



# Is there a role for aromatic plants in blue tit (*Cyanistes caeruleus*) nests? Results from a correlational and an experimental study

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## Abstract

The utility of fresh green material in avian nests is still not fully understood. Potential explanations include the effects of plants' volatile compounds on parasite reduction (nest protection hypothesis) or direct beneficial effects on nestling condition (drug hypothesis). We used correlative data collected during 2020 and 2021 in a Mediterranean population of blue tits (*Cyanistes caeruleus*) as well as experimental data (aromatic nest content manipulation) to assess the effects of aromatic plant use on nestling physiological condition and survival, nest-dwelling ectoparasitic pressure and its relationship with breeding parameters. We found that aromatic plants were disproportionally used in relation to their abundance in the environment and that their use was positively related to egg mass (but only in 2020). Blowflies and facultative parasitic mites were more frequent in nests with aromatics compared to nests without aromatics, but obligatory parasitic mites were less abundant in nests with aromatics. However, no effects of aromatic plants were observed on nestling haemoglobin levels nor erythrocyte sedimentation rate or other physiological health metrics, but the heterophil to lymphocyte ratio was higher in nests with the highest quantity of aromatics. The artificial addition of mint reduced the flea abundance, but 7-day old nestlings showed significantly lower colour saturation and brightness in the mouth flange. Nestling survival to fledging was not related to aromatic plant use. Therefore, our results partially support a beneficial effect of aromatic plants in blue tit nests because some ectoparasite groups were reduced. Immediate effects on nestling physiology or survival could not be established.

## Significance statement

Some avian species place fresh aromatic plant material in their nests, and several non-mutually exclusive hypotheses have been proposed to explain its potential functions. In this study, we use both correlational and experimental data from a blue tit population to test two hypotheses, namely the 'nest protection hypothesis' and the 'drug hypothesis'. The first one proposes that aromatic plants have direct repellent effects against ectoparasites, while the latter poses that these plant components benefit nestling condition through the stimulation of some components of the immune system. Our results suggest that some ectoparasites, such as fleas and obligatory parasitic mites, were less abundant in nests where aromatic plants were artificially or naturally added, respectively, but no relationships were detected with nestling physiology or survival, which could be partially explained by our small sample size. Our study partially supports a beneficial effect of aromatic plants in avian nests.

**Keywords** Aromatic plants · Avian nests · Nest protection hypothesis · Drug hypothesis

## Introduction

Several bird species place in their nests, apart from twigs, moss, fur and feathers, fresh green plant materials such as conifer green branches, sprigs (e.g. raptors) and leaves of

aromatic plants (passerine birds; Gwinner et al. 2000; Lambrechts and dos Santos 2000; Dykstra et al. 2009; reviewed in Dubiec et al. 2013). These plant materials generally constitute a non-random fraction of the plants available in the environment surrounding the nest, and they usually have a high content of volatile secondary metabolites (Wimberger 1984; Clark and Mason 1985; Dubiec et al. 2013). The most common substances present in such additional materials are monoterpenes: volatile oils, generally with a strong odour

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that may have insecticide and antibiotic properties (Carmo and Vieira 2016). In fact, several of those plants are used in human medicine and also by some mammals and birds for auto-medication and protection against parasites (Clayton and Wolfe 1993; Hart 1997; Yang et al. 2020). However, the function of green plant material in avian nests is still not fully understood, and several non-mutually exclusive hypotheses have been proposed to explain their potential functions (Dubiec et al. 2013).

As some of these plants have been shown to have a repellent effect against parasites, the ‘nest protection hypothesis’ has been advanced to explain the use of these green, fresh plant materials in avian nests. This protection against parasites may be attained by plants’ secondary volatile compounds repelling pathogens and ectoparasitic arthropods (Clark and Mason 1985; Clark 1991; Lafuma et al. 2001; Yang et al. 2020). However, comparisons of parasite infestation between nests with and without aromatics in which aromatic plants were either added or removed from nests revealed contradictory results. For example, nests of blue tits (*Cyanistes caeruleus*) with aromatics have lower number of fleas but no effects on blowflies or mites have been detected (Mennerat 2008; Tomás et al. 2012), and blue tit nestlings in nests with aromatics show reduced skin and feather bacteria (Mennerat et al. 2009a), but have higher enterobacteria loads (Scott-Baumann et al. 2022). Some contradictory results have been found in other bird species, such as the European starling (*Sturnus vulgaris*; Clark and Mason 1985; Fauth et al. 1991; Gwinner et al. 2000; but see Yang et al. (2020) on sparrows *Passer cinnamomeus*). Because arthropod ectoparasites, especially blood-sucking ectoparasites, are known to impact nestling growth and health (Merino and Potti 1995; Hurtrez-Boussès et al. 1997; Simon et al. 2004) and, consequently, may provoke nestling death (reviewed in Møller et al. (2009)), it is important to clarify the relationships between the aromatic plant use in avian nests and parasitism.

On the other hand, the ‘drug hypothesis’ proposes that fresh plant materials in avian nests have direct beneficial effects on nestling condition and development, as they may stimulate some components of their immune system (Gwinner et al. 2000; but see Tomás et al. (2012)). In line with the predictions of this hypothesis, it has been reported that aromatic plants in nests favour body mass and feather growth (Gwinner et al. 2000; Gwinner and Berger 2005; Mennerat et al. 2009b) as well as indicators of aerobic capacity (haemoglobin and haematocrit; Gwinner et al. 2000; Mennerat et al. 2009b; Gładalski et al. 2020). Lastly, other hypotheses have been proposed to explain the phenomenon of green material in avian nests but they attracted less attention, such as the ‘crypsis hypothesis’, which refers to the function of aromatic plants in masking the scent of the brood, the ‘water loss hypothesis’ in which plants function to reduce water

loss and the ‘courtship hypothesis’, which suggests that the input of aromatic plants is implied in post-mating sexual selection (Dubiec et al. 2013; Scott-Baumann and Morgan 2015).

The frequency of green plant materials rich in volatile compounds is expected to be high in nests from cavity-nesting bird species because these nests are exposed to a wide variety of ectoparasites and pathogens. In this sense, the blue tit offers a good model to study this subject because females frequently place fresh aromatic plant materials in the nest (Banbura et al. 1995; Lambrechts and dos Santos 2000; Tomás et al. 2012), and aromatic plants can be found in blue tit nests during all nest stages, from incubation to fledging. Undoubtedly, the effort that blue tits divert to search and collect these materials suggests that they may bring some benefits (Lambrechts and dos Santos 2000; Mennerat et al. 2009a, b). However, both the nest protection hypothesis and drug hypothesis have been tested in different blue tit populations, but studies have revealed contradictory results (see above).

In this study, we aimed to characterise aromatic plant use in blue tit nests in a sub-urban mixed deciduous wood in Portugal and to evaluate the relationships between aromatic plant use and reproductive performance, nestling physiology, colouration and behaviour, as well as ectoparasite infestation. For this purpose, we used correlative data collected during two consecutive years and performed a cross-fostering experiment between synchronous nests in which an aromatic plant, *Mentha* sp., often used by blue tits in their nests in this study area, was either added or removed from the nests during the nestling period.

## Methods

### General fieldwork and blue tit sample collection

This study was performed during the breeding seasons of 2020 and 2021 at Mata Nacional do Choupal (40°130N, 8°270W), Coimbra, Portugal. This suburban mixed wood is composed by a wide variety of native and exotic tree species, including *Fraxinus angustifolia*, *Platanus* sp., *Acer negundo*, *Alnus glutinosa*, *Populus nigra*, *Laurus nobilis* and *Eucalyptus* sp. The understorey is composed mainly by *Tradescantia fluminensis*. We placed 99 (2020) to 114 (2021) nest boxes, monitored from the onset of the breeding season. In total, 56 nest boxes (2020: 27; 2021: 29) were occupied by blue tit pairs, considering only first clutches. In 2020, we assessed the presence of aromatic plants around nests occupied by blue tits by walking four 10-m transects departing from each nest in the direction of the four cardinal points. The presence of any aromatic plants or *L. nobilis* shoots and branches within 1.5 m of each side of each transect was registered.

The following reproductive parameters were recorded: laying date (date of the first egg laid, standardised as Julian date), egg mass (mean of 2–3 unincubated eggs using a 10-g Pesola balance; accuracy: 0.1 g), clutch size, brood size (number of nestlings at 2 days of age) and number of fledglings (number of nestlings that successfully left their nests, at approximately 18–22 days of age). Hatching success was calculated as the number of 2-day-old nestlings divided by clutch size, while fledgling success was calculated as the number of fledglings divided by brood size. At each nest visit, we registered the presence and the number of aromatic plant fragments (detected by their obvious odour), which were visible in the top of the nest and nest cup. In 2020, an identification of each aromatic plant fragment species present in the nest was performed. In 2021, we registered only if there was aromatic plant material present or not in the nest and the number of fragments, independently of their species. All nests were visited at least once during the incubation period and twice during the nestling period, and maximum number of aromatic plant fragments was annotated. When nestlings were 14 days old (hatching day = day 0), they were weighed with a 10-g Pesola balance (accuracy: 0.1 g) or with a 50-g Pesola balance (accuracy: 0.5 g), depending on their body mass, and their tarsus was measured with a calliper to the nearest 0.01 mm. A blood sample was collected from the brachial vein: the vein was pricked with a 27G needle, and the blood was collected into a heparinised capillary tube. All nestlings were individually marked with aluminium rings. During the ringing, we recorded the presence of louse flies (Hippoboscidae) inhabiting the nestling bodies—these blood-sucking flies often fly off the nestling bodies, nest box and holding bags when nestlings are being manipulated. Part of the collected blood was used to make a blood smear, following Owen (2011), which was then air-dried and fixed in methanol within 48 h. Capillary tubes were immediately placed in vertical position at 4 °C for 4 h in order to determine the erythrocyte sedimentation rate (ESR), an inflammation indicator, following Heylen and Matthysen (2008). After this period, blood samples were expelled into microtubes and frozen at –20 °C until laboratory procedures (see below). In 2021, once all fledglings left their nest, we collected the nest material into a sealed plastic bag, which was transported to the laboratory.

### Cross-fostering experiment: quantification of behaviour and colouration

In 2021, we performed a cross-fostering experiment to assess the effects of the use of aromatic plants in nests on nestling morphology, physiology, colouration and both nestling and parental behaviour (begging and feeding rate, respectively). Nests with the same hatching date and similar brood size ( $\pm 2$  nestlings) were matched in pairs ( $n = 8$

pairs; mean brood size  $\pm$  standard error (SE) =  $5.81 \pm 0.06$ ). At day 2 (hatching day = day 0), we swapped half of the brood between nest pairs and started the aromatic nest content manipulation. Exchanged nestlings were weighed with a 10-g Pesola balance (accuracy: 0.1 g), and ranked according to body mass within their brood of origin. The heaviest nestling was randomly assigned to stay in the nest of origin or to be moved to the foster nest. Then, the cross-foster treatment (stay/move) was alternated through the weight-based rank list of nestlings. Nestlings assigned to be moved were marked with a nail clip and moved to the foster nest. No brood size manipulations were performed, and nestlings assigned to be moved or remain did not differ in body mass (moved nestlings mean  $\pm$  SE:  $1.85 \pm 0.01$  g; remain nestlings:  $1.72 \pm 0.02$  g;  $t$ -test,  $t_{91} = 1.33$ ,  $p = 0.19$ ). This experimental design allowed us to control for any genetic or maternal influence on nestling parameters. We added approximately 0.5 g (fresh weight) of *Mentha* sp. leaf fragments to one member of the nest pair every 3 days until day 15 and added grass in a similar weight to the other member of the nest pair. Nests that did not match with others were thus not manipulated and served for correlative analyses. We chose *Mentha* sp. because is the most commonly used aromatic plant by blue tits in their nests in our study area and because this aromatic plant has been shown to have repellent activity against mosquitoes (Kamatou et al. 2013) caused by menthol, its main essential oil component. Insecticidal properties of *Mentha*-derived oil were shown to be caused by inhibition of neurotransmission in insects (Singh and Pandey 2018). Menthol also shows biocidal properties against some mites, inhibits the growth of fungi and has antibacterial activity (Kamatou et al. 2013).

At day 7, action microcameras GoPro® Hero 5 were placed below the roofs of the nest boxes to record begging and parental feeding rate during approximately 2 h. Microcameras were placed in the morning, always at approximately 9:00 h (GMT), and a box of similar size and appearance was placed the previous day to ensure parental habituation. For the feeding rate measurements, the recording time in a nest lasted from the first to the last feed. We analysed the number of feeds and standardised the feeding rate as the number of feeds per hour in each nest. Also, we measured the total time spent begging by the whole brood over this recording period using the 'JWatcher 1.0' software (Blumstein and Daniel 2007). The total time spent begging per nest was calculated as the sum of individual begging duration divided by the brood size and was expressed as time spent begging in seconds per hour of video recording (s/h).

Once all recordings were finished, we weighed nestlings with a 10-g Pesola balance (accuracy: 0.1 g), and we photographed their mouth flanges with a Canon 700D camera equipped with a Canon 18–135 mm lens. Nestling mouths were gently opened using tweezers to visualise

and photograph flanges. All photographs were taken at  $f/8.0$  aperture, 1/200 s exposure, ISO 200, in RAW format, between 12:00 and 14:00 h (GMT) and using a LED-based ring flash to ensure the same lighting conditions. Photographs were taken from above, at a height of approximately 30 cm, and nestlings were placed under a shadow, on a dark background and next to a standard grey reference (ColorChecker Classic target, X-Rite, MI). The digital images were then normalised and linearised according to the grey scale using the 'SpotEgg' software (Gómez and Liñán-Cembrano 2017). This produced TIFF format images whose RGB values were linearly equivalent to the actual reflectance of the colours (Stevens et al. 2007; Gómez and Liñán-Cembrano 2017). Carotenoids produce colours rich in long wavelengths via the absorption of short-wavelength light (Andersson and Prager 2006). Therefore, variation in carotenoid-based colouration in nestling flanges can be captured even if we restrict the analysis to the human visible range provided by digital cameras (Dugas and McGraw 2011). Saturation, or chroma, was chosen as a carotenoid quantity proxy because yellow saturation has been empirically demonstrated to predict the carotenoid content in nestling flanges (Dugas and McGraw 2011). From the normalised and linearised TIFF images mentioned above, we measured the mean saturation values at four points ( $5 \times 5$  pixels) on the nestling flange (left and right of the maxilla, and left and right of the mandible) to calculate yellow saturation values using Adobe Photoshop 8.0 software. The mean saturation of those four points per nestling flange was used for further analysis. Dirty flange areas were excluded when selecting the points to analyse the saturation. At day 14, the nestlings were ringed and measured, and blood samples were collected and processed as described above for control nests. To minimise observer bias, blinded methods were used when all behavioural data were recorded and/or analysed.

### Laboratory procedures

Nest material was placed in a McFadyen extractor where the nest-dwelling arthropods were collected into 70° ethanol tubes at 45 °C during at least 48 h. After this period, the nest material was visually inspected to collect and count the number of blowfly (*Protocalliphora azurea*) pupae. Nest material was then dried in an oven at 40 °C for 48 h in order to obtain its dry weight with a digital balance (accuracy: 0.01 g). Collected arthropods were sorted into major groups, and the number of fleas (larvae and adults), blowfly larvae and mites were counted with a stereomicroscope. Since gamasid mites (Mesostigmata: Dermanysoidea) may reach very high abundances in nests, their number was estimated following Pacejka et al. (1996) when samples had more than 200 mites and total counts were not possible. This method consists of bringing the sample to 20 mL, taking five

consecutive 2-mL subsamples while stirring the sample, and counting mites in each subsample. Then, the average number of mites for the subsamples is calculated, and it is multiplied by 10 to estimate the average total number of mites in the original 20-mL sample. Mites were divided into two groups according to their feeding habits: facultative parasitic mites for *Androlaelaps* sp. (Mesostigmata: Laelapidae), and obligatory parasitic mites, which include two genera: *Dermanyssus* sp. (Mesostigmata: Dermanyssidae) and *Ornithonyssus* sp. (Mesostigmata: Macronyssidae). To verify the classification of mites in each group carried out under the stereomicroscope, a proportion of mites was also cleared in lactophenol and slide-mounted in Hoyer's medium to ease morphological identification under an optical microscope following specific literature (Till 1963; Krantz 1978; Moss 1978; Radovsky 2010). Both groups of mites were analysed separately in subsequent analyses.

Haemoglobin concentration (Hb, g/L) was assessed with a commercial kit following the manufacturer's instructions (Sigma-Aldrich, St Louis, MO). Blood smears were stained with Giemsa diluted 1:9 for 45 min, and we obtained the number of white blood cells (WBC) per approximately 10,000 erythrocytes (counted at the microscope in 2020, and with the software Mizutama (Ochoa et al. 2019) in 2021) and the heterophil to lymphocyte ratio (H/L ratio), assessed by identifying 100 white blood cells at the microscope at  $\times 1000$  magnification. White blood cells were identified according to Campbell and Ellis (2007). The number of polychromatic erythrocytes per approximately 10,000 erythrocytes was also counted. Immature, polychromatic erythrocytes were identified by their shape, cytoplasm colour and shape and appearance of the nucleus following Maceda-Veiga (2015). High numbers of this type of erythrocytes typically indicate an elevated erythropoietic activity. Because this is considered a relatively crude assessment of haematological dynamics and cell maturity, we also evaluated the erythrocyte maturation index (EMI) in a random sample of 125 erythrocytes distributed by 10 microscope fields at  $\times 1000$  magnification, following Castro et al. (2018). Photographs were obtained with a LEICA ICC50 W camera. Erythrocytes were measured using the software ImageJ and classified according to 10 maturity classes, with higher classes representing erythrocytes that are more immature than those of a lower class (Castro et al. 2018). For each nestling, we obtained the mean EMI and the frequency of occurrence of each EMI class.

We also counted the number of erythrocytes presenting nuclear abnormalities per approximately 10,000 erythrocytes. Nuclear abnormalities (micronuclei, budding, bilobed, kidney-shaped, binucleated, notched, vacuolated and tailed nuclei, presence of nucleo-plasmatic bridges and unknown nuclear malformations) indicate DNA damage, and they likely result from oxidative stress and exposure to macromolecules' aggressive agents (Zúñiga-González et al. 2000;



Pereda-Solis et al. 2019). The number of micronuclei (MN) was analysed separately from the rest of nuclear abnormalities (NAE).

## Statistical analyses

We compared the use of aromatic plants in blue tit nests in 2020 with their availability in the nest surroundings using the Jacob's index (Jacob 1974) to assess the selectivity of each of the aromatic plant species. This index varies from  $-1$  to  $1$  and provides a range between strong avoidance ( $-1$ ) to strong selection ( $1$ ), the value of zero corresponding to a use of a particular plant species proportional to its availability in the environment (i.e. no selection). For these analyses, we used first as well as second/replacement clutches.

To have a general overview of the co-variation among nestling physiological health indicators and between those and ectoparasite pressure, we used the Pearson product-moment correlation between physiological parameters measured in 14-day-old nestlings and the Spearman rank correlation between mean brood colouration (in 7-day old nestlings) and physiological (in 14-day old nestlings) parameters and nest ectoparasite abundance.

All analyses from the subsequent sections were run in R software 4.2.2 (R Development Core Team 2022), using the packages 'lme4' (Bates et al. 2022), 'lmerTest' (Kuznetsova et al. 2020), 'pscl' (Jackman 2020) and 'car' (Fox et al. 2022), and the performance of such linear models was assessed using the package 'performance' (Lüdtke et al. 2023). To achieve normality of model residuals and error variance homoscedasticity, ESR, EMI, WBC, H/L ratio, number of polychromatic erythrocytes, total number of erythrocytic nuclear abnormalities and number of micronuclei in erythrocytes were log-transformed. Only first clutches were used in this study, except for the Jacob's index analyses. The basic statistics are presented as the mean  $\pm$  SE.

## Correlative data

To assess the relationships between the use of aromatic plants in nests (presence and quantity) and reproductive parameters, we used unmanipulated nests from 2020 and 2021 (i.e. in which neither *Mentha* sp. nor grass was artificially added in nests). For each reproductive parameter, measured at the brood level, we ran two different linear models (LM): the first one was used to check the relationships between the presence of aromatics and reproductive parameters, and the second one was used to check associations between the quantity of aromatics and reproductive parameters. The first linear model included the standardised laying date, mean egg mass, hatching success (arcsine-transformed), clutch size, brood size, number of fledglings and fledging success (arcsine-transformed) as the dependent

variables in separate models; while the presence of aromatics (two levels: yes and no), year (two levels: 2020 and 2021) and their interaction were included as the predictors. We included the standardised laying date (except when modelling laying date) and clutch size (except when modelling laying date and clutch size) as the covariates. The second linear model included the same dependent variables in separate models, while the maximum number of aromatic pieces in the nest, year and their interaction were included as the predictors. We included the standardised laying date (except when modelling laying date) and clutch size (except when modelling laying date and clutch size) as the covariates.

To assess the relationships between the use of aromatic plants in nests (presence and quantity) and physiological parameters of the 14-day-old nestlings, we used linear mixed-effects models (LMM) using unmanipulated nests from 2020 and 2021. For each physiological parameter, measured at the nestling level, we run two different LMM. The first LMM included the body mass (log-transformed), haemoglobin, ESR, mean EMI, WBC, H/L ratio, number of polychromatic erythrocytes, MN and NAE as the dependent variables in separate models, while the presence of aromatics, year and their interaction were the predictors. We included the hour of ringing, body mass (log-transformed) and tarsus length (log-transformed) as the covariates (body mass excluded when modelling itself), and nest identity was included as the random factor (random intercept). The second LMM included the same dependent variables in separate models, while the maximum number of aromatic plant pieces in the nest, year and their interaction was included as the predictors. We included the hour of ringing, body mass (log-transformed) and tarsus length (log-transformed) as the covariates (body mass excluded when modelling itself), and nest identity was included as the random factor (random intercept). We used the Mann–Whitney *U*-test to evaluate the influence of aromatic presence in nests on the frequency of each erythrocytic maturity class (classes from 1 to 10; Castro et al. 2018).

To evaluate the effects of the presence and quantity of aromatic plants in nests on the abundance of blowflies and fleas in 2021, we used zero-inflated generalised models. For each ectoparasite group, we run two different zero-inflated generalised Poisson models (ZIGP). The first one included the abundance of blowflies and fleas as the dependent variables in separate models, while the presence of aromatics was included as the predictor. The second one included the same dependent variables in separate models, while the maximum number of aromatic pieces in the nest was included as the predictor. To check for variation in the abundance of facultative parasitic mites (*Androlaelaps* sp.) and obligatory parasitic mites (Macronyssidae and Dermanyssidae) in relation to the presence and quantity of aromatic plants in nests in 2021, we used generalised linear models (GLM) with a

Poisson distribution and a logit-link function. For each mite group, we run two different GLM. The first one included the abundance of facultative and obligatory parasitic mites as the dependent variables in separate models, while the presence of aromatics was included as the predictor. The second one included the same dependent variables in separate models, while the maximum number of aromatic pieces in the nest was included as the predictor. We did not include the dry nest weight as a covariate in any model because it did not correlate with ectoparasite abundance (Spearman rank correlations; blowflies:  $\rho = -0.09$ ,  $p = 0.77$ ,  $n = 12$ ; fleas:  $\rho = -0.23$ ,  $p = 0.52$ ,  $n = 10$ ; facultative parasitic mites:  $\rho = -0.01$ ,  $p = 0.98$ ,  $n = 11$ ; obligatory parasitic mites:  $\rho = -0.12$ ,  $p = 0.72$ ,  $n = 11$ ).

### Experimental data

We evaluated the effects of the experimental manipulation in the presence of aromatic plants in nests (i.e. nests with mint versus nests with grass) on 7-day old nestlings' condition (body mass and mouth colouration metrics) using generalised linear mixed-effects models (GLMM) and LMM. The body mass, saturation, hue and brightness were included as the dependent variables in separate models. We included the treatment (two levels: mint and grass) as the fixed factor, while we included both pair of nests and nest of origin nested within pair of nests as the random factors. We included the body mass as the covariate in all models except the one modelling body mass. Brood size and standardised laying date were not included as the covariates because they were not significant in any model. We used GLMM fitted with a gamma distribution and the identity link function when modelling saturation and brightness, while we used LMM fitted with a normal distribution when modelling hue and body mass.

We also assessed the effects of treatment (mint versus grass) on begging and feeding rate on day 7, both measured at the brood level, using linear models. The total time spent begging by broods and feeding rate were included as dependent variables in separate models. Treatment was included as the fixed factor, and brood size was included as covariate but only when modelling the feeding rate. We assessed whether feeding rate was correlated with total time spent begging with the Spearman rank correlation. We assessed the effect of the experimental manipulation on nestling survival using linear models: brood size at day 7, number of fledglings and fledgling success were included as dependent variables in separate models, while treatment was included as the predictor, and standardised laying date was included as the covariate. We could not perform models for 14-day-old nestling physiology because half of the experimental broods died because of predation by great spotted woodpeckers (*Dendrocopos major*) (4 out of 16 nests) and starvation (4 out of 16 nests), thus most of paired nests were lost.

Finally, we assessed the effects of treatment (mint versus grass) on the abundance of ectoparasites using GLM fitted with a Poisson distribution and a logit-link function. The abundance of blowflies and fleas was included as the dependent variables in separate models, while treatment was included as the predictor. We did not include the dry nest weight as a covariate because it did not show an association with ectoparasite abundance (blowflies:  $\rho = 0.21$ ,  $p = 0.45$ ,  $n = 16$ ; fleas:  $\rho = -0.38$ ,  $p = 0.14$ ,  $n = 16$ ). We used the Wilcoxon rank sum test to check for the variability of facultative and obligatory parasitic mites with treatment (mint versus grass).

## Results

### Aromatic plant use in blue tit nests

In 2020, the prevalence of aromatic plants in nests was 68.2% (30 out of 44 nests had aromatic plant fragments in their composition at least in one visit to the nest from incubation to fledging), and 72.4% (21 out of 29) in 2021 considering only unmanipulated nests, i.e. nests not involved in cross-fostering in which aromatic plants were neither added or removed by the observer. The most common plant found in blue tit nests was *Mentha* sp., which was detected in 36.4% of the nests monitored in 2020. Other identified plant fragments were *Erigeron* sp. (4.5%), followed by *Melissa officinalis*, *Foeniculum vulgare*, *Lavandula* sp., *Santolina* sp. and *L. nobilis* (all with a prevalence of 2.3%). We could not identify fragments of plants in 14 nests. From the 7 plant-genus/species detected in blue tit nests in 2020, blue tits showed high selectivity ( $E_i$  ranging from 0.77 to 1) in all of them, with the exception of *L. nobilis* and *M. officinalis*, for which blue tits showed either an utilisation proportional to the availability (*M. officinalis*,  $E_i = 0$ ) or avoidance (*L. nobilis*,  $E_i = -0.92$ ). *Laurus nobilis* was present in the vicinity of 27 of the monitored nests but was only included in the nest material of one nest.

### Correlations among physiological metrics and ectoparasitism

Correlations among nestling physiological parameters and with ectoparasite abundances in control nests are found in Tables S1 and S2, respectively. Correlations among colour metrics and ectoparasite abundances are reported in Table S3. Nestlings with higher ESR had lower haemoglobin concentrations ( $r = -0.50$ ,  $p < 0.001$ ) and lower number of polychromatic erythrocytes ( $r = -0.40$ ;  $p < 0.001$ ). The number of polychromatic erythrocytes was also negatively associated with MN ( $r = -0.32$ ;  $p = 0.004$ ), but positively correlated with NAE ( $r = 0.29$ ;  $p = 0.009$ ) and the H/L ratio

( $r = 0.26$ ;  $p = 0.021$ ). Nestlings with higher mean EMI showed lower haemoglobin concentrations ( $r = -0.27$ ,  $p = 0.014$ ) and NAE ( $r = -0.29$ ,  $p = 0.010$ ). The ESR, mean EMI and the number of polychromatic erythrocytes were positively correlated with blowfly abundance ( $r = 0.83$ ,  $p = 0.021$ ;  $r = 0.73$ ,  $p = 0.038$ ;  $r = 0.90$ ,  $p = 0.037$ , respectively). Blowfly abundance was also negatively correlated with haemoglobin concentrations ( $r = -0.75$ ,  $p = 0.033$ ). The abundance of facultative parasitic mites was positively correlated with the number of polychromatic erythrocytes ( $r = 0.90$ ,  $p = 0.037$ ), whereas, in experimental nests, higher numbers of obligatory parasitic mites were correlated with lower nestling flange colour saturation ( $r = -0.75$ ,  $p = 0.019$ ).

### Unmanipulated nests: relationship between aromatic plant use and reproductive parameters

There were no differences in reproductive parameters (laying date, hatching success, clutch size, brood size, number of fledglings and fledging success) between nests with and without aromatic plants ( $p > 0.08$  in

all cases; Table 1). However, in nests with aromatic plants, the eggs tended to be heavier, but only in 2020 (LM: interaction year  $\times$  presence of aromatics: estimate =  $-0.21 \pm 0.10$ , reference categories = without aromatics; 2021,  $t_{32} = -2.02$ ,  $p = 0.051$ , Table 1). The maximum number of aromatic fragments present during the nestling period was also not correlated with brood size, number of fledglings nor fledging success ( $p > 0.46$  in all cases).

### Unmanipulated nests: relationship between aromatic plant use and ectoparasites abundance

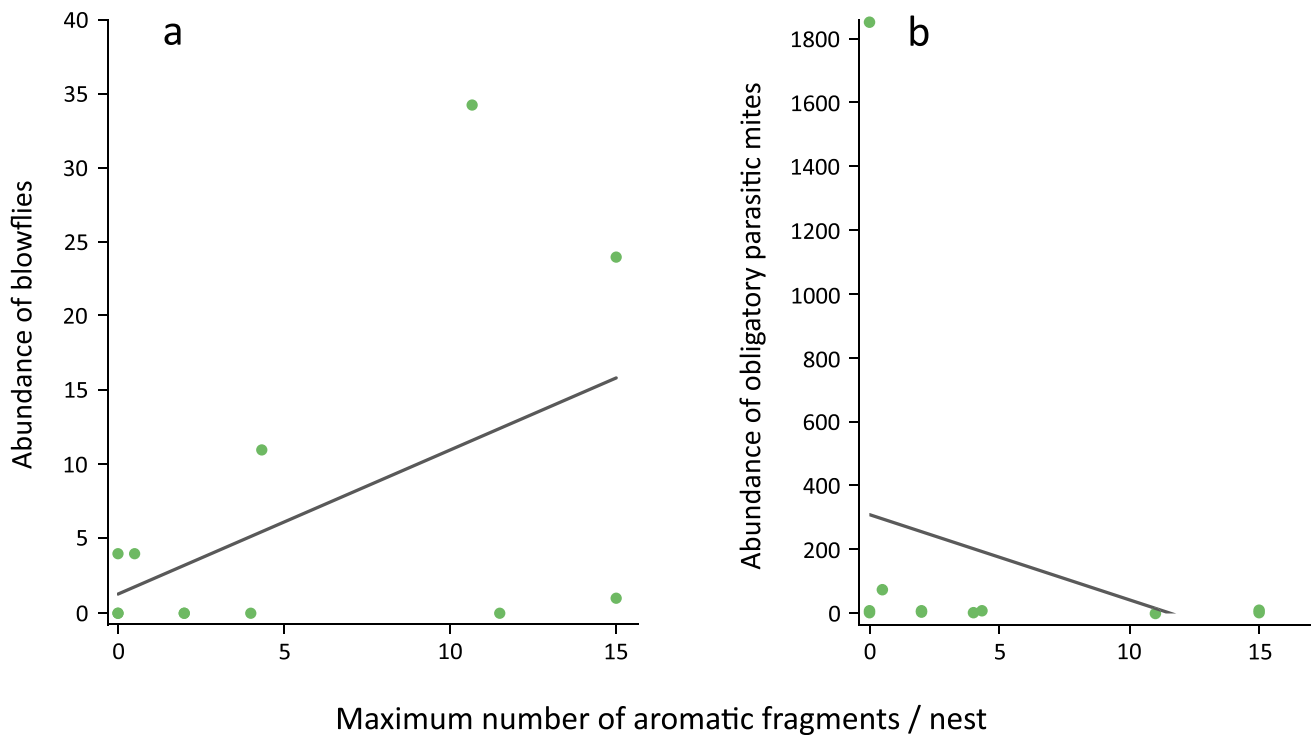
Nests with aromatic plants harboured more blowflies than nests without aromatic plants (ZIGP: estimate =  $1.34 \pm 0.53$ , reference category = without aromatics,  $z$ -value =  $2.51$ ,  $p = 0.012$ ; Table 1; Fig. S1). Also, the abundance of blowflies increased with the maximum number of aromatic plant fragments in the nest (ZIGP: estimate =  $0.07 \pm 0.02$ ,  $z = 3.57$ ,  $p < 0.001$ ; Fig. 1a). Nests with aromatic plants also had more fleas (ZIGP: estimate =  $2.48 \pm 0.42$ , reference category = without

**Table 1** Reproductive and physiological parameters in unmanipulated nests and nestlings and ectoparasite abundance in nests of blue tits (*Cyanistes caeruleus*) in Mata Nacional do Choupal, Portugal. Data included unmanipulated nests (without aromatic plant manipulation) and 2020 and 2021, except for ectoparasite groups which were analysed only in 2021

	With aromatics			Without aromatics		
	Mean	SE	<i>N</i>	Mean	SE	<i>N</i>
Laying date (Julian)	105.09	1.37	11	103.31	0.99	29
Mean egg mass (g)	1.19	0.01	10	1.15	0.02	28
Hatching success (%)	63.70	0.05	11	76.80	0.04	29
Clutch size	6.13	0.20	23	6.06	0.26	17
Brood size	4.52	0.27	23	4.63	0.33	17
Number of fledglings	2.04	0.28	23	2.18	0.33	17
Fledging success (%)	47.40	0.06	23	44.54	0.06	17
Abundance of blowflies (larvae and pupae)	<b>8.33</b>	<b>3.55</b>	<b>9</b>	<b>1.00</b>	<b>0.55</b>	<b>4</b>
Abundance of fleas (larvae and adults)	<b>11.57</b>	<b>7.28</b>	<b>7</b>	<b>2.33</b>	<b>1.02</b>	<b>3</b>
Abundance of facultative parasitic mites ( <i>Androlaelaps</i> sp.)	<b>42.00</b>	<b>19.07</b>	<b>8</b>	<b>23.33</b>	<b>9.69</b>	<b>3</b>
Abundance of obligatory parasitic mites (Macronyssidae/ Dermanyssidae)	<b>13.50</b>	<b>7.45</b>	<b>8</b>	<b>620.67</b>	<b>321.52</b>	<b>3</b>
Body mass (at 14 days old) (g)	8.74	0.14	58	8.52	0.16	38
Haemoglobin concentration (g/L)	78.85	2.40	54	80.34	3.05	29
ESR	15.06	0.92	51	13.42	0.81	26
EMI	0.21	0.01	53	0.22	0.01	32
WBC	28.41	2.23	53	26.67	1.36	32
H/L ratio	0.97	0.07	53	0.81	0.06	32
Number of polychromatic erythrocytes	23.51	3.16	45	12.18	1.82	32
NAE	10.65	0.85	46	7.12	0.83	32
MN	1.63	0.23	46	2.50	0.38	32

Values in bold refer to significant differences between nests with and without aromatics

Abbreviations: *ESR* erythrocytic sedimentation rate, *EMI* erythrocytic maturation index, *WBC* white blood cells, *H/L ratio* heterophil to lymphocyte ratio, *NAE* number of erythrocytic nuclear abnormalities other than micronuclei, *MN* number of micronuclei in erythrocytes



**Fig. 1** Relationships between the abundance of blowflies (a) and obligatory parasitic mites (Macronyssidae/Dermanyssidae) (b) and the maximum number of aromatic plant fragments in blue tit (*Cyanistes caeruleus*) unmanipulated nests at Mata Nacional

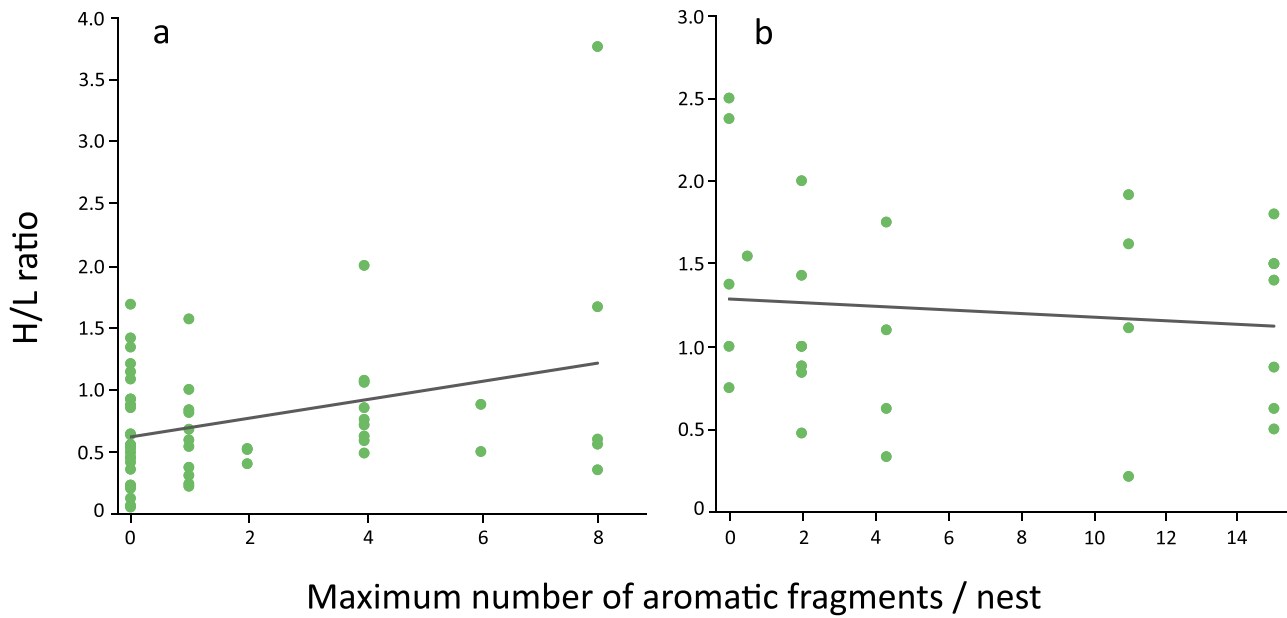
aromatics,  $z = 5.94$ ,  $p < 0.001$ ; Table 1; Fig. S1), but this was independent of the maximum number of aromatic plants used (ZIGP: estimate =  $-0.002 \pm 0.02$ ,  $z = -0.08$ ,  $p = 0.94$ ). Regarding mites, nests with aromatics also had more facultative parasitic mites (GLM: estimate =  $0.59 \pm 0.13$ , reference category = without aromatics,  $z = 4.47$ ,  $p < 0.001$ ; Table 1; Fig. S1), but showed lower abundances of obligatory parasitic mites (GLM: estimate =  $-3.83 \pm 0.02$ , reference category = without aromatics,  $z = -38.68$ ,  $p < 0.001$ ; Table 1; S1). The higher the maximum number of aromatics in the nests, the lower the abundance of obligatory parasitic mites (GLM: estimate =  $-1.39 \pm 0.05$ ,  $z = -26.16$ ,  $p < 0.001$ ; Fig. 1b). The statistical significance of this result is, however, greatly influenced by one nest without aromatics that harboured over 1800 obligatory parasitic mites. This relationship should, therefore, be considered with caution because it is impossible to ascertain if this one observation is causal or occurred only by chance, and we cannot state with certainty that the absence of aromatics or its sparse use by blue tits contributes to higher obligatory mite parasitism. The prevalence of louse flies was very low (1 out of 8 nests with louse flies) and, therefore, no comparison between nests with and without aromatic plants was made.

do Choupal, Portugal, in 2021. Linear regression for blowflies:  $y = 1.293 + 0.971x$ ,  $R^2 = 0.26$ ,  $p = 0.09$ ; linear regression for obligatory parasitic mites:  $y = 309.700 - 26.690x$ ,  $R^2 = 0.08$ ,  $p = 0.39$

### Unmanipulated nests: relationship between aromatic plant use and nestling health indicators

There were no effects of aromatic plant use (presence or quantity) in blue tit nests on most physiological metrics evaluated—body mass, haemoglobin concentration, ESR, mean EMI, WBC, number of polychromatic erythrocytes, MN and NAE ( $p > 0.06$  in all cases; Table 1). Although the H/L ratio did not differ significantly between nests with and without aromatic plants ( $p = 0.28$ ), nestlings from nests with higher maximum number of aromatic plant fragments had higher H/L ratios (LMM: estimate =  $0.04 \pm 0.02$ ,  $t_{15,2} = 2.25$ ,  $p = 0.039$ ), but only in 2020 (LMM: interaction maximum number of aromatics  $\times$  year: estimate =  $-0.043 \pm 0.02$ , year reference category = 2021,  $t_{15,9} = -2.27$ ,  $p = 0.04$ ; Fig. 2). The mean EMI did not differ between nests with and without aromatic plants, but this was because the maturity class 2 was more frequent in nestlings from nests with aromatic plants, whilst maturity class 3 occurred more often in nests without aromatic plants (Mann–Whitney  $U$ -test: class 2:  $W = 1073.00$ ,  $p = 0.04$ ; class 3:  $W = 521.00$ ,  $p = 0.003$ ; the rest of classes were not significant,  $p > 0.05$  in all cases).





**Fig. 2** Relationship between the heterophil to lymphocyte ratio (H/L ratio) in blue tit (*Cyanistes caeruleus*) nestlings and the maximum number of aromatic plant fragments in unmanipulated nests at Mata

Nacional do Choupal, Portugal, in 2020 (a) and 2021 (b). Linear regression for 2020:  $y=0.074x+0.619$ ,  $R^2=0.11$ ,  $p=0.01$ ; linear regression for 2021:  $y=-0.011x+1.286$ ,  $R^2=0.01$ ,  $p=0.57$

**Effects of nests’ aromatic plant manipulation on nestling condition, survival, nestling begging and parental feeding behaviour**

Nestlings from nests with added mint did not differ in body mass at the age of 7 days from nestlings of nests with added grass (LMM: estimate =  $0.061 \pm 0.30$ , reference category = nests with mint,  $t_{68,3} = 0.20$ ,  $p = 0.84$ ;

Table 2). However, nestlings in nests with added mint showed lower flange colour saturation (GLMM: estimate =  $1.33 \pm 0.64$ , reference category = nests with mint,  $\chi^2 = 4.35$ ,  $p = 0.037$ ; Table 2; Fig. S2) and brightness (GLMM: estimate =  $5.93 \pm 1.59$ , reference category = nests with mint,  $\chi^2 = 13.78$ ,  $p < 0.001$ ; Table 2; Fig. S2), but higher colour hue (LMM: estimate =  $-0.62 \pm 0.29$ , reference category = nests with mint,  $t_{65,6} = -2.15$ ,  $p = 0.035$ ;

**Table 2** Reproductive, feeding, begging and mouth colouration parameters in nestlings and ectoparasite abundance in nests of blue tits (*Cyanistes caeruleus*) subjected to aromatic treatment (addition of *Mentha* sp. fragments) and controls (addition of grass fragments) in Mata Nacional do Choupal, Portugal. Data included: 2021

	Mint			Grass		
	Mean	SE	N	Mean	SE	N
<b>Nestling level</b>						
Body mass (at 7 days of age) (g)	5.82	0.14	40	5.88	0.17	40
Saturation (%)	83.81	0.36	40	85.10	0.46	40
Hue (°)	29.65	0.17	40	29.03	0.20	40
Brightness (%)	42.53	1.18	40	49.29	1.29	40
<b>Brood level</b>						
Brood size (at day 7)	5.00	0.30	8	5.25	0.22	8
Number of fledglings	1.88	0.45	8	1.25	0.46	8
Fledging success (%)	0.27	0.06	8	0.19	0.07	8
Abundance of blowflies (larvae and pupae)	1.75	0.95	8	1.00	0.61	8
Abundance of fleas (larvae and adults)	2.00	1.41	8	13.88	9.41	8
Abundance of facultative parasitic mites ( <i>Androlaelaps</i> sp.)	100.40	35.19	5	105.00	63.79	4
Abundance of obligatory parasitic mites (Macronyssidae/ Dermanyssidae)	6.00	1.94	5	1.50	0.63	4
Feeding rate (feeds/hour)	38.99	3.00	7	44.24	3.12	6
Time spent begging by broods (seconds/hour)	4.35	0.58	7	4.10	0.41	5

Table 2; Fig. S2) than nestling in nests with added grass. Time spent begging by broods was not related with aromatic plant nest content manipulation (LM:  $F_{1,10} = 0.18$ ,  $p = 0.82$ ; Table 2). Parental feeding rate was not affected by aromatic plant nest content manipulation (LM:  $F_{1,10} = 0.01$ ,  $p = 0.93$ ; Table 2) but increased with the brood size (LM:  $F_{1,10} = 10.66$ ,  $p = 0.008$ ). Feeding rate was not related with time spent begging by broods (Spearman rank correlation;  $\rho = 0.51$ ,  $p = 0.09$ ,  $n = 12$ ). Aromatic plant manipulation did not affect nestling survival up to day 7 nor the number of fledglings or fledging success ( $p > 0.05$  in all cases, data not shown for simplicity); however, our sample size of 14-day-old nestlings was very low.

### Effects of nests' aromatic plant manipulation on ectoparasite abundance

Our limited dataset for experimental nests suggests that those nests with added mint had a significantly lower abundance of fleas (GLM: estimate =  $1.94 \pm 0.27$ , reference category = nests with mint,  $z = 7.24$ ,  $p < 0.001$ ; Table 2; Fig. S3), but no significant effects were found on the abundance of blowflies (GLM: estimate =  $-0.56 \pm 0.44$ , reference category = nests with mint,  $z = -1.26$ ,  $p = 0.21$ ; Table 2). Regarding mites, there were no differences in the abundance of any of the two mite groups between nests with added mint and those with added grass (Wilcoxon rank sum test: facultative parasitic mites:  $W = 12.00$ ,  $p = 0.73$ ; obligatory parasitic mites:  $W = 15.00$ ,  $p = 0.26$ ).

## Discussion

Our study suggests that aromatic plants may have different effects on different nest-dwelling ectoparasite groups: correlative data suggested that they may protect against obligatory parasitic mites (Macronyssidae and Dermanyssidae) either directly, or indirectly by favouring *Androlaelaps* sp. mites that may have preyed on them (see below), but not against blowflies (nests with aromatics had, inclusively, higher levels of blowflies). Mennerat et al. (2008) also did not find a repellent effect of aromatic plants (*Lavandula* sp. and *Helichrysum* sp. both naturally brought by the birds to the nest or artificially added to the nests) on blowfly infestation intensity in blue tits from populations where blowfly parasitic pressure is reported to be the highest in Europe (Corsica). Although our correlative data did not show a relationship between aromatics and fleas, the experiment suggests that aromatics have a beneficial effect protecting against fleas. However, the results of the experimental manipulation need to be considered cautiously because sample size was relatively small due to a low survival of blue tit nestlings, given adverse weather conditions and predation by woodpeckers.

The opposite effect of the presence of aromatics in the nest material of blue tits on the different groups of mites evaluated in our study emphasises the relevance of analysing nidicolous mites differentially according to their ecological relationships with their hosts (Roy et al. 2013). The occurrence of blood-sucking genera *Ornithonyssus* and *Dermanyssus* has been linked to a reduction in nestling condition and fitness (e.g. Møller 1990; Clayton and Tompkins 1995; Merino and Potti 1996; Berggren 2005; Cantarero et al. 2013a). Although *Androlaelaps casalis* is able to feed on vertebrate blood and sera (Radovsky 1994), it is also considered an opportunistic feeder, preying on other mites and their eggs (Barker 1968). It has been demonstrated that this mite preys on *Dermanyssus gallinae*, thus being considered as a potential biological pest control for this species in poultry facilities (Lesna et al. 2012). Further confirmation of *Androlaelaps* sp. feeding upon other blood-sucking mites was observed in the current study, as some of the facultative parasitic mites' morphology found in our samples matched the description of *A. casalis* when having recently fed upon *D. gallinae* reported by Lesna et al. (2009). Therefore, the increase in the abundance of this (facultatively predatory) mite in nests where aromatic plants were present might have had a beneficial effect by reducing obligatory parasitic mite abundance. However, even if *A. casalis* might be indirectly beneficial to blue tit nestlings by preying on parasitic mites, all the three mite families together (Dermanyssidae, Macronyssidae and Laelapidae) may have a detrimental effect up to a level by potentially transmitting pathogenic viruses and bacteria (Valiente Moro et al. 2005; Chaisiri et al. 2015). Nevertheless, even if parasite pressure was different in nests with and without aromatic plants, we did not detect significant differences in blue tit nestlings' physiology between nests with and without aromatics, except that a higher number of aromatics in the nests was related with an higher heterophil to lymphocyte ratio in one of the years of the study. In addition, no differences on nestling survival were detected, in accordance with a nearly lack of differences on nestling physiological health metrics. Contrastingly, Mennerat et al. (2009b) reported positive effects of aromatic plants on nestling blue tit body mass, haematocrit and feather development, and they suggested that discrepancy of results among studies may be due to the beneficial effects of aromatic plants only being noticeable when rearing conditions are sub-optimal—in fact, in their study, the effects on body mass were visible only in experimentally enlarged broods. Given the generally low breeding success of blue tits in our study area in 2021 (PM-R et al. unpublished data), which was likely partially driven by adverse meteorological conditions, this explanation seems not to apply in our population. Also, the ability of parents to compensate high parasite infestation levels (either by bringing higher quantity or quality of food) may mask the effects of ectoparasites on

nestling physiology and survival (Simon et al. 2004; DeSimone et al. 2018).

However, we detected significant effects of aromatic plant manipulation on nestling mouth flange colouration at day 7. Mouth colouration is generally considered a predictor of nestling condition (de Ayala et al. 2007; Ewen et al. 2008; Dugas and McGraw 2011) and survival (PM-R et al. unpublished data), given that it reflects the access to higher quality of food resources rich in carotenoids. Carotenoids, among other functions, have been related to the prevention of oxidative stress (Pérez-Rodríguez (2009) but see Costantini and Møller (2008)). But, contrary to expected, nestlings from the mint treatment had duller flanges, characterised by a lower hue and less saturated colour, with higher reflectance. This finding suggests a lower level of circulating carotenoids in nestlings from nests with added mint, which could indicate that carotenoids were being traded-off with physiological functions other than communication and signalling (e.g. feather growth, as body mass did not differ between treatments). The trade-offs between competing physiological systems for dietary antioxidants need further study. Larcombe et al. (2008) showed that the yellow plumage colouration in blue tit nestlings was positively related with lipid peroxidation levels, suggesting that colouration reflected the oxidative damage rather than resistance to oxidative stress. In our study, nests with added grass had more fleas, which has been related to increased oxidative stress in female great tit (*Parus major*) nestlings (De Coster et al. (2012) but see Maronde et al. (2018)). Hence, stronger carotenoid-based colouration in nestling' flanges could reflect oxidative damage as a consequence of flea infestation and related inflammation processes (Owen et al. 2010).

We do not know to what extent the modification of the nest microclimatic conditions by inclusion of mint, especially in opposition to grass (which has lower water content), could have affected ectoparasite communities, since we did not measure temperature or humidity inside the nest boxes. As the water loss hypothesis suggests that plants may reduce water loss, potentially maintaining higher humidity in nests (Scott-Baumann and Morgan 2015), this could have benefited blowflies, whose abundance increased with humidity in pied flycatchers (*Ficedula hypoleuca*) (Cantarero et al. 2013b). However, our experimental results, in which nests with grass had higher flea abundance, could be partially explained if lower humidity levels favoured the invasion of nests by immigrant fleas (Heeb et al. 2000). Given the opposite trends suggested in our study between aromatic plant use and different parasite groups' abundance in nests (mites and fleas versus blowflies), the beneficial effects against parasitism may have cancelled each other. Anyway, blowflies are suggested to have stronger negative effects than mites (by this study and others, e.g. Merino and Potti (1995)). In our study, correlative analyses among physiological parameters

and infestation in control nests suggested that ESR increased with blowfly parasitism. This shows that the blood-sucking feeding of these ectoparasites, besides causing blood loss, also provokes a decrease in the haemoglobin levels (Whitworth and Bennett 1992; Hurtrez-Boussès et al. 1997) and an increase in the production of erythrocytes (higher polychromasia) and may also lead to inflammatory responses in the host, as in louse flies infesting barn swallows (*Hirundo rustica*) (Saino et al. 1998).

We found that in nests with higher numbers of facultative parasitic mites, the nestlings also had higher polychromasia (but with no apparent effects on haemoglobin), supporting the facultative ectoparasitic blood sucking habits of this mite genus; however, its impacts on chick anabolic capacity and anaemia are low. Our correlative analyses also suggest that nestlings with high number of polychromatic erythrocytes were more stressed, showing higher H:L ratios and having higher number of erythrocytic nuclear abnormalities, but not micronuclei as they may tend to occur in older erythrocytes (Sommer et al. 2020), which could have been reduced by blood loss.

Polychromasia occurs when the bone marrow compensates for blood loss or erythrocyte destruction by producing more immature erythrocytes. When blood loss is severe, further effects on haemoglobin concentration, which is reduced, are detected and they suggest anaemia and an inability of the host to compensate for the blood loss (Martinho 2009). Therefore, polychromasia, besides clinically helping the diagnosis of regenerating versus non-regenerating anaemia (which usually have different causes—e.g. blood loss versus chronic disease), could be considered an early and more sensitive warning of an impaired health status related with the loss of anabolic capacity. In our study, this could be the case for the effects of facultative parasitic mites, for which only effects on polychromasia were detected with no effects on haemoglobin concentration. More recently, the assessment of the EMI has been suggested as a less crude assessment of the regenerative status in comparison with polychromasia (Maceda-Veiga et al. 2010; Castro et al. 2018). Evaluation of EMI may be less prone to observer error as it is based on direct measurements of red blood cell size, instead of a classification of an erythrocyte as mature or immature, which can be relatively ambiguous and requires validation among observers. This metric has been applied mostly to toxicological studies (Maceda-Veiga et al. 2010; Castro et al. 2018). In our study, EMI correlated with haemoglobin concentration, number of erythrocytic nuclear abnormalities and blowfly parasitism levels. Different classes of EMI occurred with different probabilities in nests with and without aromatics.

Micronuclei and other nuclear abnormalities occur as result of oxidative damage to DNA, which can be a consequence of exposure to a toxicant, but also other situations where reactive oxygen metabolites are produced—e.g.

inflammation and developmental problems, among others. For example, during growth, if the endoreticular system is underdeveloped, it may fail to remove aged and damaged erythrocytes from circulation, increasing the prevalence of nuclear abnormalities, especially micronuclei (Ceyca-Contreras et al. 2014). Because infestation by blowflies was found to increase the metabolic rates of nestling tree swallows (*Tachycineta bicolor*) (Sun et al. 2020), probably through the activation of the immune system, we would expect higher number of erythrocytic nuclear abnormalities in infested nests (Nilsen 2003), but such evidence was not found.

In conclusion, our results partially support the nest protection hypothesis against some blood-sucking ectoparasites. Female blue tits investing in looking for aromatic plants in the surrounding environment, even if those are present in disproportionately lower availability in the nest surroundings, may be more experienced, invest more effort in the reproductive attempt or be of higher quality. This was partially supported by the higher egg mass found in nests where females used aromatic plants. Because we found no relationship between the presence of aromatic plants in blue tit nests and physiological parameters of nestlings, our correlative data does not support the drug hypothesis. Nevertheless, drawing of robust conclusions from our experimental manipulation which intended to clarify causal relationships between aromatic plant presence and ectoparasite pressure and health and condition of nestlings was impaired by the reduced sample size, due to the high brood mortality in one of breeding seasons, which prevented the evaluation of physiological parameters in experimental nests. Such poor breeding conditions may have also biased our overall nestling sampling (including unmanipulated nests), as those nestlings in poorer condition could have died before sampling occurred. Overall, our data showed did not show evidence that aromatic plant use affected short-term survival of nestlings. Therefore, in order to arrive at more solid conclusions, it would be useful to perform further studies on the effects of aromatic plant use on nestling parasitism and health, encompassing larger sample sizes and increased quantities of mint artificially added in different sets of environmental conditions and breeding seasons. Also, studying if aromatic plant use has long-term effects on nestlings, such as on their recruitment would be relevant. Moreover, the effects of microclimatic conditions in ectoparasite abundance should also be further scrutinised in nests with and without aromatic plants.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s00265-023-03393-9>.

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**Data availability** All data generated or analysed during this study are included in this published article.

## Declarations

**Ethics approval** This research involved animals, and the authors assert that all procedures contributing to this work comply with the ethical standards of the European and Portuguese guidelines for animal welfare (EU Directive 2010/63/EU and Portuguese Decree-Law no113/2013) and were performed under licence of the competent authorities regulating the use of animals for research (ICNF Licence N°04/2020/CAPT). Approval from ethics committee was not required for this study.

**Conflict of interest** The authors declare no competing interests.

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