434744552	FALSE
40,60606061	0,524206478	1,012487742	2,508682452	-1,460269496	FALSE
41,88888889	0,565463577	1,047642716	2,6188433	-1,487916145	FALSE
43,17171717	0,614515411	1,089077232	2,749106786	-1,520075964	FALSE
44,45454545	0,671215781	1,1383133	2,902309848	-1,559878287	FALSE
45,73737374	0,735259468	1,196846723	3,081079045	-1,610560109	FALSE
47,02020202	0,806171954	1,265924942	3,28738484	-1,675040931	FALSE
48,3030303	0,883296318	1,346354087	3,522150328	-1,755557692	FALSE
49,58585859	0,96575831	1,438373304	3,784969986	-1,853453365	FALSE
50,86868687	1,052563694	1,541619027	4,074136988	-1,9690096	FALSE
52,15151515	1,142687804	1,655178929	4,386838505	-2,101462897	FALSE
53,43434343	1,235163189	1,777747902	4,719549077	-2,249222699	FALSE
54,71717172	1,329186908	1,907900706	5,068672293	-2,410298477	FALSE
56	1,423984794	2,04412545	5,430470676	-2,582501088	FALSE



Figure S1: Picture of the opaque screen in climate-controlled aviaries.

Two trees or two pots were placed behind the opaque screen to prevent birds from seeing or eating buds or caterpillars. They could only smell them.

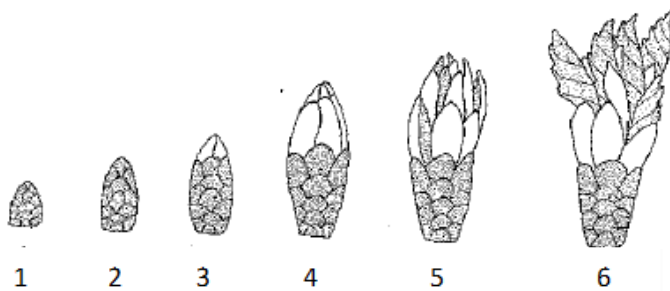


Figure S2: Illustration of bud phenological stages of deciduous oaks (*Quercus sp.*).

Caterpillars can enter the buds during the whole period from stipule lengthening (stage 4) until bud bursting (stage 6). Adapted from Du Merle & Mazet, 1983.

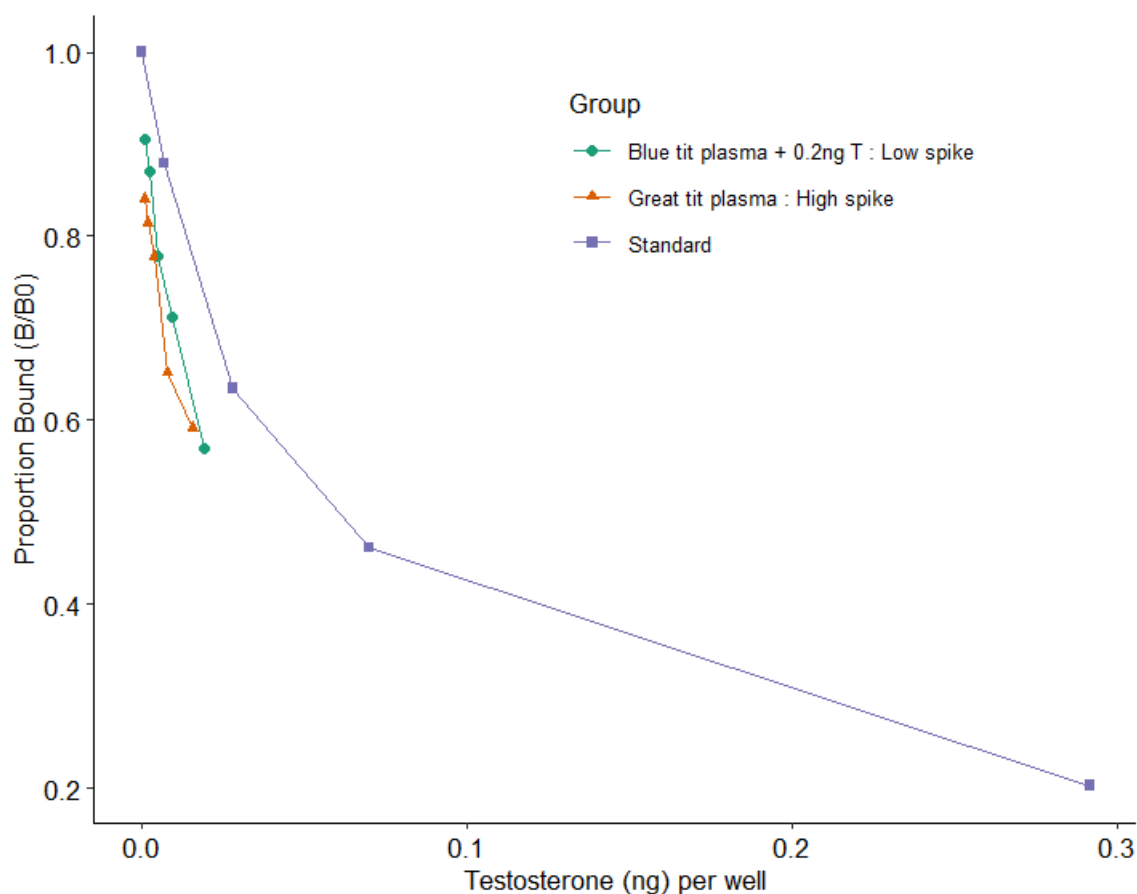


Figure S3: Testosterone assay validation for great tit and blue tit plasma.

To validate the assay in great tits, two plasma pools were created from samples collected from great tits in April 2021 and from blue tits in December 2015. The first pool was spiked without exogenous testosterone per well (high spike) because in April, birds are supposed to have high testosterone level, and the second pool was spiked with 0.2 ng of exogenous testosterone per well (low spike). Each plasma pool was then serially diluted to create a 5-point curve. During validation, testosterone was extracted from 50 μ L of plasma with DEE and methanol/dry ice bath, dried under nitrogen gas at 40°C, and resuspended overnight with 120 μ L assay buffer. Recovery after extraction was 91%.

Supplementary material of Article 6 (Chapter 4)

Olfactory communication from trees to birds: Effects of herbivore-induced plant defences on a songbird behaviours and reproductive decisions.

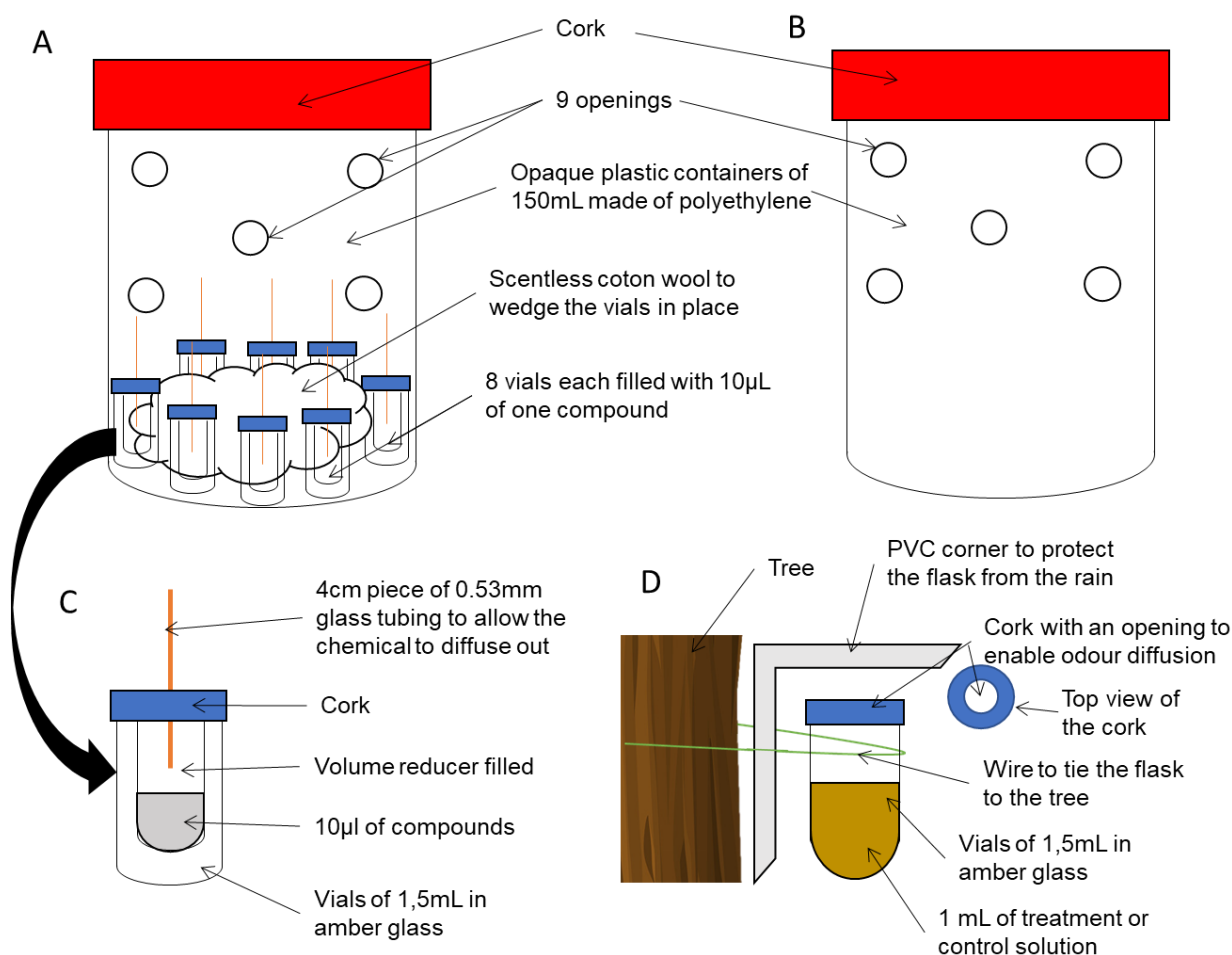


Figure S1. HIPV flasks design.

A, B, C: In 2019-2020 and for the Y-maze experiment, we made flasks to diffuse the artificial HIPVs out with 150 mL opaque plastic containers (diameter 37 mm, Corning, France), previously pierced by 9 mm of diameter holes at the high part. HIPV treatment flasks contained eight 1.5 mL amber glass vials (32 x 11.6 mm, Chromoptic, France) (A) while control flasks were empty (B). Each amber vial contained a volume reducer of 0.1 mL (31 x 6 mm, Chromoptic, France) that was filled with 10 µL of one of the selected compounds. A 4 cm piece of 0.53 mm diameter glass tubing (Chromoptic, France) was pushed through the top seal of the cap to allow the chemical to diffuse out over time (C). At the end of the 2019 breeding season, a random selection of flasks ($n = 3$ control, $n = 3$ HIPV) were returned to the lab to confirm odours were still being emitted. In 2019-2020, three flasks were placed around each nest-box. D: In 2021-2022, each flask was a 1.5 mL amber glass vial filled with 1 mL of treatment (paraffin oil + a defined quantity of each one the eight compounds (see table S1)) or control solution (paraffin oil only). Odour diffused through a wide opening in the vial cork. Five flasks were placed around each nest-box.

Table S1: Quantities of the compounds introduced in 300 mL of paraffin oil to create the treatment flasks in 2021-2022. Control flasks were filled with 300 mL of paraffin oil.

Compounds	Mass (mg)	
	2021	2022
Aromadendrene	244.72	274.16
(E)- β -ocimene	2854.31	2843.22
Farnesene	3034.89	3157.23
(Z)-hex-3-enyl benzoate	3459.13	3455.43
DMNT	3563	3596.73
α -humulene, (E)-caryophyllene	2317.21	2266.40
Methyl salicylate	3051.6	3056.82
	590.74	595.02

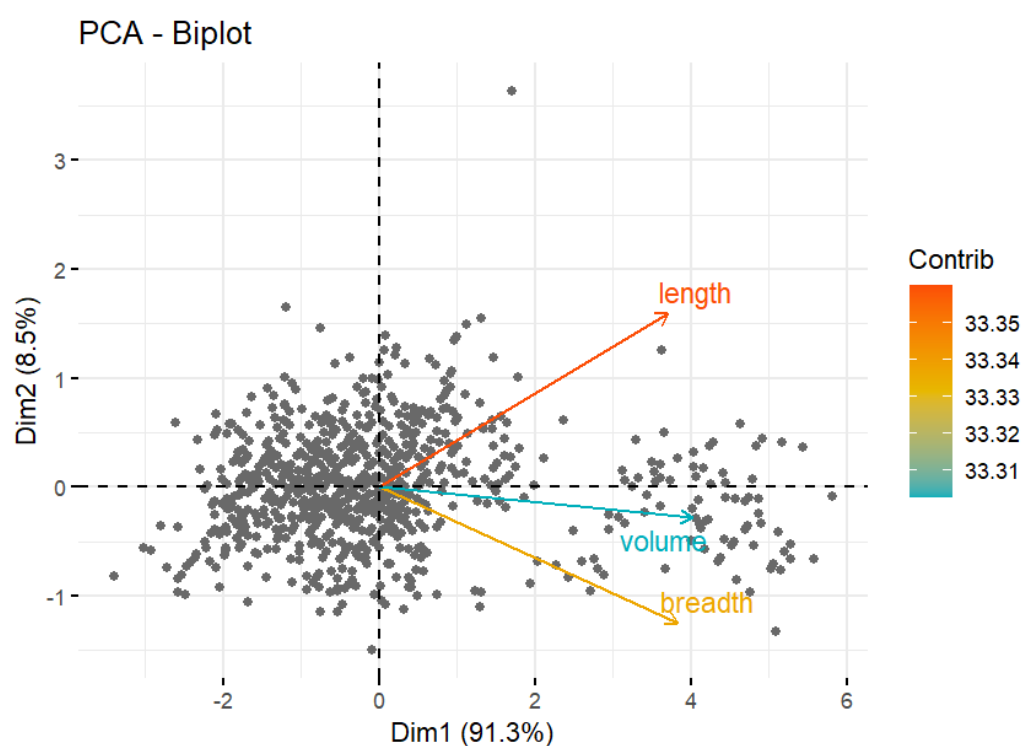


Figure S2: Biplot of the PCA run for egg size. ($n = 847$)

High value of the first axis represents bigger eggs.

Table S2. Results from generalised linear model exploring the variables that influence the first side chosen by a blue tit in a Y maze experiment with HIPV *versus* control smells and generalised linear mixed models exploring the variables that influence the number of dishes visited, and the time spent in each arm during 30 minutes of maze exploration ($n = 29$). Individual identity was included as a random effect in the mixed models. Variables in bold had a significant effect on the response variable ($p < 0.05$), italicized variables depict trends ($p < 0.10$).

Response variable	Explanatory variable	Estimate	std. Error	Statistic	p
<i>First side</i>	(Intercept)	1.91	0.69	z value	
	Sex	0.55	0.99	0.56	0.579
	Exploration score	0.12	0.54	0.22	0.824
	<i>Wind</i>	<i>0.21</i>	<i>0.11</i>	<i>1.84</i>	<i>0.066</i>
	Exploration score *sex	0.09	0.98	0.09	0.931
<i>Dish</i>	(Intercept)	1.83	0.20	z value	
	Sex	-0.37	0.31	-1.19	0.234
	Exploration score	0.07	0.18	0.38	0.702
	Treatment	-0.13	0.16	-0.82	0.414
	Wind	-0.16	0.16	-0.96	0.336
	Exploration score*Sex	-0.29	0.33	-0.91	0.365
	Treatment*Sex	0.62	0.25	2.52	0.012
	Treatment* Exploration score	0.01	0.15	0.08	0.936
	Treatment*Wind	0.44	0.12	3.75	<0.001
Treatment*Exploration score*Sex	0.81	0.26	3.07	0.002	
<i>Time</i>	(Intercept)	681.02	121.82	t value	
	Sex	-239.23	179.08	-1.34	0.845
	Exploration score	146.30	109.13	1.34	0.409
	Treatment	-263.26	172.28	-1.53	0.687
	Wind	-243.01	97.10	-2.50	0.364
	Exploration score *Sex	-387.02	183.46	-2.11	0.282
	<i>Treatment*Sex</i>	<i>428.59</i>	<i>253.25</i>	<i>1.69</i>	<i>0.097</i>
	Treatment* Exploration score	-40.93	154.33	-0.27	0.129
	Treatment*Wind	360.26	137.31	2.62	0.011
	<i>Treatment* Exploration score*Sex</i>	<i>491.94</i>	<i>259.46</i>	<i>1.89</i>	<i>0.064</i>

Table S3. Effect of the treatment and population on counts of occupancy (at least one egg laid), egg number, number of 15 days old nestlings, number of fledglings.

A: Results of generalised linear model exploring a year or population effect and a treatment effect with HIPV *versus* control smells on the Explanatory variables in bold have a significant effect on the response variable ($p < 0.05$), italicized variables depict trends ($p < 0.10$).

B: Results of the post-hoc test run for the significant interactions Treatment*Year and Treatment*Population found in the GLM of number of fledglings. Explanatory variables in bold have a significant effect on the response variable ($p < 0.05$), italicized variables depict trends ($p < 0.10$).

A: Results of the generalised linear model.

Response variable	Explanatory variable	Estimates	std. Error	z-value	p
<i>Occupancy</i> (n=253)	(Intercept)	2.39	3.72		
	Treatment	0.05	0.49	0.10	0.920
	Year [2020]	0.12	0.39	0.34	0.731
	Year [2021]	0.49	0.54	1.47	0.142
	Year [2022]	0.49	0.54	1.47	0.142
	Population	0.17	0.23	0.88	0.380
	Treatment × Year [2020]	0.1	0.52	0.21	0.832
	Treatment × Year [2021]	-0.03	0.44	-0.06	0.954
	Treatment × Year [2022]	0.04	0.47	0.09	0.925
	Treatment × Population	0.09	0.30	0.36	0.720
<i>Egg number</i> (n=2265)	(Intercept)	4.62	10.94		
	Treatment	-0.005	0.15	-0.04	0.972
	Year [2020]	-0.06	0.11	-0.51	0.611
	Year [2021]	0.37	0.15	3.53	<0.001
	Year [2022]	0.35	0.15	3.38	0.001
	Population	0.32	0.09	4.74	<0.001
	Treatment × Year [2020]	0.24	0.19	1.56	0.118
	Treatment × Year [2021]	0.04	0.15	0.30	0.766
	Treatment × Year [2022]	0.09	0.16	0.65	0.515
	Treatment × Population	0.07	0.10	0.74	0.457
<i>Number of 15 days old nestlings</i> (n=1715)	(Intercept)	3.83	7.21		
	Treatment	0.22	0.26	1.10	0.272
	Year [2020]	0.47	0.26	2.88	0.004
	Year [2021]	1.12	0.47	7.34	<0.001
	Year [2022]	0.91	0.39	5.86	<0.001
	<i>Population</i>	0.14	0.09	1.88	0.060
	Treatment × Year [2020]	0.08	0.23	0.36	0.721
	Treatment × Year [2021]	-0.25	0.16	-1.25	0.213

	Treatment x Year [2022]	-0.08	0.19	-0.40	0.688
	Treatment x Population	0.17	0.12	1.71	0.087
Number of fledglings (n=1248)	(Intercept)	4.07	9.86		
	Treatment	-0.46	0.15	-1.94	0.052
	Year [2020]	-0.09	0.16	-0.51	0.613
	Year [2021]	0.58	0.30	3.50	<0.001
	Year [2022]	0.52	0.28	3.10	0.002
	Population	-0.16	0.08	-1.70	0.090
	Treatment x Year [2020]	0.92	0.62	3.70	<0.001
	Treatment x Year [2021]	0.39	0.35	1.66	0.097
	Treatment x Year [2022]	0.73	0.49	3.08	0.002
	Treatment x Population	0.34	0.17	2.80	0.005

B: Results of the post-hoc test ran for the significant interactions in the GLM of number of fledglings.

<i>Interaction tested</i>	<i>Pairwise comparison</i>	<i>Estimates</i>	<i>std. Error</i>	<i>z- ratio</i>	<i>p</i>
<i>Treatment x Year</i>	Control 2019 - HIPV 2019	0.29	0.22	1.38	0.868
	Control 2020 - HIPV 2020	-0.62	0.12	-5.01	<0.001
	Control 2021 - HIPV 2021	-0.09	0.09	-0.98	0.978
	Control 2022 - HIPV 2022	-0.43	0.09	-4.51	<0.001
<i>Treatment x Population</i>	Control Corsica - HIPV Corsica	-0.05	0.10	-0.45	0.970
	Control Corsica - Control Mainland	0.16	0.09	1.69	0.326
	<i>Control Corsica - HIPV Mainland</i>	-0.23	0.09	-2.45	0.069
	HIPV Corsica - Control Mainland	0.20	0.09	2.15	0.137
	<i>HIPV Corsica - HIPV Mainland</i>	-0.18	0.08	-2.34	0.089
	Control Mainland - HIPV Mainland	-0.38	0.08	-4.59	<0.001

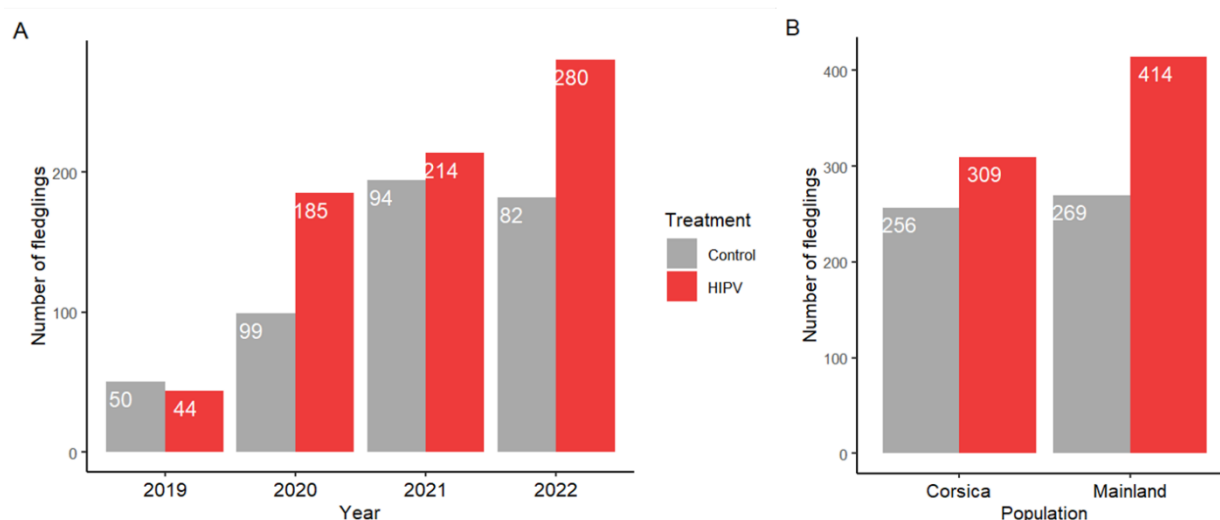


Figure S3: Number of fledglings across years and populations

A: Wild blue tits and great tits produced more fledglings in HIPV in 2020 ($p < 0.001$) and 2022 ($p < 0.001$) compared to control nest-boxes.

B: Wild blue tits and great tits produced more fledglings in HIPV in the Mainland ($p < 0.001$) compared to control nest-boxes.

Table S4: Effect of the odour treatment at the individual level.

A: Results of mixed models analyses exploring factors (including the HIPV versus control treatment) influencing laying date, clutch size, reproductive success, nestling weight, and eggs size. Explanatory variables in bold have a significant effect on the response variable ($p < 0.05$), italicized variables depict trends ($p < 0.10$).

Reponse variable	Explanatory variable	Estimates	std. Error	Statistic	p
<i>Lay date</i> (n = 253)	(Intercept)	31.43	3.52	t value	
	Treatment	7.34	4.81	1.52	0.129
	Year [2020]	4.44	3.66	1.21	0.226
	Year [2021]	3.52	3.30	1.07	0.288
	Year [2022]	11.19	3.43	3.26	0.001
	Population	5.22	2.22	2.35	0.019
	Species	-2.55	3.48	-0.73	0.463
	Treatment × Year [2020]	-8.21	5.15	-1.60	0.112
	Treatment × Year [2021]	-5.35	4.56	-1.17	0.241
	Treatment × Year [2022]	-5.24	4.89	-1.07	0.285
	Treatment × Population	-5.56	2.76	-2.01	0.045
Treatment × Species	7.57	4.61	1.64	0.102	
<i>Clutch size</i> (n=253)	(Intercept)	2.23	0.11	z value	
	Treatment	-0.06	0.14	-0.37	0.711

	Year [2020]	-0.16	0.10	-1.41	0.160
	Year [2021]	-0.13	0.09	-1.25	0.213
	Year [2022]	-0.14	0.09	-1.34	0.181
	Population	0.16	0.08	2.25	0.024
	Species	-0.09	0.10	-0.83	0.408
	Treatment x Year [2020]	0.13	0.18	0.86	0.390
	Treatment x Year [2021]	0.08	0.16	0.53	0.597
	Treatment x Year [2022]	0.05	0.15	0.36	0.720
	Treatment x Population	-0.02	0.09	-0.26	0.796
	Treatment x Species	-0.02	0.15	-0.12	0.902
	(Intercept)	9.64	0.40	t value	
	Treatment	0.17	0.51	0.32	0.747
	Year [2020]	0.73	0.40	1.85	0.064
	Year [2021]	1.02	0.37	2.73	0.006
	Year [2022]	0.98	0.39	2.50	0.013
	Population	-0.11	0.25	-0.45	0.650
	Species	4.74	0.43	11.02	<0.001
<i>Nestling weight (n = 1349)</i>	Treatment x Year [2020]	-0.23	0.59	-0.39	0.694
	Treatment x Year [2021]	-0.62	0.52	-1.19	0.233
	Treatment x Year [2022]	-0.34	0.56	-0.61	0.545
	Treatment x Population	0.24	0.22	1.13	0.261
	Treatment x Species	1.13	0.56	2.02	0.043
	(Intercept)	7.26	5.77	z value	
	Treatment	1.31	1.56	0.23	0.818
	Year [2020]	0.55	0.44	-0.75	0.453
	Year [2021]	0.67	0.48	-0.56	0.579
	Year [2022]	1.16	0.85	0.20	0.845
	Population	0.18	0.10	-3.14	0.002
	Species	4.61	3.91	1.80	0.071
	Treatment x Year [2020]	1.68	1.83	0.48	0.634
	Treatment x Year [2021]	1.19	1.17	0.17	0.862
	<i>Treatment x Year [2022]</i>	10.13	13.98	1.68	0.093
	Treatment x Population	1.30	1.16	0.30	0.768
<i>Reproductive success (number of fledglings/ number of hatchlings) (n = 235)</i>					

	<i>Treatment x Species</i>	0.12	0.13	-1.88	0.060
<i>Egg size (PCA) (n = 847)</i>	(Intercept)	-0.52	0.14	t value	
	Treatment	0.02	0.20	0.12	0.906
	Year	0.01	0.20	0.06	0.953
	Species	4.66	0.18	26.13	<0.001
	Treatment x Year	0.06	0.38	0.16	0.875
	Treatment x Species	-0.26	0.2	-1.32	0.19

B: Results of the post-hoc test ran for the significant interactions in the linear model of laying date and nestling weight. Explanatory variables in bold have a significant effect on the response variable ($p < 0.05$), italicized variables depict trends ($p < 0.10$). BT: Blue tit, GT: Great tit.

<i>Response variable</i>	<i>Interaction tested</i>	<i>Pairwise comparison</i>	<i>Estimates</i>	<i>std. Error</i>	<i>t-ratio</i>	<i>p</i>
<i>Lay date</i>	Treatment x Population	Control Corsica - HIPV Corsica	-6.43	3.22	-1.99	0.192
		<i>Control Corsica - Control Mainland</i>	-5.22	2.22	-2.35	0.091
		Control Corsica - HIPV Mainland	-6.09	2.99	-2.04	0.178
		HIPV Corsica - Control Mainland	1.21	2.90	0.42	0.976
		HIPV Corsica - HIPV Mainland	0.34	2.07	0.16	0.999
		Control Mainland - HIPV Mainland	-0.87	2.34	-0.37	0.982
		<i>Nestling weight</i>	Treatment x Species	Control BT – HIPV BT	0.01	0.15
Control BT – Control GT	-4.74			0.43	-10.95	<0.001
Control BT – HIPV GT	-5.86			0.42	-14.11	<0.001
HIPV BT – Control GT	-4.75			0.42	-11.44	<0.001
HIPV BT – HIPV GT	-5.87			0.41	-14.41	<0.001
Control GT – HIPV GT	-1.12			0.53	-2.11	0.156

Additional analyses in the general discussion

- To study the potential interactive effects of the exposure to HIPVs and social relationships within a pair on gonadal size (data used in **Chapter 3, article 4**) in the part of the discussion entitled “Multimodal cues: integrating multiple sensory signals in reproductive decision-making“, I ran a linear mixed-effect model for each measurement with the male gonadal size as response variable. I included in these models an interaction between the gonadal size of the female and the treatment that the birds received (control air or HIPVs) as a fixed effect, and the year and the identification of the breeding pair as a random effects.
- To study the potential interactive effects of the exposure to HIPVs and female mate preference on female reproduction (data described in **Chapter 1, article 2** and **Chapter 3, article 5**) in the part of the discussion entitled “Multimodal cues: integrating multiple sensory signals in reproductive decision-making“, I studied the female oestradiol level, laying date and clutch size in relation to the female mate preference and exposure of birds to HIPVs. I ran linear models on mean oestradiol levels and laying dates and a generalized linear model on clutch size. In all models, I included an interaction between the female mate preference (time spent with the male in the preference test (s)) and the treatment that the birds received (control air or HIPVs) as fixed effect.

RESUMÉ EN FRANÇAIS

Contexte

L'aptitude des organismes à survivre et à se reproduire réside dans leur capacité à adapter leur comportement et leur physiologie aux variations de l'environnement. Les individus doivent donc adapter leur phénologie, c'est-à-dire la chronologie de chacun des événements biologiques de leur cycle de vie, aux conditions environnementales de chacune des saisons⁹⁻¹¹. Dans le cas de la reproduction, qui est l'étape du cycle de vie que j'ai étudiée dans cette thèse, le moment optimal pour donner naissance à une nouvelle génération correspond à la période pendant laquelle la nourriture est abondante. Les organismes s'assurent ainsi que la période pendant laquelle leur progéniture aura le plus de besoins énergétiques coïncide avec une période de forte disponibilité alimentaire¹⁶. Cependant, les conditions environnementales varient d'une année à l'autre, entraînant alors une variation du moment du pic d'abondance de nourriture. Les individus doivent donc utiliser des facteurs, également appelés indices, pour prédire et anticiper la phénologie de leurs proies, et ainsi adapter leur cycle de reproduction en conséquence^{22,26-31}. Les facteurs utilisés peuvent être nombreux et, dans cette thèse, j'en ai étudié deux de différentes natures : un facteur social lié à la préférence pour le partenaire, et un indice environnemental lié à la phénologie de la végétation.

Dans les couples monogames, les individus partagent de nombreux comportements qui sont exclusivement dirigés vers leur partenaire. Ces comportements nécessitent une grande coordination⁴⁴ et contribuent à renforcer leurs liens. Chez les oiseaux, qui sont le modèle d'étude de ma thèse, les stimulations mutuelles entre les partenaires peuvent favoriser, voire accélérer la mise en place du système reproducteur et ainsi permettre une flexibilité temporelle quant au moment de la reproduction⁴⁸. Le choix d'un partenaire, ainsi que le temps que les partenaires passent ensemble, affectent leur capacité à coordonner leurs comportements et à améliorer le succès de leur reproduction^{66,67}. Par conséquent, le degré de préférence que la femelle exprime pour son partenaire peut influencer sa reproduction⁶⁸⁻⁷⁰. La théorie de l'allocation différentielle^{71,72} suggère que la qualité du mâle module l'investissement des femelles en matière de reproduction⁷³⁻⁷⁶, ainsi que la viabilité de la progéniture^{77,78}. La théorie de l'allocation des sexes, quant à elle, prédit que les femelles ajustent le sex-ratio de leur couvée en fonction de la qualité du mâle avec lequel elles sont en couple^{82,84-87}. Elles produiraient alors plus de mâles si celui-ci est de bonne qualité. Le fait d'être accouplé à un partenaire non

désiré peut par conséquent réduire la motivation d'un individu à se reproduire, ou causer un stress physiologique altérant les performances reproductives ^{70,89-92}.

Si la reproduction saisonnière se déroule bien dans un contexte social, les membres d'un couple reproducteur ne doivent pour autant pas seulement se coordonner entre eux ; ils doivent également se coordonner avec leur environnement. Par conséquent, les organismes s'appuient sur des indices environnementaux, pour tenter de prédire les conditions environnementales au moment de l'élevage de leur progéniture. Chez la plupart des vertébrés, la photopériode, c'est-à-dire le changement de la longueur du jour, est le principal indice que les individus utilisent pour initier et réguler les cascades physiologiques et comportementales qui régissent la reproduction ⁹⁴⁻⁹⁶. Cependant, les changements saisonniers de photopériode étant les mêmes d'une année à l'autre, des indices supplémentaires aident les individus à prédire la période optimale de reproduction, qui, elle, varie annuellement. Il existe de nombreux indices environnementaux supplémentaires qui modulent l'activité reproductive des organismes, tels que la température, la disponibilité en nourriture ou encore les précipitations. Dans cette thèse, je me suis intéressée à un indice en particulier, qui est la phénologie de la végétation.

La végétation constitue le premier niveau de la plupart des chaînes alimentaires, y compris celles des oiseaux insectivores que j'ai étudiés, à savoir la mésange bleue et la mésange charbonnière. La survie de nombreuses espèces de chenilles, qui constituent la nourriture idéale pour les poussins des mésanges ¹⁶⁹⁻¹⁷¹, dépend de la synchronisation entre leur émergence et l'ouverture des bourgeons de leurs arbres hôtes ^{125,174,179,181-185}. La phénologie de la végétation, et en particulier le développement des bourgeons, a ainsi été suggérée comme un indice supplémentaire que les oiseaux pourraient utiliser pour prédire la période du futur pic d'abondance de chenilles, et ainsi ajuster le moment de l'élevage des poussins ^{186,187}. Cette hypothèse a été étayée par plusieurs observations de terrain ^{187,195,196} mais voir ²⁰³. Par exemple, une population de mésanges bleues corses vivant dans une forêt de chênes à feuilles persistantes, dont la phénologie est caractérisée par un débourrement tardif, se reproduit un mois plus tard qu'une population vivant dans une forêt de chênes à feuilles caduques, dans laquelle le débourrement a lieu un mois plus tôt ^{186,191-194}. Une question qui reste en suspens est de savoir par quel canal sensoriel les oiseaux pourraient utiliser la phénologie de la végétation comme indice prédictif de la disponibilité en chenilles pour orchestrer leur reproduction. Les plantes produisent un large éventail de Composés Organiques Volatils (COVs) impliqués dans la communication végétale et les fonctions écophysiologiques ^{205,206}. Les oiseaux pourraient alors utiliser leur sens de l'odorat pour évaluer le développement de la végétation.

Les oiseaux possèdent un système olfactif qui leur permet de détecter des odeurs à de très faibles concentrations et dans des contextes variés²⁰⁴. Lorsque des insectes herbivores se nourrissent de feuilles, les plantes émettent un type particulier de COVs d'alarme, que l'on appelle en anglais les « Herbivore-Induced Plant Volatiles » (HIPVs), afin d'attirer les prédateurs de ces insectes^{208–210}. De nombreuses études ont montré que les oiseaux pouvaient utiliser les HIPVs pour différencier des arbres infestés de chenilles d'arbres non infestés^{202,203,267,269,271,272,276,277,258–261,263–266} et ainsi optimiser leur recherche de nourriture. Étant donné que les émissions de HIPVs reflètent le moment de l'émergence et l'abondance des chenilles présentes dans l'environnement^{211–213,322}, en utilisant l'information fournie par les HIPVs au début du printemps, les oiseaux pourraient d'une part ajuster le moment de leur reproduction afin de synchroniser la période de nourrissage de leurs poussins avec le moment où l'abondance de chenilles est maximale, et d'autre part ajuster la taille de leur couvée pour qu'elle corresponde à la quantité de chenilles qui sera disponible au moment de l'élevage des poussins.

Objectifs et méthodes

Cette thèse vise à clarifier la manière dont les signaux sociaux et environnementaux influencent les décisions reproductives de deux oiseaux insectivores, la mésange bleue et la mésange charbonnière. Plus spécifiquement, j'ai étudié l'effet de la préférence de la femelle pour son partenaire, ainsi que l'importance du développement de la végétation et des HIPVs sur la période reproductive et l'investissement reproducteur de ces oiseaux.

Dans le **Chapitre 1**, j'ai étudié l'effet de la préférence de la femelle pour son partenaire sur son entrée en reproduction et son niveau d'investissement. De nombreuses études sur la préférence des femelles ont été menées dans la nature^{295–299}, où il est souvent impossible de démêler les rôles respectifs des différentes composantes impliquées dans le choix du partenaire^{300–303}. Les études en captivité, quant à elles, impliquent fréquemment des manipulations expérimentales des caractéristiques des mâles et les femelles n'ont généralement le choix qu'entre peu de mâles^{304,305,314–320,306–313}. Dans ce premier chapitre, j'ai adopté une approche intermédiaire, combinant les avantages des études sur le terrain et en captivité. J'ai mené deux expériences distinctes avec des mésanges bleues (**article 1**) et des mésanges charbonnières (**article 2**). Dans un premier temps, j'ai évalué la préférence des femelles devant un panel de six mâles et j'ai analysé quels traits phénotypiques avaient motivé leurs choix. Dans un second temps, j'ai apparié ces mêmes femelles avec soit un mâle qu'elles ont plébiscité, soit un mâle qu'elles ont évité, et j'ai testé l'impact de ces appariements sur leurs paramètres reproducteurs.

J'ai émis l'hypothèse que les femelles appariées avec leur mâle préféré montreraient un développement physiologique plus rapide, qu'elles pondraient plus tôt, des œufs plus nombreux et plus gros, et qu'elles produiraient davantage d'œufs fécondés (théorie de l'allocation différentielle) et de descendants mâles (théorie de l'allocation des sexes).

Dans les chapitres suivants, j'ai étudié si et comment les oiseaux utilisent la phénologie de la végétation comme un indice pour décider de quand se reproduire et de combien s'investir dans la reproduction. Pour étudier ces questions, j'ai combiné des approches descriptives (**Chapitre 2**) et expérimentales, à la fois en laboratoire (**Chapitres 3 et 4**) et en milieu naturel (**Chapitre 4**).

Dans le **Chapitre 2**, j'ai tout d'abord décrit la corrélation existant sur le terrain entre le débourrement des bourgeons de chênes et la reproduction des mésanges. Cette relation a été décrite dans plusieurs populations^{186,187,195,321}, mais jamais à l'échelle du territoire d'un seul couple reproducteur, c'est-à-dire à l'échelle individuelle. À partir d'images satellites à haute résolution, j'ai quantifié le développement de la végétation autour de chaque nichoir. J'ai ensuite analysé si les dates de ponte des oiseaux étaient corrélées aux dates de verdissement de la végétation, et si les couples dont la reproduction était la mieux synchronisée avec le verdissement avaient un meilleur succès reproducteur (**article 3**). J'ai émis l'hypothèse qu'un développement plus tardif de la végétation serait associé à des dates de ponte plus tardives, et que les individus dont la phénologie de reproduction serait mieux synchronisée avec celle des arbres locaux auraient un meilleur succès reproducteur.

Dans le **Chapitre 3**, j'ai étudié en captivité l'effet des HIPVs sur le comportement reproducteur et la physiologie reproductive des oiseaux. Dans une première expérience, des couples de mésanges bleues ont été hébergés dans des compartiments hermétiques dans lesquels l'air provenait soit d'enceintes contenant des chênes infestés de chenilles, soit d'enceintes vides (**article 4**). La croissance des gonades des oiseaux a été suivie durant le printemps, en émettant l'hypothèse que la perception des HIPVs induirait une croissance plus rapide des ovaires et des testicules. Dans une deuxième expérience, des couples de mésanges charbonnières ont été hébergés dans des volières climatisées et exposés à des chênes infestés de chenilles, cachés derrière un écran opaque, afin que les oiseaux puissent sentir les HIPVs sans voir les arbres (**article 5**). Toutes les deux semaines, des échantillons de sang ont été prélevés sur les oiseaux pour déterminer leurs niveaux d'hormones sexuelles. J'ai émis l'hypothèse que les oiseaux exposés aux HIPVs présenteraient une augmentation plus précoce et plus importante de leurs concentrations de stéroïdes sexuels. Dans les deux expériences, j'ai également suivi la

reproduction des oiseaux (date de ponte, taille de la couvée, taille des œufs), en supposant que les oiseaux exposés au signal olfactif pondraient plus tôt, plus d'œufs et de plus grande taille.

Enfin, dans le **Chapitre 4**, j'ai examiné l'effet d'HIPVs artificiels imitant les émissions de bourgeons de chênes infestés de chenilles sur la reproduction des mésanges dans des populations sauvages. J'ai d'abord testé, en captivité et sur des mésanges bleues, l'attractivité d'un mélange artificiel d'HIPVs élaboré en amont de ma thèse (**article 6**), afin d'une part de valider si ce bouquet d'odeurs pouvait être utilisé sur le terrain, et d'autre part de tester si la perception des HIPVs par les oiseaux était innée ou acquise. J'ai ensuite utilisé le bouquet artificiel dans des populations naturelles pour tester si la présence de ces odeurs au début du printemps modifiait l'investissement reproducteur des oiseaux. Puisque ce signal olfactif fournit un signal de présence précoce des chenilles et à des concentrations plus élevées que naturellement, j'ai émis l'hypothèse que les oiseaux installés dans les nichoirs entourés de HIPVs artificiels pondraient plus tôt, plus d'œufs et de plus grande taille.

Principaux résultats

Dans le **Chapitre 1**, j'ai étudié l'entrée et l'investissement dans la reproduction de mésanges femelles en fonction de leur degré de préférence pour le mâle avec lequel elles étaient appariées. J'ai trouvé que les femelles de mésanges charbonnières sélectionnaient leur partenaire en fonction du comportement exploratoire du mâle, tandis que les femelles de mésanges bleues les sélectionnaient en fonction de caractéristiques liées aux couleurs du plumage. J'ai ensuite apparié ces femelles à des mâles plus ou moins désirés pour la saison de reproduction. Les femelles de mésange charbonnière appariées avec leur mâle préféré ont avancé leur date de ponte (**article 2**), tandis que les mésanges bleues n'ont pas adapté leur reproduction, qu'elles soient appariées à leur mâle préféré ou évité (**article 1**).

Dans le **Chapitre 2**, j'ai étudié la corrélation entre le débourrement des arbres et l'investissement reproducteur des mésanges dans différentes populations méditerranéennes. Je n'ai trouvé aucune preuve claire que la dynamique de développement de la végétation influençait les dates de ponte ou le succès reproducteur des oiseaux à l'échelle individuelle. Cependant, à l'échelle de la population, j'ai mis en évidence une corrélation positive entre la date de ponte des oiseaux et le verdissement de la végétation (**article 3**).

Dans le **Chapitre 3**, j'ai utilisé des approches expérimentales en captivité pour étudier l'effet des odeurs émises par les arbres infestés de chenilles sur la physiologie reproductive des

oiseaux. Dans les deux expériences constituant ce chapitre, les mésanges n'ont pas pondu plus tôt, ni plus d'œufs lorsqu'elles étaient exposées aux HIPVs. En présence d'odeurs d'arbres infestés de chenilles, chez les mésanges bleues, les femelles les plus exploratrices ont développé des follicules ovariens plus gros que dans le groupe contrôle (**article 4**), alors que chez les mésanges charbonnières, les mâles ont présenté des taux de testostérone plus élevés pendant la période de la ponte (**article 5**) par rapport aux oiseaux non exposés aux odeurs.

Dans le **Chapitre 4**, au cours d'une expérience de quatre ans, j'ai examiné l'effet d'un mélange artificiel d'HIPVs mimant les odeurs émises par des bourgeons de chênes infestés de chenilles sur les décisions de reproduction de mésanges en populations naturelles. Dans une expérience préliminaire en captivité testant l'attractivité des mésanges bleues naïves aux HIPVs, j'ai trouvé que les oiseaux pouvaient détecter et s'orienter vers le bouquet artificiel d'odeurs de manière innée (**article 6**). Sur le terrain, les oiseaux dont les nichoirs étaient entourés de HIPVs n'ont pas avancé leurs dates de ponte, ni augmenté la taille de leur couvée, mais davantage de nichoirs équipés de HIPVs ont été occupés par rapport aux nichoirs témoins. Par conséquent, un plus grand nombre d'œufs et d'éclosions ont été constatés dans ces nichoirs et un plus grand nombre de poussins se sont envolés à partir des nichoirs entourés de HIPVs (**article 6**).

Discussion

Le rôle des préférences sexuelles et de la phénologie végétale dans la reproduction des oiseaux.

Les effets observés de la végétation sur la reproduction des oiseaux se sont, d'une façon générale, révélés moindres qu'attendus. Je n'ai par exemple jamais pu mettre en évidence un effet de la végétation sur les dates individuelles de ponte, ni sur les tailles de ponte, et ce aussi bien dans mes études corrélatives (**Chapitre 2**), que dans mes approches expérimentales manipulant les odeurs émises par les arbres (**Chapitres 3 et 4**). On serait tentés d'en conclure que le développement de la végétation au printemps ne constitue pas une source d'information dont les oiseaux font usage pour moduler leur effort reproducteur. Cela semble pourtant prématuré dans la mesure où plusieurs résultats de ma thèse montrent que les mésanges sont sensibles à ce facteur environnemental, bien que nous ne comprenions encore pas dans les détails les processus en jeu. J'ai notamment pu montrer que les mésanges bleues étaient attirées par les HIPVs, et ce sans même les avoir appris, suggérant un mécanisme inné de

reconnaissance de l'importance de ces signaux (**Chapitre 4**). Au-delà de cet effet comportemental des HIPVs, j'ai également mis en évidence des effets sur la physiologie de la reproduction, avec des effets stimulants des odeurs de bourgeons infestés de chenilles sur les taux de testostérone chez les mâles (**Chapitre 3 article 5**) et sur la croissance des follicules ovariens des femelles les plus exploratrices (**Chapitre 3 article 4**). Ces effets stimulants des odeurs végétales sur les traits comportementaux et physiologiques des mésanges se sont accompagné d'effets mesurés à l'échelle des populations en milieu naturel. J'ai trouvé que les dates moyennes de ponte étaient corrélées avec les dates moyennes de débourrement des arbres au cours de quatre des cinq années du suivi effectué (**Chapitre 2**). De plus, un nombre plus important d'œufs et de poussins ont été produits dans les zones où j'ai artificiellement augmenté les émissions de composés volatils d'alarme (**Chapitre 4**). La détection d'un effet du développement de la végétation sur la période de reproduction ou l'investissement des oiseaux à l'échelle individuelle nécessite donc probablement des études à plus long terme avec des échantillons de taille plus importante.

Le développement de la végétation est, à juste titre, classé parmi les "indices supplémentaires" que les oiseaux peuvent utiliser pour orchestrer leur reproduction. Cela suggère donc bien que les potentiels effets de cet indice sur le calendrier saisonnier des animaux sont subtils et complémentaires d'autres indices. De la même façon, les facteurs sociaux représentent aussi des indices supplémentaires. J'ai trouvé un effet de la préférence de la femelle pour son partenaire chez les mésanges charbonnières (**Chapitre 1 article 2**) mais pas chez les mésanges bleues (**Chapitre 1 article 1**). Une des raisons pourrait être que les femelles de mésange bleue sont indifférentes à leurs partenaires, mais il se pourrait aussi que chez cette espèce, les facteurs sociaux interagissent avec d'autres facteurs environnementaux qui masqueraient l'effet de la préférence pour le partenaire sur la reproduction. De telles interactions entre les indices environnementaux sont probablement très répandues dans la nature. Cependant, les étudier dans des conditions contrôlées est un défi en raison de la complexité des plans expérimentaux et de la taille importante des échantillons requis.

En définitive, cette thèse souligne que nous avons encore peu de connaissances sur les mécanismes physiologiques impliqués dans l'utilisation des signaux environnementaux et sociaux pour réguler la reproduction. La meilleure preuve de l'impact de ces signaux sur la reproduction est de détecter les mécanismes que les femelles utilisent pour identifier et combiner ces indices avec les cascades neuroendocrines régulant les étapes finales de la ponte.

Conclusion

Répondre à de multiples indices est nécessaire pour que les organismes puissent ajuster leur phénotype dans des environnements complexes. Dans cette thèse, j'ai combiné des approches descriptives et expérimentales, à la fois en conditions contrôlées et sur le terrain, pour explorer l'effet d'un facteur social, la préférence des femelles pour leur mâle, et d'un indice environnemental, le développement de la végétation et ses signaux olfactifs d'alarme, sur les décisions reproductives et les mécanismes sous-jacents de la reproduction saisonnière aviaire. Ces stratégies complémentaires m'ont permis de mettre en évidence un avancement de la ponte liée à la préférence du partenaire, un investissement reproductif plus important et des ajustements physiologiques des hormones sexuelles et du développement gonadique suite à l'exposition aux signaux olfactifs émis par des bourgeons d'arbres infestés de chenilles. Les résultats de cette thèse suggèrent tout de même que nous sommes en présence de mécanismes dont les effets sont relativement subtils. Cependant, les expériences peuvent peiner à rendre compte avec précision de la complexité des conditions réelles. Comme suggéré dans une précédente thèse, la décision d'initier la reproduction peut être conceptualisée comme une série de feux de signalisation. Les expériences menées sur un seul indice isolé des autres peuvent faire passer le feu au vert pour ce signal particulier, mais pas pour les autres, de sorte que la reproduction ne peut pas commencer. La multitude de facteurs environnementaux susceptible d'avoir un effet sur la reproduction, la complexité de leur intégration, les multiples interactions qui se produisent entre eux et les nombreuses « inconnues » font du comportement reproductif un mystère difficile à élucider.

Abstract

For most seasonal organisms, successful reproduction requires synchronising the rearing period of the offspring with the timing of food availability. Individuals must therefore use cues present in the environment to predict the timing of the food peak and organise their reproduction accordingly. In monogamous species sharing parental care, reproductive success also relies on the strong coordination between partners. In this thesis, I examine whether and how two passerine birds, the blue and great tits, use social and environmental cues to orchestrate their reproduction. Females of these species engage in mate selection, but whether their preference for a partner influences their reproductive decisions has not been explored yet, and this constitutes the first research avenue of this thesis. Although breeding pairs must coordinate with each other, they also have to coordinate with their environment. Birds rely on environmental cues to adapt their reproductive timing with the period of food abundance. Since caterpillars, the main food source used by blue and great tits to feed their nestlings, hatch synchronously with tree budburst, tree phenology has been suggested to be a supplementary cue used by birds to predict caterpillar phenology and thus adapt their reproduction accordingly. Furthermore, tree buds emit olfactory alarm signals when they are grazed by caterpillars. A second research line of this thesis thus explores whether and how birds use vegetation development and olfactory signals from buds to coordinate their reproductive activities with their prey phenology. Combining descriptive and experimental approaches, both in captivity and in the field, this thesis sheds light on how seasonal species adapt to a constantly changing world, ingeniously matching their life cycles to environmental fluctuations.

Keywords: Reproduction, insectivorous birds, supplementary cues, vegetation phenology, Herbivore-Induced Plant Volatiles, mate choice

Résumé

Pour la plupart des animaux saisonniers, une reproduction réussie nécessite la synchronisation de la période d'élevage des jeunes avec la disponibilité en nourriture. Les individus doivent alors prédire le moment du pic de nourriture et organiser leur reproduction en conséquence. Néanmoins, chez les espèces partageant les soins parentaux, le succès de la reproduction repose également sur une forte coordination des deux partenaires. Dans cette thèse, j'examine si et comment deux passereaux, les mésanges bleue et charbonnière, utilisent des signaux sociaux et environnementaux pour orchestrer leur reproduction. Dans un premier temps, cette thèse explore si la préférence de la femelle pour son partenaire influence ses décisions en matière de reproduction. En ce qui concerne les signaux environnementaux, les chenilles, essentielles à la survie des poussins, éclosent de manière synchrone avec le débourrement des bourgeons des arbres et ces derniers émettent des signaux olfactifs d'alarme lorsqu'ils sont attaqués par ces mêmes chenilles. Un deuxième axe de recherche de cette thèse explore ainsi si, et comment, les oiseaux utilisent le développement de la végétation, notamment via ces signaux olfactifs, pour coordonner leurs activités de reproduction avec la phénologie de leurs proies. Combinant des approches descriptives et expérimentales, en captivité et sur le terrain, cette thèse met en lumière la façon dont les espèces saisonnières s'adaptent à un monde en constante évolution, faisant ingénieusement correspondre leurs cycles de vie aux fluctuations environnementales.

Mot-clés : Reproduction, oiseaux insectivores, indices supplémentaires, phénologie végétale, composés organiques volatiles, choix de partenaire