Heterogeneidad ambiental y evolución del tamaño de puesta en el herrerillo común *(Cyanistes caeruleus)*

Testando la hipótesis de la adaptación local

Jorge Garrido Bautista

Tesis doctoral

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Memoria presentada por el graduado Jorge Garrido Bautista para optar al título de Doctor por la Universidad de Granada

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Los científicos son mezquinamente celosos,
siguen apegados a sus ideas mucho después de que hayan quedado obsoletas,
y se incomodan cada vez que surge algo nuevo que no fueron capaces de anticipar.

Frans de Waal

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General discussion

Abstract

Environmental heterogeneity creates a range of habitats of different quality for species, which exert distinct selective pressures that will shape the genotype and, consequently, the phenotype of organisms. Traditionally, evolutionary models considered both the large-scale environmental heterogeneity and geographical barriers as the main promoters for adaptive population divergence. However, recent research shows that small scale environmental heterogeneity, together with non-random dispersal, can promote genotypic and phenotypic population differentiation, sometimes even leading to local adaptation.

In this thesis, I document the effect of fine-scale environmental heterogeneity on the evolutionary dynamics of a wild population of blue tits (*Cyanistes caeruleus*). I characterized various abiotic and biotic factors in different adjacent forest formations, which form a continuous woodland extending across the two opposing slopes of a valley, and analysed how such environmental heterogeneity promotes the adaptive divergence between populations and shapes a habitat-dependent optimal clutch size. To this end, this thesis has been divided into two parts. The first part shows how environmental heterogeneity influences the offspring's phenotype; while the second part demonstrates how such heterogeneity, together with non-random dispersal, has promoted the genotypic and phenotypic divergence within the blue tit population, as well as the evolution of a habitat-dependent and locally adapted optimal clutch size.

The forest formations extending along the valley exhibited distinct selective pressures for blue tits during reproduction, as they differed in a wide variety of abiotic and biotic factors. The forest formations of the east-facing slope of the valley (a Holm oak forest and a dry Pyrenean oak forest), unlike the forest formations of the west-facing slope (a Scots pine forest and a humid Pyrenean oak forest), received more solar radiation, had more insolation time and temperature, and showed lower humidity, tree cover and food availability. Moreover, the presence of ectoparasites (prevalence of *Ceratophyllus gallinae* fleas and *Protocalliphora azurea* blowflies, and abundance of *Culicoides* biting midges) was higher in nests of the east-facing slope of the valley, while the presence of haemoparasites (microfilariae) infecting adult blue tits did not vary along the continuous woodland. The presence of *Nasonia vitripennis* parasitoids, which in turn parasitize *P. azurea* pupae, was higher in nests of the west-facing slope of the valley.

Blue tits adjusted their reproductive effort to the forest type where they bred. The laying date was earlier in deciduous (dry and humid Pyrenean oak forests) than in evergreen (Scots pine and Holm oak forests) forests, whilst the clutch size was the lowest in the Scots pine forest and the highest in the humid Pyrenean oak forest, with intermediate values found in the Holm oak and dry Pyrenean oak forests. The clutch size determined the number of offspring a blue tit pair raised in a single reproductive event, since there were no habitat-dependent differences in hatching success nor fledgling success. Although the oxidative status and immune system of nestlings (measured at 13 days after hatching) varied between forest types, the offspring quality, in terms of mean body mass and body condition, was similar across the woodland. No effects of ectoparasites on blue tit reproduction, nestling oxidative status or nestling immune system were detected; however, the presence of fleas in nests was associated with a lower body mass for small, marginal nestlings and a higher number of nestlings in a nest. For their part, the presence of biting midges and microfilariae was negatively related to nestling and adult body mass, respectively.

Results from natal dispersal showed a strong philopatry, with first-year blue tits being recaptured as reproductive adults in their natal slope of the valley. Accordingly, population genetic analyses revealed the existence of two genetic clusters, with blue tits from each cluster associated with the slope they inhabit. The two genetic populations showed divergence in clutch size, exceeding the level of differentiation expected based on genetic drift, hence suggesting that natural selection has favoured different clutch sizes in the two slopes of the valley. A brood size manipulation experiment demonstrated the existence of a habitat-dependent optimal clutch size; an experimental increase of the brood size provoked a decrease in the offspring condition, in terms of mean body mass and body condition, in the two forest types where the experiment was carried out (dry and humid Pyrenean oak forests), mainly because parents did not increase their feeding effort.

Overall, the results of this thesis evidence that environmental heterogeneity at a fine scale can promote the genotypic and phenotypic population divergence, even in organisms with a potentially high dispersal capacity, such as birds. The different chapters presented in this thesis demonstrate how, even in a continuous and heterogeneous woodland, natural selection may favour adaptive divergence and, consequently, local adaptation for life-history traits, such as clutch size.

Resumen

La heterogeneidad ambiental es responsable de la existencia de hábitats de distinta calidad para las especies, los cuales ejercen presiones selectivas diferentes que acabarán moldeando el genotipo y, por *ende*, el fenotipo de los organismos. Los modelos evolutivos clásicos consideran la heterogeneidad ambiental a gran escala, junto a la existencia de barreras geográficas, como uno de principales promotores para la divergencia adaptativa. Sin embargo, las últimas investigaciones muestran que la heterogeneidad ambiental a pequeña escala espacial y la dispersión no aleatoria también promueven la diferenciación genética y fenotípica de las poblaciones, conduciendo incluso a situaciones de adaptación local.

El objetivo de esta tesis es documentar el efecto de la heterogeneidad ambiental a pequeña escala espacial sobre la dinámica evolutiva de una población salvaje de herrerillo común (*Cyanistes caeruleus*). Se caracterizaron diversos factores abióticos y bióticos de distintas formaciones boscosas adyacentes, que forman un continuo de hábitats y se sitúan a lo largo de las dos laderas enfrentadas de un valle, y se analizó cómo dicha heterogeneidad ambiental a tan pequeña escala espacial promueve la divergencia adaptativa de las poblaciones y modula un tamaño de puesta óptimo dependiente del hábitat. Para ello, esta tesis se ha dividido en dos bloques. El primero versa sobre cómo la heterogeneidad ambiental modula el fenotipo de la descendencia, mientras que el segundo bloque trata sobre cómo dicha heterogeneidad ambiental, junto a una dispersión no aleatoria, ha promovido la divergencia genética y fenotípica de las poblaciones de herrerillo común, además de demostrar la evolución de un tamaño de puesta óptimo distinto y localmente adaptado según el tipo de hábitat.

Los bosques que se extienden a lo largo del valle supusieron diferentes presiones selectivas para los herrerillos reproductores, ya que variaron en numerosos factores abióticos y bióticos. Los bosques de la loma oeste del valle (un encinar y un robledal seco), en comparación con los bosques de la loma este (un pinar y un robledal húmedo), recibieron una mayor radiación solar y tiempo de exposición a dicha radiación, su temperatura ambiente fue mayor, su humedad relativa menor, tuvieron una menor cobertura arbórea y mostraron una menor disponibilidad de alimento. Además, la presencia de ectoparásitos (prevalencia de pulgas *Ceratophyllus gallinae* y larvas de mosca *Protocalliphora azurea*, y abundancia de ceratopogónidos *Culicoides*) fue mayor en los nidos ubicados en los bosques de la loma este del valle,

mientras que la presencia de hemoparásitos (microfilarias) en los herrerillos reproductores fue constante a lo largo del continuo de hábitats. La presencia de parasitoides (*Nasonia vitripennis*) de pupas de *P. azurea* fue mayor en los nidos ubicados en los bosques de la loma este del valle.

Los herrerillos ajustaron su esfuerzo reproductivo al tipo de bosque donde se reprodujeron. La fecha de puesta estuvo adelantada en los bosques caducifolios (robledales) y retrasada en los bosques perennifolios (encinar y pinar), mientras que el tamaño de puesta fue menor en el pinar y mayor en el robledal húmedo de la loma este del valle, con valores intermedios en el encinar y el robledal seco. Al no existir diferencias dependientes del tipo de bosque en la tasa de eclosión o supervivencia de los pollos hasta el abandono del nido, el tamaño de puesta predijo el número de descendientes que una pareja de herrerillo común produce por evento reproductivo. Aunque el estado oxidativo y el sistema inmunitario de los volantones (medidos a los 13 días de edad desde la eclosión) varió según el tipo de bosque, la calidad de la descendencia, en términos de tamaño corporal y condición física, fue similar a lo largo del continuo de hábitats. Los ectoparásitos no afectaron a la reproducción de los herrerillos, ni al estado oxidativo o sistema inmunitario de los volantones; sin embargo, la presencia de pulgas en los nidos estuvo relacionada con un menor peso corporal de los volantones más pequeños de la nidada y un mayor número de volantones en el nido. Por otro lado, la presencia de *Culicoides* y microfilarias se relacionó negativamente con la masa corporal de los volantones y los adultos reproductores, respectivamente.

Los resultados de dispersión natal mostraron una fuerte filopatría de los herrerillos, con los volantones siendo anillados en su primer año como reproductores en la misma loma del valle donde nacieron. En consecuencia, los análisis de genética de poblaciones revelaron la presencia de dos clústeres genéticos, cada uno situado en una loma del valle. Las dos poblaciones genéticas mostraron además una divergencia en el tamaño de puesta mayor que la que se esperaría según la deriva genética, sugiriendo así que la selección natural ha favorecido distintos tamaños de puesta en las dos lomas del valle. Un experimento de alteración del tamaño de la nidada demostró la existencia de un tamaño de puesta óptimo distinto según el tipo de bosque; un aumento del tamaño de nidada causó una disminución de la condición física de la descendencia en los bosques donde se realizó el experimento (robledal seco

de la loma este y robledal húmedo de la loma oeste), debido a que los parentales no incrementaron su esfuerzo de ceba.

En conjunto, los resultados de esta tesis demuestran que la heterogeneidad ambiental a micro-escala puede promover la divergencia genética y fenotípica de las poblaciones, incluso en organismos con una capacidad de dispersión potencial tan alta como las aves. Los capítulos presentados en esta tesis demuestran cómo, bajo un escenario de heterogeneidad ambiental dentro de un continuo de hábitats, la selección natural puede favorecer la divergencia adaptativa y, en consecuencia, la adaptación local para rasgos de estrategias vitales como el tamaño de puesta.

GENERAL INTRODUCTION

Challenging the traditional view. From large to fine scale population divergence and adaptation

Spatiotemporal variation in environmental factors is ubiquitous, so the existence of environmental heterogeneity implies that habitats are of different quality for a given species. The degree of environmental heterogeneity creates the range of habitats available in which to reproduce (Sparrow 1999), forming a mosaic of optimal and suboptimal habitats or a gradient of habitats (Hansson et al. 1995). Understanding which mechanisms underlie population divergence and adaptation in response to such environmental variation is a central issue in evolutionary biology. When facing habitat heterogeneity, organisms are expected to match their phenotype to local environmental conditions in order to maximize fitness. In other words, a species inhabiting a wide variety of habitats would face distinct selective pressures (e.g. food availability, parasite presence) that will, under the proper conditions and through several generations, conduct to adaptation (Darwin & Wallace 1858; Darwin 1959). Therefore, in heterogeneous environments, natural selection is expected to promote a range of phenotypes, as organisms can access to different habitats that are optimal for each phenotype (Edelaar & Bolnick 2019). By contrast, in homogeneous environments, natural selection should favour a common, well-matched phenotype (Edelaar et al. 2017). However, the combined effects of several evolutionary processes, including stochastic (genetic drift and mutation) and deterministic (selection and gene flow) processes, actually explain the population divergence and adaptation (Slatkin 1987; Lenormand 2002).

Geographical barriers have traditionally viewed as the main cause for population adaptive divergence (Slatkin 1987), together with the fact that genetic differentiation between populations typically increases with geographical distance (Slatkin 1993; Hutchinson & Templeton 1999; Clegg & Phillimore 2010). This is because geographical barriers and large geographical distances restrict gene flow between populations, thus leading to an isolation by environment and isolation by distance pattern, respectively. This isolation may promote the evolution of local adaptations, which evolve across large geographical distances (e.g. Laugen et al. 2003; Antoniazza et al. 2010; Stelkens et al. 2012), and may be, in certain conditions, the initial stage in the process of ecological speciation (Lowry 2012). Local adaptation occurs when a population evolves traits that confer higher survival and reproduction (i.e. fitness) in

the home environment relative to populations from other environments (Kawecki & Ebert 2004; Blanquart et al. 2013; Wadgymar et al. 2022). Local adaptation is generally a result of divergent selection over one or more traits which exceeds the homogenizing effects of gene flow (García-Ramos & Kirkpatrick 1997; Hendry et al. 2002; Lenormand 2002; Nosil & Crespi 2004). It is thus expected that local adaptation would occur across broader geographical scales because gene flow between populations should be restricted.

However, this traditional view has changed during last decades. Population adaptive divergence at fine spatial scales occurs more commonly than once expected. In fact, geographical barriers and large distances are no longer considered essential for population divergence and adaptation (see, for example, Richardson et al. 2014). Research into population divergence and adaptation at small spatial scales has received little attention because gene flow, considered as an opposing evolutionary force to natural selection, has been assumed to homogenize gene pools and prevent adaptive divergence at the small scale. Nonetheless, genetic and adaptive population divergence may occur at spatial scales much smaller than previously thought because gene flow can be non-random (Edelaar & Bolnick 2012) and other ecological and evolutionary mechanisms besides natural selection might initiate adaptive divergence (Richardson et al. 2014). Also, genetic differentiation can be observed at the small scale depending on local environmental and ecological conditions (Ferrer et al. 2016), which can restrict gene flow among populations when organisms match habitat selection to natal conditions (Edelaar et al. 2008). Moreover, intermediate levels of gene flow can maintain small scale adaptive divergence (Garant et al. 2007; Fitzpatrick et al. 2016, 2017). In sum, local adaptation can occur at the fine spatial scales at which populations should experience high gene flow based on the expected levels of dispersal. Different not-mutually exclusive mechanisms may promote the small scale local adaptation (e.g. natural selection, habitat choice or selective barriers against migrants; summarised in Richardson et al. 2014), and these processes act by either increasing the selection strength or reducing the maladaptive gene flow. During last years, evidence from a wide variety of taxa, from plants to invertebrates and vertebrates, suggest that fine scale population divergence and adaptation are in fact more common than previously thought (Hargeby et al. 2004; Skelly 2004; Antonovics 2006; Laine 2006; Shine et al. 2012; Snowberg & Bolnick 2012; McDevitt et al. 2013; Richardson & Urban 2013; Langin et al. 2015; Izen et al. 2016; Herrera et al. 2017).

Box 1. Implications of local adaptation for conservation biology

Nature is facing a rapid global change nowadays, including global warming, changes in land uses, habitat fragmentation or the introduction of alien species in local environments. Human-induced changes in local environmental conditions influence evolutionary processes and, ultimately, biodiversity (Candolin & Wong 2012). Thus, understanding processes of fine scale adaptation to environmental heterogeneity is essential to predict how species' distributions and abundances will be affected by climate change (Smith et al. 2019). Information on local adaptation is increasingly becoming pivotal for policy, management and conserving biodiversity (Meek et al. 2023), as well as to prepare populations for future conditions under climate change (Capblancq et al. 2020; Meek et al. 2023). For example, information on local adaptation may be used to improve the adaptive potential of populations to maximize their long-term persistence (Flanagan et al. 2018), identify management units and design conservation plans (Flanagan et al. 2018; Peterson et al. 2019), manage gene flow to incorporate locally adaptive alleles into populations when conducting translocation and reintroduction plans (Catullo et al. 2019; Thurman et al. 2022) and preserve locally adapted genotypes through seed banking and cryopreservation (Howard et al. 2016; Tunstall et al. 2018). Still, despite the last methodological advances for studying local adaptation, information on local adaptation is missing for many endangered species and, as noted by Meek et al. (2023): "*conservation practitioners need to identify the best combination of strategies, tools, and resources for adaptively managing species with local adaptation in mind*".

In this respect, it is worth highlighting that fine scale population genetic and phenotypic divergence have been evidenced even in highly motile organisms, such as birds, in some cases with important consequences for local adaptation (Garant et al. 2005; Postma & van Noordwijk 2005a; Blondel et al. 2006; Senar et al. 2006; Postma et al. 2007, 2009; Björklund et al. 2010; Ortego et al. 2011; Porlier et al. 2012a; Arnoux et al. 2014; García-Navas et al. 2014a; Camacho et al. 2016; Ferrer et al. 2016; Menger et al. 2017; Recuerda et al. 2023). For example, non-random gene flow and divergent

selection can generate small scale genetic differentiation and local adaptation of several avian traits, such as clutch size (Postma & van Noordwijk 2005a; Postma et al. 2007, 2009) or genetic variance in offspring's body mass (Garant et al. 2005). In addition, different ecological barriers may promote the small scale genetic differentiation among bird populations within habitat mosaics (Ferrer et al. 2016), which ultimately may promote the phenotypic divergence and local adaptation (García-Navas et al. 2014a). Lastly, restricted gene flow and selection against immigrant genes, together with assortative mating, can generate fine scale local adaptation of breeding and morphological traits in some insular bird populations (Blondel et al. 1999, 2006; Charmantier et al. 2016).

Nonetheless, other evolutionary scenarios besides local adaptation may emerge when populations inhabit nearby and ecologically different habitats. When the homogenizing effects of gene flow overcomes the diversifying effects of selection, the evolution of local adaptation will be restricted. This is because gene flow across habitat patches or boundaries can introduce maladapted alleles into locally adapted populations, preventing local populations from expressing optimal phenotypes for their habitats (Slatkin 1987; Orr 2000), thus conducing to local maladaptation. Local maladaptation occurs when a local population have reduced survival and reproduction in the home environment in relation to foreign populations (Brady et al. 2019a). Accordingly, maladapted populations are expected to show a phenotypic deviation from its optimal adaptive peak within a particular habitat. Besides gene flow from populations well-adapted to other environments (Bolnick & Nosil 2007), maladaptation may be caused by several not-mutually exclusive processes, such as genetic drift, genetic trade-offs (antagonistic pleiotropy and genetic linkage) or selection on correlated traits (summarised in Brady et al. 2019b).

Maladapted populations have been described in several taxa (e.g. Brady 2013, 2017; Rolshausen et al. 2015; Rogalski 2017; Negrín-Dastis et al. 2019; Singer & Parmesan 2019), including birds. For example, high levels of gene flow between close habitats of different quality can maintain maladapted clutches in tits (Paridae), with populations exhibiting deviations from their optima (Dhondt et al. 1990; Blondel et al. 1998), or may provoke a mismatch in the optimal time of reproduction (Dias & Blondel 1996; Blondel et al. 2001). In some cases, a source–sink population structure can be established when migrants from a large, well-adapted population (the source)

carry alleles that are less fit in a small, local environment (the sink). Consequently, realized gene flow will displace small populations from their local optima (Bolnick & Nosil 2007), as demonstrated in close bird populations (Dias & Blondel 1996; Blondel et al. 2001, 2006). On the other hand, when gene flow homogenizes gene pools among populations, the phenotypic variation observed in populations inhabiting ecologically different conditions may be a consequence of adaptive phenotypic plasticity (Chevin & Hoffmann 2017), which can provide the potential for organisms to rapidly respond to environmental changes (Przybylo et al. 2000; Charmantier et al. 2008; Biquet et al. 2022), in contrast to genetically microevolution. Also, the selection of some breeding traits may affect the variation of phenotypic plasticity in populations found in heterogeneous habitats (Porlier et al. 2012b). Overall, these plastic responses to habitat variation can occur simultaneously with local adaptation within the same population (Blondel 2007; Blondel et al. 2006).

Clutch size as the focal trait. Identifying the optimal clutch size

Clutch size was used as the focal trait in this thesis. Clutch size is considered to be an ecologically relevant trait which plays a major role in determining reproductive rates and population regulation. The determinants of clutch size have been long studied and are well-known (Klomp 1970; Murphy & Haukioja 1986), with the spatiotemporal variation in food availability having a strong influence on clutch size determination (Lundblad & Conway 2021). In the blue tit (*Cyanistes caeruleus*), for example, the clutch size and habitat-dependent variation has been studied extensively in recent decades (see the next section). Blue tit clutch size varies from large to small geographical scales, with female blue tits laying larger clutches in deciduous forests than in evergreen, coniferous forests (e.g. Blondel et al. 1987, 1993, 2006; Dias et al. 1994; Tremblay et al. 2003; Lambrechts et al. 2004). This is because deciduous forests typically contain more food resources (caterpillars and other invertebrates) (Tremblay et al. 2003), but also because the peak of caterpillars appears earlier in the season in deciduous patches (Blondel et al. 1991; Tremblay et al. 2003, 2005). Also, the blue tit clutch size increases with increasing latitude, mainly as consequence of habitat-specific constraints in food availability (Blondel et al. 2006; Ziane et al. 2006; Charmantier et al. 2016). Besides food availability, clutch size may be determined by other factors, such as breeding population density (Dhondt et al. 1992) or parasite presence (Martin et al. 2001).

Clutch size has proven a suitable trait for micro-evolutionary studies. Specifically, clutch size has an important heritable component in natural populations of several passerine birds (reviewed in Postma & van Noordwijk 2005b), including the blue tit (García-Navas et al. 2014a), suggesting the existence of a substantial additive genetic variance for this life history trait. This allows clutch size to provide options and responses for selection to act on (e.g. Gibbs 1988; Price & Liou 1989; Sheldon et al. 2003). The optimal clutch size, defined as that which maximizes the fitness (Charnov & Krebs 1974), reflects the parent's ability to rear offspring surviving to the maturity and is determined by both female phenotypic quality (Perrins & Moss 1975; Nicolaus et al. 2015) and local environmental variation (Perrins & Moss 1975; Högstedt 1980; Török et al. 2004). When facing habitat heterogeneity, individuals are expected to adjust their clutch size to local circumstances. When the maladaptive gene flow is reduced or absent, populations will show different and habitat-specific adaptive optima, suggesting the existence of local adaptation for clutch size. Indeed, clutch size optimization occurs in several bird populations (Perrins & Moss 1975; Gustafsson & Sutherland 1988; Pettifor et al. 1988, 2001; Tinbergen & Daan 1990; Pettifor 1993; Knowles et al. 2010), even at the small spatial scales at which populations should experience high gene flow. For example, a genetically distinct island population of great tits (*Parus major*) can maintain small and optimal clutches due to strong selection against immigrant genes for large clutches from outside the island, despite its closeness to other island populations (Postma & van Noordwijk 2005a; Postma et al. 2009). The divergence in clutch size may exceed the neutral genetic differentiation in close populations of blue tits separated by few kilometres, suggesting the existence of local adaptation (García-Navas et al. 2014a). Also, island populations of blue tits inhabiting nearby and ecologically different habitat patches may exhibit clutch size optimization due to non-random dispersal and the existence of small scale selective barriers (Blondel et al. 2006; Charmantier et al. 2016).

However, populations might fail to optimize the clutch size (Nur 1984; Dhondt et al. 1990; Blondel et al. 1998; Sanz & Tinbergen 1999; Tinbergen & Both, 1999; Rytkönen & Orell 2001; Tinbergen & Sanz 2004; Török et al. 2004), mainly because gene flow between close (Dhondt et al. 1990; Blondel et al. 1998) or distantly (Postma & van Noordwijk 2005a) populations with different optima, which might preclude habitat-dependent local adaptation for clutch size; but also because of unpredictable environmental fluctuations (McNamara 1998). For all these reasons, clutch size

represents an ideal life history trait to examine potential patterns of adaptive divergence at the small scale, and thus it was used as a focal trait in this thesis.

Model organism

The Eurasian blue tit (*Cyanistes caeruleus*) is a small (body mass: 9–12 g, length: 11–12 cm), forest-dwelling and insectivorous passerine. It is a small tit (Paridae) characterized by its short bill, rounded head and singular coloration: a head with a blue crown patch surrounded by white cheek patches outlined with a blackish throat bib, together with bright blue wings and tail as well as yellow underparts (Fig. 1a). The blue tit is widely distributed in the western Palearctic and has recently been separated from its close relative in north Africa and the Canaries islands, these nowadays constituting a separate species (Shirihai & Svensson 2018; Stenning 2018).

There is slight and clinal variation in its morphology, mainly affecting plumage colouration and being indicative of the subspecies. *Cyanistes caeruleus caeruleus* extends over much of Europe except west and south-west, but including most of Mediterranean large islands; *C. c. obscurus* is confined to British Isles; *C. c. ogliastrae* is distributed in the southern Iberia; *C. c. balearicus* resides in Balearics; and *C. c. orientalis*, *C. c. raddei* and *C. c. persicus* are distributed in the eastern part of Europe, from south-east Russia to the surroundings of the Caspian sea (Shirihai & Svensson 2018). Mitochondrial control region DNA sequences from several European populations of blue tits have revealed that this species colonized central Europe from the Iberian Peninsula and the Balkans after the last glaciation during the Pleistocene (Kvist et al. 1999, 2004).

The blue tit is mainly resident in the Iberian Peninsula, although few populations may display migrant movements in some years or seasons (reviewed in Salvador 2016). It is a hole-nesting species, nesting in natural and secondary cavities in trees (Stenning 2018). However, the blue tit may readily use nest boxes when provided (Serrano-Davies et al. 2017; Fig. 1b), which makes individual monitoring and sample collection easy. For this reason, the blue tit has been a popular model organism for ecological and evolutionary research (e.g. Blondel et al. 2006; Charmantier et al. 2016; Mueller et al. 2016; Perrier et al. 2020).

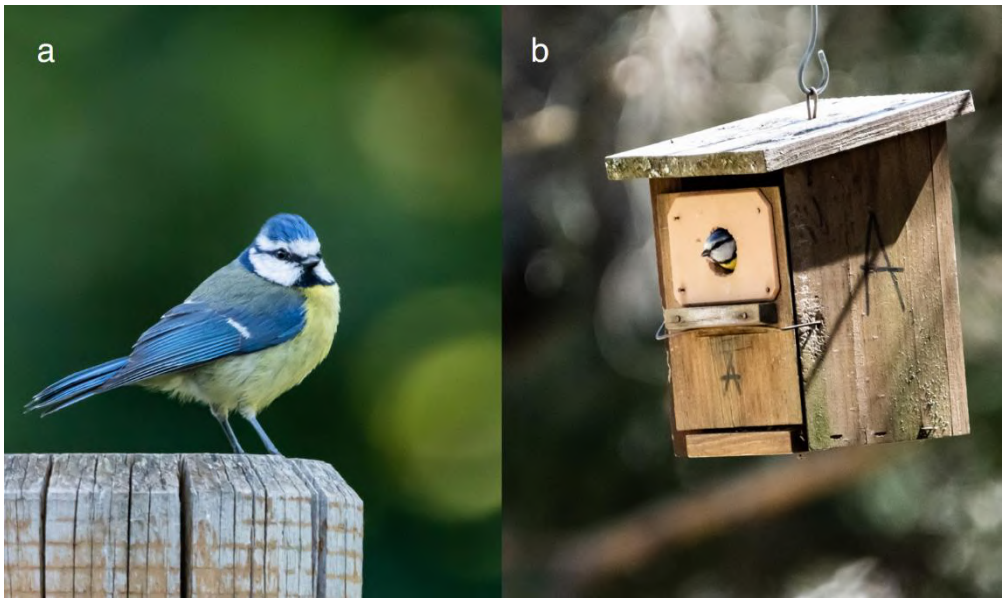

Figure 1. Eurasian blue tit (*Cyanistes caeruleus*) posed in a wood post (a) and leaving the nest box (b). Photos by David Ochoa Castañón.

Sexes are similar, but they can be separable by slight differences in plumage: the male has intense blue marginal coverts edges, while the female has a more bluish grey marginal covert edges. Females produce a mean clutch of 10 eggs (6–9 eggs in the Iberian Peninsula) in a single brood, which she incubates alone for 12–14 days while being fed by the male (Fargallo 2004; Salvador 2016). Eggs generally hatch asynchronously (hatching spread of 2 days; Slagsvold et al. 1995; Stenning 2008), leading to within-brood size hierarchy and resulting in a brood comprising core and marginal nestlings (Stenning 2018). Blue tits are socially monogamous, but extra-pair paternity is relatively common (Kempenaers et al. 1992; Schlicht & Kempenaers 2013; Schlicht et al. 2014). Both female and male provision nestlings in the nest, although the degree of provisioning rate may depend on prey size, brood size and nestling age (García-Navas & Sanz 2011a, 2011b; García-Navas et al. 2014b). Fledglings leave the nest between 16–22 days after hatching (Salvador 2016).

Box 2. Blue tit non-random dispersal and local (mal)adaptation

The blue tit is mostly sedentary, showing a limited and non-random dispersal. Both sexes disperse over short distances, typically less than 1 km from their natal territory, but females disperse over long distances than males (Matthysen et al. 2005; Foerster et al. 2006; Parejo et al. 2007; Ortego et al. 2011; García-Navas et al. 2014a). Females may decrease their breeding dispersal distances if they had a high hatching success in the preceding breeding season (García-Navas & Sanz 2011c), which in turn can increase their offspring recruitment rate (García-Navas et al. 2014a). This restricted and sex-biased dispersion makes the blue tit an excellent model organism to examine processes of genetic and phenotypic population divergence at a small scale. Several studies have revealed that this limited dispersion reduces the gene flow among neighbouring blue tit populations and promote genetic differentiation (Ortego et al. 2011; Ferrer et al. 2016), as well as local adaptation for morphological and reproduction traits (Blondel et al. 1999; Porlier et al. 2012a; García-Navas et al. 2014a; Charmantier et al. 2016). Nevertheless, other studies have reported maladaptation in some close populations of blue tits as a consequence of high gene flow (Blondel et al. 1998), leading to a source-sink population structure (Dias & Blondel 1996; Blondel et al. 2001, 2006). The blue tit has been proved to be a model species to examine evolutionary processes at a very small spatial scale, and it was used in this thesis to examine fine scale patterns of genetic and phenotypic divergence.

The blue tit can be found in a wide variety of habitats, ranging from urban areas (Vaugoyeau et al. 2016) to natural woodlands. The breeding biology of this species and the habitat-dependent variation in reproductive parameters within Europe has been studied extensively during last decades. In natural habitats, studies have found that deciduous forests are the preferred habitat for blue tits as they correspond to the highest reproductive and rearing parameters, including clutch size, feeding effort, nestling growth or the number and quality of offspring (e.g. Blondel et al. 1987, 1991, 1999, 2006; Gil-Delgado et al. 1992; Dias et al. 1994, Fargallo & Johnston 1997; Tremblay et al. 2003, 2005; Lambrechts et al. 2004; Blondel 2007; Garrido-Bautista et al. 2023). Deciduous forests offer a higher quality habitat for blue tits during the

breeding season than coniferous, evergreen forests because they typically contain more caterpillars and other invertebrates (Huhta et al. 1998; Tremblay et al. 2003), but also because the peak of caterpillars usually appears earlier in the season in deciduous forests (Blondel et al. 1991; Tremblay et al. 2003, 2005). Also, in contrast to deciduous forests, coniferous and evergreen forests impose foraging costs to blue tits (Díaz et al. 1998). As in many other passerines, blue tits feed on small invertebrates, with caterpillars being the most important prey consumed during the reproduction period. Indeed, caterpillars are a substantial part of the nestling diet in deciduous and evergreen woodlands, followed by spiders and other small invertebrates (Blondel et al. 1991; Tremblay et al. 2005; García-Navas & Sanz 2011b).

Study system

Sierra Nevada is the most important mountain range of the Baetic system, being located in the south-east of the Iberian Peninsula. It extends mostly over the Spanish province of Granada, although its easternmost part extends over the Spanish province of Almería. Sierra Nevada runs along almost 90 km, has a maximum width of 30 km and its surface is about 2,000 km^2. Sierra Nevada was declared as nature reserve by the United Nations Educational, Scientific and Cultural Organization (UNESCO) in 1986. In 1989, most of its area was declared as Natural Park by the Andalusian government, while, 10 years later, also a large part of its area was declared as National Park by the Spanish Parliament due to its singular flora, fauna and geomorphology.

The study area is located 1700–1800 m above the sea level (a.s.l.) in the south-facing slope of Sierra Nevada National Park (south-eastern Spain; 36^{o}57'N, 3^{o}24'W). Although Sierra Nevada comprises several biogeographic sectors ("malacitano-almijarense", "alpujarreño-gadorense" and "nevadense"), the study area belongs to the "nevadense" biogeographic sector, characterized by the presence of metamorphic siliceous substrate of Palaeozoic origin (e.g. schists, quartzites, phyllites, slates and micas). The study area is located in the superior meso-mediterranean bioclimatic zone, thus exhibiting hot and dry summers and cold winters with snowfalls and frosts (Valle et al. 2004; Olivares et al. 2011). The potential vegetation of the study area is dominated by "nevadense" siliceous vegetation series, including shrubs (*Genista* sp., *Cystus* sp., etc.) and Holm oak (*Quercus ilex*) and Pyrenean oak (*Quercus pyrenaica*) woodlands (Valle et al. 2004).

The study area, with 800 ha in total, consists of a Mediterranean heterogeneous and continuous woodland located along a valley, with two mountain slopes, one facing each other and being separated by approximately 1.5 km (Fig. 2). The east-facing slope of the valley is composed by two contiguous main forest formations, one of Holm oaks and another of Pyrenean oaks, while the west-facing slope of the valley is composed by other two main forest formations of Scots pines (*Pinus sylvestris*) and Pyrenean oaks (Fig. 2). The Pyrenean oak forests from the east- and west-facing slopes of the valley are referred as dry and humid, respectively, thorough this thesis because the latter is traversed by a stream. In the west-facing slope of the valley, there is a river (río Chico) crossing the Scots pine forest and a stream (acequia Almiar) that cross both the Scots pine and the humid Pyrenean oak forests (Fig. 2). Note that thorough this thesis the humid Pyrenean oak forest is sometimes referred as mixed or humid forest (chapters 1, 2, 3, 4 and 5), mainly due to the idiosyncrasies of the scientific publication system. This is because the humid Pyrenean oak forest consists mainly of Pyrenean oaks along with some Holm oaks and, as aforementioned, is traversed by the Almiar stream.

The four forest formations represent different environmental pressures for blue tits during reproduction, as they differ markedly in a wide variety of factors, such as solar radiation, insolation time, temperature, humidity, canopy cover, vegetation quality (estimated as normalized difference vegetation index, NDVI), food availability and parasite presence. To characterize forest heterogeneity and estimate parasite pressure, several image analysis techniques and nest collection, trapping and molecular-based methods were performed during the course of this thesis. Quantum GIS 3.10.5 (QGIS Development Team 2020) and ArcGIS Desktop 10.3.1 (ESRI, Redlands, CA, USA) software were used to analyse solar radiation, insolation time, canopy cover and NDVI, whilst dataloggers iButtom® were used to measure the ambient temperature and humidity. Food availability was estimated by shooting oak branches in standardized conditions, following Zandt (1994). Caterpillars are the key food source for blue tits during spring (Blondel et al. 1991, Tremblay et al. 2005, García-Navas & Sanz 2011b), including the blue tits from the population studied in this thesis (72% of all identified prey items consumed by nestlings were caterpillars and Lepidoptera pupae; see Chapter 8). Thus, caterpillar availability was used as a proxy for food availability.

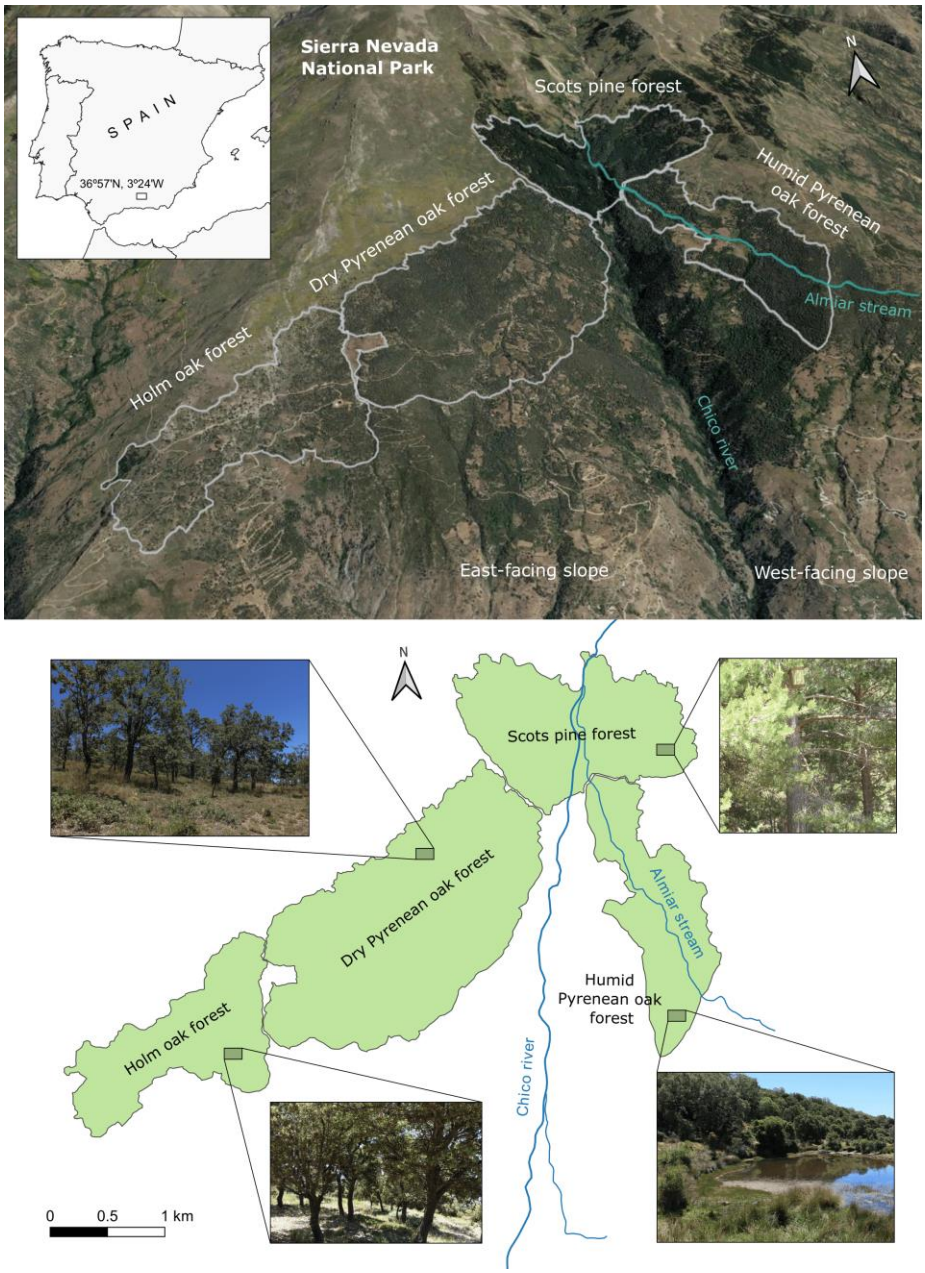

Figure 2. Map of the study area showing the locations of the four forest formations in the Sierra Nevada National Park (above: satellite composition, below: vector composition with photographs). The east-facing slope of the valley is composed by the Holm oak and dry Pyrenean oak forests, while the west-facing slope is composed by the Scots pine and humid Pyrenean oak forests. Notice that the Chico river separates the two mountain slopes and crosses the Scots pine forest, while the Almiar stream traverse both the Scots pine and humid Pyrenean oak forests. Maps created with QGIS 3.10.5 software, connected to Google Earth. Photos by Jorge Garrido Bautista.

On the other hand, flying ectoparasites (*Culicoides* sp. biting midges and *Simulium* sp. black flies) were collected and identified by using a trapping method specifically developed for nest boxes. Nest-dwelling ectoparasites (*Protocalliphora azurea* blowflies and *Ceratophyllus gallinae* hen fleas) were collected and examined by sorting through nest material once blue tit nestlings fledged and left the nest. The presence of haemoparasites (filarial worms) in the bloodstream of adult blue tits was checked using the PCR.

The specific GIS and remote sensing analyses and results (solar radiation, insolation time, canopy cover, NDVI) are given in Chapter 6, although partial results can also be found in the supplementary material of Chapters 2, 4, 5 and 7. The methodology, statistical analyses and results relative to dataloggers (temperature and humidity) are given in the supplementary material of Chapters 2, 4, 5 and 7, as well as in Chapter 8. The specific methodology used to estimate food (caterpillar and other arthropods) availability and the respective results are given in Chapter 8 and the supplementary material of Chapters 4 and 5. Chapters 1, 4, 5 and 6 include the description of the protocol followed to collect and identify the nest-dwelling ectoparasites (blowflies and fleas), as well as the results obtained. Chapters 2 and 6 include the description of the protocol followed to trap flying ectoparasites (biting midges and black flies) and the respective results. Lastly, Chapter 3 delves with the identification of haemoparasites (filarial worms) infecting adult blue tits across the woodland.

In short, the two forest formations of the east-facing slope (Holm oak and dry Pyrenean oak forests) receive more solar radiation, have more time of insolation per day, lower percentage of dense tree cover and lower NDVI than the two forest formations of the west-facing slope (Scots pine and humid Pyrenean oak forests). The food (caterpillar) availability is lower in the dry Pyrenean oak forest of the east-facing slope than in the humid Pyrenean oak forest of the west-facing slope. The frequency of nest infestation by nest-dwelling ectoparasites (blowflies and fleas) is lower in the east-facing slope than in the west-facing slope, although there are no differences between forests in the frequency of nest infestation by flying ectoparasites, neither by biting midges nor black flies. However, the abundance of biting midges in nest boxes is higher in the east-facing slope of the valley than in the west-facing slope. There is no forest-dependent variation in the presence of haemoparasites (filarial worms)

infecting adult blue tits. The reader is encouraged to read the respective aforementioned chapters to find the specific analyses and results relative to forest characterization and parasite presence.

General methods

The correlative and experimental study and data collection for this thesis was carried out during springs of 2016–2019 and 2021 using blue tits breeding in nest boxes. During all breeding seasons, between 100 and 175 nest boxes (number of nest boxes per year; 2016: 175, 2017: 170, 2018: 120, 2019: 100, 2021: 150) were placed throughout the four forest formations, and their geographical position was recorded with a GPS device. The location of nest boxes remained constant throughout the study. In 2019 and 2021, nest boxes were installed only in the dry and humid Pyrenean oak forests because experiments were carried out in these two forests (see Chapters 2 and 8). Nest boxes were all of the same type (ICONA C model; basal area: 196 cm^2, height: 20 cm, hole diameter: 3 cm, material: painted wood; more details in Moreno-Rueda 2003). Nest boxes were hung from a tree branch using a metal hook at a height of 3–4 m, and the average separation between nest boxes was 96.56 ± 53.20 m (mean ± s.d.). The occupation rate of nest boxes by blue tits was high in all years (2016: 57.7%, 2017: 60.0%, 2018: 68.3%, 2019: 74.0%, 2021: 66.0%).

Nest boxes were monitored throughout each year's breeding season to determine basic reproductive parameters of blue tits, namely the laying date (date of the first egg laid), clutch size, hatching date (date of the first egg hatched), brood size (nestlings counted at 3 days from hatching), fledgling number (nestlings counted at 13 days from hatching) and the number of fledglings that successfully left the nest (successful fledging assumed if fledglings were not found dead in their nests; blue tit fledging period of the studied population: 20–22 days). Brood size was counted at 3 days given that blue tits practice asynchronous hatching and so it may take 2–3 days for all the eggs to hatch (Stenning 2008). The fledgling number was recorded when nestlings were 13 days old because they have reached their asymptotic body size by this age (Björklund 1996). The specific analyses and results of reproductive parameters are summarized in Chapter 6.

Besides recording basic reproductive parameters, several sampling procedures were performed during the fledging and post-fledging periods. Firstly, parents were

captured in their nests when the nestlings were between 8 to 11 days old using scuttles that closed the nest box opening when the bird entered to feed the nestlings. This age range was chosen to ensure the nestlings would not be harmed, given that the parents do not return to their nest box immediately (Schlicht & Kempenaers 2015), as tit nestlings develop endothermy from day 8 (Andreasson et al. 2016). Once captured, parents were sexed by examining for brood patches in females. Their tarsus length was measured with a digital calliper (accuracy: 0.01 mm) and they were weighed with a digital, portable scale (accuracy: 0.1 g). The parents were banded with aluminium rings for further identification. A 100 μL blood sample (approximately 1% of adult body mass, which is about 10 g) was collected from the jugular vein of birds using heparinized insulin syringes in sterile conditions, following Owen (2011). The blood sample was preserved in absolute ethanol and used for further genetic analyses to examine the presence of haemoparasites (Chapter 3) and genetic population structure (Chapter 7). Parents were liberated within 10 m of their nest boxes.

Secondly, when the nestlings were 13 days old, their tarsus length and body mass were recorded as explained above for adults. The nestlings were also banded with aluminium rings for further identification (see Chapter 7). When the nestlings were 12 days old, we inoculated 0.1 mg of phytohaemagglutinin (PHA-P; Sigma-Aldrich, L-8754) diluted in 0.02 mL of isotonic phosphate-buffered saline in their left wing web to measure the cutaneous immune response to PHA, following Smits et al. (1999) (see Chapter 5 for more details). Also, a 100 μL blood sample was taken from the largest and smallest nestlings within each brood, as explained above for adults. A drop of blood was smeared on a slide and air-dried, following Owen (2011). Blood smears were fixed in absolute methanol and stained with a Wright-Giemsa combination stain to obtain several immune parameters (see Chapters 5 and 8 for more details). The rest of blood samples were preserved in a portable fridge and then transported to the laboratory for further analyses of oxidative stress (Chapters 4 and 8). When manipulating both parents and nestlings, the handling time was kept to a minimum to reduce bird stress (de Jong 2019).

Thirdly, the estimation of presence and numbers of ectoparasites in nest boxes was performed using two procedures. Flying ectoparasites (biting midges and black flies) were captured by placing a 60 mm petri dish layered with a drop of body gel oil in each nest box. The petri dishes were placed in the inner roof of nest boxes when nestlings

were 12 days old and collected the next day. In the laboratory, flying insects were removed using xylene, counted and identified (see Chapters 2 and 6 for more details). On the other hand, the nest-dwelling ectoparasite pressure (blowflies and hen fleas) was estimated by carefully revising the nest material once all fledglings left their nests. The presence and numbers of blowfly larvae and pupae and the presence of hen flea larvae and adults were recorded (see Chapters 1, 4, 5 and 6 for more details).

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OBJECTIVES

Objectives

The main objective of this thesis is to understand the ecological and evolutionary mechanisms underlying the evolution of optimal clutch size at the microgeographic scale. To address this issue, I used as a model system a monitored (2016–2021) population of blue tits (*Cyanistes caeruleus*) breeding across a continuous and heterogeneous woodland, formed by different and adjacent forest formations located along the two slopes of a valley. The main hypothesis of this thesis is that blue tit clutch size is locally adapted to each habitat (i.e. forest type). If so, parents should rear a habitat-dependent clutch size that will maximize the quantity and quality of offspring, given that natural selection has favoured such clutch size. This thesis consists of eight chapters, each of which aims to find the answers to the different questions raised by the specific objectives. The specific hypotheses and objectives are:

1. Forest formations extending along the valley will differ in its environmental factors, which would exert different selective pressures for breeding blue tits. To test this hypothesis, I quantify the variation in abiotic and biotic factors across the woodland. **Chapters 1**, **2**, **3**, **4**, **5** and **6** examine the variation in the presence of ectoparasites, vectors and haemoparasites between forest formations, while **Chapter 6** examines the variation in abiotic and biotic factors along the woodland.

2. The offspring condition will not differ between the forest formations if parents optimize their clutch size. To test this hypothesis, I examine how the forest type modulates the offspring's physiology and morphology. **Chapter 4** explores the variation in the blue tit nestling oxidative status with forest type and ectoparasite presence, while **Chapter 5** examines the variation in the blue tit nestling immune response with forest type and ectoparasite presence. For its part, **Chapter 6** investigates the variation in blue tit nestling body size and condition with forest type and ectoparasite presence, as well as with parental quality.

3. Breeding blue tits will adjust their clutch size to the forest type where they reproduce, as a result of the environmental heterogeneity along the valley. To test this hypothesis, I examine the variation in adult reproductive performance along the woodland. **Chapter 6** investigates the variation in blue tit breeding biology between

the forest formations along the valley, in addition to explore the relationships between the blue tit reproduction and the ectoparasite presence.

4. If clutch size is locally adapted, I expect that blue tits from the different forest formations along the valley will form differentiated genetic clusters, mainly as a consequence of non-random dispersal, with natural selection favouring different clutch sizes between these clusters. To test this hypothesis, I examine the natal dispersal, the genetic population differentiation and the role of natural selection in shaping the clutch size. **Chapter 7** describes the natal dispersal of blue tits, analyses patterns of genetic (neutral markers) and phenotypic (clutch size and body size) variation between blue tits from the different forest formations, and tests the role of natural selection and genetic drift in shaping the phenotypic variation among populations.

5. If clutch size is locally adapted, then blue tit parents rearing their own original clutch size will produce highest quality offspring. To test this hypothesis, I conducted a brood size manipulation experiment in each slope of the valley and measured different parental and nestling condition parameters. **Chapter 8** describes the experimental test of this hypothesis through a brood size manipulation experiment, measuring parental provisioning effort and different condition parameters of offspring (oxidative status, immune system, body size and body condition).

PART I

Environmental and biotic heterogeneity at small scale

Chapter 1

Variation in parasitoidism of *Protocalliphora azurea* (Diptera: Calliphoridae) by *Nasonia vitripennis* (Hymenoptera: Pteromalidae) in Spain

ABSTRACT Parasitoid wasps may act as hyperparasites and sometimes regulate the populations of their hosts by a top-down dynamic. *Nasonia vitripennis* (Walker, 1836) is a generalist gregarious parasitoid that parasitizes several host flies, including the blowfly *Protocalliphora* (Hough, 1899) (Diptera, Calliphoridae), which in turn parasitizes bird nestlings. Nonetheless, the ecological factors underlying *N. vitripennis* prevalence and parasitoidism intensity on its hosts in natural populations are poorly understood. We have studied the prevalence of *N. vitripennis* in *Protocalliphora azurea* (Fallén, 1817) puparia parasitizing wild populations of pied flycatcher (*Ficedula hypoleuca*) and blue tit (*Cyanistes caeruleus*) birds in two Mediterranean areas in central and southern Spain. We found some evidence that the prevalence of *N. vitripennis* was higher in moist habitats in southern Spain. A host-dependent effect was found, since the greater the number of *P. azurea* puparia, the greater the probability and rate of parasitoidism by the wasp. Our results also suggest that *N. vitripennis* parasitizes more *P. azurea* puparia in blue tit nests than in pied flycatcher nests as a consequence of a higher load of these flies in the former. Based on the high prevalence of *N. vitripennis* in *P. azurea* puparia in nature, we propose that this wasp may regulate blowfly populations, with possible positive effects on the reproduction of both bird species.

Keywords: Blowfly, *Cyanistes caeruleus*, *Ficedula hypoleuca*, *Nasonia vitripennis*, parasitoid, *Protocalliphora azurea*

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Introduction

Hyperparasitism is a special form of parasitism in which a parasite is infested by another parasite or parasitoid (Hochberg & Ives 2000; Sullivan 2009), thus establishing a multitrophic ecological system with at least three levels: the host, the parasite, and the parasitoid (Sullivan and Völkl 1999). Hyperparasites are usually parasitoid wasps, insects of the order Hymenoptera whose larvae parasitize several life stages of other arthropods, eventually killing them (Quicke 1997). The population dynamics of host species can influence the ecology of parasitoid wasps and vice versa; hence, both the bottom-up and the top-down effects might regulate the host-parasitoid systems (Hawkins 1992). Forest and agricultural ecosystems where biological control is performed using parasitoid top-down dynamics are well documented (Rivers 2004; Foottit & Adler 2017; de Lange et al. 2018). Furthermore, parasitoid top-down dynamics in poultry and cattle farms can strongly affect host populations (Morgan 1980; Rutz & Axtell 1981; Skovgård & Nachman 2004). The success or failure of parasitoid top-down regulation may depend on optimal temperatures and moisture for the parasitoid (Skovgård & Nachman 2004), host microhabitat (Frederickx et al. 2014), host distribution, parasitoid searching behaviour (May 1978), or host density (May et al. 1981; Aung et al. 2011).

Nasonia vitripennis (Walker, 1836) (Hymenoptera, Pteromalidae, Pteromalinae), a generalist gregarious parasitoid wasp found in the Holarctic region, parasitizes several calyptrate flies (Werren & Loehlin 2009; Desjardins et al. 2010; Peters & Abraham 2010). Among the hosts of *N. vitripennis* is *Protocalliphora* (Hough, 1899) (Diptera, Calliphoridae), a group of blowflies whose larvae are obligate hematophagous ectoparasites of nestling birds (Whitworth & Bennett 1992). Several studies have shown that blood-feeding *Protocalliphora* larvae have negative effects on the physiology and survival of developing nestlings in different bird species (Merino & Potti 1995; Puchala 2004; Simon et al. 2004; Simon et al. 2005; Hannam 2006; Streby et al. 2009). Other studies have failed to find negative effects of blowflies on nestlings (Miller & Fair 1997; Thomas & Shutler 2001), perhaps, because short-term detrimental effects may be difficult to detect if parental compensation occurs through increased feeding rates to heavily parasitized nestlings (Johnson & Albrecht 1993).

Nasonia species have been widely used as model organisms (Lynch et al. 2006; Oliveira et al. 2008; Godfray 2010; Niehuis et al. 2010; Cook et al. 2018), but little is known on its ecology and prevalence of parasitoidism in their natural habitats. Most studies on the relative abundance, prevalence, and intensity of parasitoidism of this wasp have been conducted in urban areas or poultry and cattle farms, where the insect's prevalence is very low (less than 5%) (Skovgård & Jespersen 1999; Skovgård & Jespersen 2000; Marchiori 2004; Rodrigues-Guimarães et al. 2006; Marchiori et al. 2007; Oliva 2008), perhaps because all hosts parasitized by the wasp in the aforementioned studies were flies that develop on decaying matter or parasitize cattle. By contrast, birds' nests may be the primary habitat for *N. vitripennis*, which shows high intensity of parasitoidism when parasitizing *Protocalliphora* (Peters 2010). In fact, most studies on the parasitoidism of *Protocalliphora* and other blowflies puparia by *Nasonia* (Ashmead, 1904), report that 30–90% of bird nests containing blowfly puparia are infested by the wasp, with 20–50% of puparia being parasitized (Bennett & Whitworth 1991; Grillenberger et al. 2008; Grillenberger et al. 2009; Daoust et al. 2012).

Climatic conditions, habitat, host availability, or even the host species parasitized by *Protocalliphora* could play a role in the parasitoidism by this wasp and help explain the variability in its abundance. However, the ecological factors that determine parasitoid prevalence or intensity of *N. vitripennis* are still poorly understood. The present study documents patterns of parasitoidism of *N. vitripennis* on puparia of *Protocalliphora azurea* (Fallén, 1817) in wild breeding populations of pied flycatcher (*Ficedula hypoleuca*) and blue tit (*Cyanistes caeruleus*) in Spain and examines biotic and abiotic causes of variation in the prevalence and abundance of this parasitoid.

Materials and methods

Nasonia vitripennis is a gregarious ectoparasitoid whose females lay their eggs in the puparia of different calyptrate flies. After emergence of eggs, larvae feed, moult, and metamorphose into adults at 14 days of age (at 25 °C), which leave the puparium through a self-made exit holes to mate and reproduce (Whiting 1967). One female can carry hundreds of eggs (reviewed in Whiting 1967), but the number of parasitoids emerging from a puparium varies with the host species, ranging from 35–50 in flesh

flies (Rivers & Denlinger 1995) to 15–25 in *Protocalliphora* hosts (Gold & Dahlsten 1989; Draber-Monko 1995; Peters 2010). *Protocalliphora azurea* is a blowfly whose larvae parasitize cavity-nesting birds. Females of Protocalliphora lay a mean of 15–75 eggs directly in nests when nestlings hatch (Gold & Dahlsten 1989; Bennett & Whitworth 1991), but the number of eggs laid per nest varies according to bird species. Both insects are univoltine. *Nasonia vitripennis* larvae and pupae may sometimes overwinter inside the puparia of *P. azurea* in a diapause state until the emergence of adults following the spring (Gold & Dahlsten 1989), although in our study areas, the adult typically emerges during the first spring. This species shows a relatively high level of overwintering survival among pteromaline wasps (Floate & Skovgård 2004). Both sexes of *P. azurea*, however, overwinter as adults and reproduce in spring near the nesting areas of their bird hosts (Bennett & Whitworth 1991).

We studied the prevalence of the wasp *N. vitripennis* on the blowfly *P. azurea* in wild populations of the pied flycatcher and the blue tit breeding in nest boxes in two different mountainous Mediterranean areas in central and southern Spain. In each area, we sampled two well-differentiated habitats, and only nests infested by the blowfly were considered for *N. vitripennis* analyses. In 2009, we sampled pied flycatcher nests in two habitats from central Spain, an afforested Scots pine (*Pinus sylvestris*) area (n = 20 nests) and a nearby deciduous Pyrenean oak (*Quercus pyrenaica*) forest (n = 33 nests), both located in the Sierra de Ayllón (41°4'N; 3°27'W) at 1200–1400 m a.s.l. For a detailed description of this study area, see Camacho et al. (2015). Some blue tit nests were also sampled (n = 7 nests) in this area. In 2016 and 2017, we sampled blue tit nests in a mixed forest in Sierra Nevada (southern Spain; 36°57'N; 3°24'W), at 1700–1800 m a.s.l. This forest may be divided into two areas, a dry zone composed by holm oaks (*Quercus ilex*) and Pyrenean oaks (n = 29 nests) and a moist zone irrigated by the Chico river and the Almiar stream, composed by a mixed forest of Scots pines, holm oaks, and Pyrenean oaks (n = 53 nests). In the two study areas, nest boxes were of the same type (ICONA C model; detailed descriptions in Potti & Montalvo 1990; Moreno-Rueda 2003) and were cleaned at the end of each breeding season.

Once all nestlings had fledged, nests were dismantled, and their material was examined to collect blowfly puparia. In central Spain, puparia were included in plastic containers and placed in an incubator under a constant temperature of 25 °C (± 1 °C)

until emergence. After emergence, we counted the puparia from which *N. vitripennis* emerged and the puparia from which the blowfly emerged. In southern Spain, blowfly puparia were collected in labelled vials, preserved in 70% ethanol and taken to laboratory for examination. The presence or absence of *N. vitripennis* in puparia was determined using a stereo microscope by cutting the puparia in half and searching for *N. vitripennis* larvae or pupae inside or, in empty puparia (found in 6 nests), by confirming the occurrence of minute exit holes on the pupa exuviae, this being indicative that puparia had been parasitized by the wasp. In both cases, the identification of adult wasps and blowflies allowed to emerge confirmed that the only parasitoid and host species found in bird nests were *N. vitripennis* and *P. azurea*, respectively. Although not all *N. vitripennis* pupae collected in southern Spain were allowed to emerge, we are rather confident that parasitoids were *N. vitripennis*, as no other pteromaline species was found parasitizing *P. azurea* puparia in this study area.

For each nest examined, we counted the number of blowfly puparia. Based on the sampling units (nest and puparia), we defined two types of prevalence of *N. vitripennis*: (1) prevalence by nests, calculated as the percentage of nests infested by the blowfly in which at least one puparium was parasitized by *N. vitripennis*, and (2) prevalence by puparia, calculated as the percentage of blowfly puparia per nest that were parasitized by *N. vitripennis*, only nests with *N. vitripennis* being considered. Aborted blowfly puparia, defined as those in which no adults developed, were excluded from *N. vitripennis* prevalence analyses.

To test for differences in prevalence per nests in relation to habitats and years, given that these are frequencies, we used the chi-squared test (Rózsa et al. 2000). To test for differences in prevalence by puparia or number of parasitized puparia per nest in relation to habitat and year, given that they are mean values, we used Student's *t*-test (Rózsa et al. 2000). We also used the *t*-test to check for differences in the number of blowfly puparia in nests with or without *N. vitripennis*. We used a generalized linear model (GLM), linked to a binomial distribution (infested or not), to assess the probability that a nest was infested by the wasp according to the number of blowfly puparia. We checked the variables to evaluate whether they satisfied the premises of parametric statistics (following Zuur et al. 2010). When the premises were not satisfied, variables were log-transformed (total number of puparia and number of parasitized puparia per nest), or nonparametric tests were used, employing the Mann-

Whitney U-test instead of the *t*-test. Means are given with the raw data, even though all analyses regarding the number of total puparia and parasitized puparia per nest were performed with log-transformed variables. Mean values are given with the standard error. All tests were run in Statistica 8.0 (StatSoft Inc. 2007).

Results

Parasitoidism in central Spain

Overall, 86.8% (46 of 53) of pied flycatcher nests infested by the blowfly *P. azurea* were parasitized by the wasp *N. vitripennis*. In nests infested by *N. vitripennis*, an average of 78.2% of puparia per nest was infested by the wasp (in total, 252 of 312). Five of the seven blue tit nests in this study area were infested by *N. vitripennis* (prevalence per nest of 71.4%), with an average of 7.8 blowfly puparia parasitized by the wasp per nest (60.9% of puparia; Table 1). For pied flycatcher nests, no differences were found in wasp prevalence per nest between the oak (29 of 33) and pine (17 of 20) forests ($\chi^2 = 0.09$, $p = 0.76$; Table 1). Habitats did not differ in the number of blowfly puparia parasitized by the wasp per nest (5.52 ± 0.95 in oak forest and 5.41 ± 0.96 in pine forest; $t_{44} = 0.64$, $p = 0.52$) or in the prevalence per puparia (oak vs. pine: 79.0% and 77.7%; $t_{44} = 0.15$, $p = 0.88$; Table 1). Consequently, in subsequent analyses, the data from both forests were pooled together. Nests infested by *N. vitripennis* harboured significantly more blowfly puparia than did noninfested nests (infested: 6.78 ± 0.83 puparia; noninfested: 3.00 ± 1.21 puparia; $t_{51} = 2.59$, $p = 0.01$). The probability that some blowfly puparia were parasitized by *N. vitripennis* increased with the number of total puparia in the nest (GLM, $\chi^2 = 7.02$, $p = 0.008$). The number of parasitized blowfly puparia per nest rose with the total number of blowfly puparia in the nest ($r = 0.87$, $p < 0.001$). However, the percentage of blowfly puparia parasitized did not correlate with the number of puparia in the nest ($r = -0.01$, $p = 0.95$).

Parasitoidism in southern Spain

During 2016 and 2017, 65.9% (54 of 82) of blue tit nests infested by the blowfly showed parasitoidism by *N. vitripennis*. In infested nests, 53.9% of the blowfly puparia were parasitized. In total, in both infested and noninfested nests, 434 of 977

(44.4%) blowfly puparia were parasitized by *N. vitripennis*. Significant differences were found between years in the prevalence per nest of *N. vitripennis*, this being higher in 2017 (40 of 53) than in 2016 (14 of 29; $\chi^2 = 6.17$, $p = 0.013$). Wasp prevalence per nest also differed between habitats, being higher in the moist zone (41 of 53) than in the dry zone (13 of 29; $\chi^2 = 8.82$, $p = 0.003$; Table 1). Similar to pied flycatcher nests, blue tit nests with wasps had a higher number of blowfly puparia (14.81 ± 1.14 puparia, n = 54) than did the nests without the wasp (6.32 ± 1.06 puparia, n = 28; Mann-Whitney U-test, $z = 4.68$, $p < 0.001$). The number of blowfly puparia in nests infested by *N. vitripennis* did not differ between habitats (moist: 6.77 ± 1.01 puparia, n = 41; dry: 8.44 ± 1.07 puparia, n = 13; $z = -0.27$, $p = 0.78$) or between years (2016: 9.21 ± 1.80 puparia, n = 14; 2017: 7.63 ± 0.96 puparia, n = 40; $z = 0.49$, $p = 0.96$). The prevalence of *N. vitripennis* per *P. azurea* puparia in infested nests did not vary between habitats (moist vs. dry, 56.0% and 47.3%; $t_{52} = 1.03$; $p = 0.31$; Table 1), nor did the number of puparia parasitized by the wasp (moist: 8.44 ± 1.07; dry: 6.77 ± 1.00; $t_{52} = 0.16$; $p = 0.88$). Although the number of parasitized puparia increased with the number of puparia in the nest ($r = 0.74$, $p < 0.001$), the percentage of parasitized puparia did not covary with the number of puparia ($r = -0.04$, $p = 0.79$).

Table 1. Prevalence (in %) of *Nasonia vitripennis* per bird nest and per *Protocalliphora azurea* puparium (only in infested nests) in pied flycatcher and blue tit nests in two different habitats of two geographic regions of Spain. Sample size is indicated in brackets.

Geographic zone	Bird species	Prevalence per nest		Prevalence per puparium	
Central Spain	Pied flycatcher	Pine forest	Oak forest	Pine forest	Oak forest
		85.00 (20)	87.88 (33)	77.70 (19)	79.00 (29)
	Blue tit	71.40 (7)		60.90 (5)	
Southern Spain	Blue tit	Moist forest	Dry forest	Moist forest	Dry forest
		77.36 (53)	44.83 (29)	56.00 (41)	47.30 (13)

Discussion

Parasitoidism drastically reduces host fitness, in most cases to zero, mainly due to the death of the host before producing offspring (Poulin 2011). Hence, parasitoids most

often occur at low intensities and prevalences, mainly due to their extremely high virulence and impact on host survival (Poulin & Randhawa 2015). Nevertheless, unlike most parasitoid wasps, *Nasonia* registers high parasitoidism intensity and prevalence (Godfray 2010). We found that the percentage of nests with parasitoidism by the wasp *N. vitripennis* was 86.8% in pied flycatcher nests and 65.9% in blue tit nests. Furthermore, the percentage of the blowfly puparia parasitized was very high: 78.2% in pied flycatcher nests and 53.9% in blue tit nests. Few studies have been conducted on *Nasonia* hyperparasitism in wild populations, all suggesting high rates of parasitoidism on parasitic blowflies in bird nests, in contrast to the low rates of parasitoidism reported for other flies in nonnatural environments (see Introduction). In line with our results, the findings by Daoust et al. (2012) indicated that *N. vitripennis* appeared in 85.3% and 67.2% of tree swallow (*Tachycineta bicolor*) nests containing *Protocalliphora* puparia in 2 consecutive years, with 50.4% and 39.3% of the puparia being parasitized by two *Nasonia* species. Grillenberger et al. (2008) reported that 60% of Central European bird nests contained fly puparia parasitized by *Nasonia*, with 46.8% of these puparia being parasitized. In another study in North America, Grillenberger et al. (2009) reported that 91% of the bird nests contained *Nasonia* and that a mean of 48% of puparia per nest were parasitized by these wasps. By contrast, Bennett & Whitworth (1991) recorded lower values: 29.5% of nests of several bird species contained parasitized puparia of *Protocalliphora*, and only 16.7% of all the puparia were parasitized by *N. vitripennis*. A similar low prevalence of *N. vitripennis* in both blowflies and flesh flies hosts was reported by Werren (1983). These results show that the prevalence of *Nasonia* is generally high in nature, implying a high mortality rate of *Protocalliphora* puparia due to this wasp. Therefore, *Nasonia* can be considered an important selective agent on *Protocalliphora* that likely regulates its natural populations.

This raises the question as to why the prevalence of *Nasonia* species in the puparia of *Protocalliphora* blowflies is so high. Although *N. vitripennis* is a generalist parasitoid, its parasitoidism rate decreases when it competes with other parasitoid wasps and attacks fly hosts that do not parasitize birds (Rutz & Scoles 1989; Skovgård & Jespersen 1999; Skovgård & Jespersen 2000; Kaufman et al. 2001). The level of parasitoidism of *N. vitripennis* on *Protocalliphora* in North America, when occurring in mixed infestations with other *Nasonia* species (e.g. *N. giraulti*), is slightly lower than the rate found in Europe, where *N. vitripennis* is the only *Nasonia* species found

(Bennett & Whitworth 1991; Grillenberger et al. 2008; Grillenberger et al. 2009; Daoust et al. 2012; our study). Moreover, the host range of this wasp includes puparia which are large, lack appendages and have flat surfaces (Peters 2010), and these attributes all match to *Protocalliphora*. The above results suggest that *Protocalliphora* constitutes one of the most suitable hosts of *N. vitripennis*.

The high rates of parasitoidism reported by the literature may also be due to the artificial nature of nests (i.e. nest boxes). However, nest material was removed each year once the breeding season of birds ended, thus reducing ectoparasite loads (Møller 1989), including *N. vitripennis* wasps that can overwinter inside blowfly puparia (Bennett & Whitworth 1991). Nevertheless, nesting in cavities seems to increase the probability of parasitization by *N. vitripennis* (72% vs. 22–32% of cavity and non-cavity nests, respectively; Bennett & Whitworth 1991), perhaps because the cavity nest microclimate is more benign for wasps. If this is so, the microclimate created by nest boxes could enhance the establishment of *N. vitripennis*.

We found that *N. vitripennis* infested bird nests with a high host puparia load (on average, 6.78 puparia in flycatcher nests and 14.81 in blue tit nests). Both the probability of infestation of the blowfly puparia by *N. vitripennis* and the number of parasitized puparia increased with the number of puparia in bird nests, suggesting that the more puparia in the nest, the easier for wasps to detect them, possibly by chemoreception (Peters 2011). Some studies have shown a positive correlation between parasitoidism intensity of *N. vitripennis* and host pupal size (Wylie 1967; Rivers & Denlinger 1995), but the correlation between host density and the parasitoidism rate is less clear. Indeed, only 25% of host-parasitoid systems appear to work in a host density-dependent manner. In the superfamily Chalcidoidea, to which *Nasonia* belongs, a host-density-dependent increase in the parasitoidism rate has been reported for less than 30% of parasitoids (Stiling 1987). However, we noted a clear density-dependent effect of the blowfly on the incidence of wasp parasitoidism, with higher rates of puparia being parasitized as their number increased. A plausible explanation for the discrepancy in parasitoidism rates is the spatial scale of the studies. Most studies examining density-dependent effects are conducted at small scales, where parasitoids show a negative density-dependence pattern. However, this pattern may change at larger scales, where parasitoids need to search different – and sometimes distant – habitat patches to find their hosts, resulting in massive

parasitoidism after a profitable habitat (i.e. holding numerous hosts) is found (Heads & Lawton 1983). In fact, in southern Spain, we found a trend for parasitoidism rate to be higher in the moist zone than in the dry zone, given that the blowfly prevalence was higher in the moist (89.7%) than in the dry (38.6%) zone. This finding supports the idea that the higher host density in a plot, the higher the parasitoidism rate (but see below for alternative explanations for the difference between habitats). However, in infested nests, the percentage of parasitized puparia did not covary with the number of puparia available. A possible explanation for this result is that wasps may parasitize a limited number of puparia within the nest.

The prevalence of *N. vitripennis* was higher in central than in southern Spain (see Table 1). These differences might be due to different environmental factors between the two study areas or to variation between years, given that the two areas were sampled in different years. Alternatively, these differences could be due to the different bird species infested by the blowfly – pied flycatchers in central Spain and blue tits in southern Spain –, a possibility suggested by the fact that the rate of wasp parasitoidism in blue tit nests in central Spain was most similar to that found in the moist forest of southern Spain (see Table 1). The intensity of blowfly parasitism was higher in blue tit nests (mean 14.7 blowflies per nest) than in pied flycatcher nests (mean 6.95 blowflies per nest), probably due to larger brood sizes in blue tit nests. Hence, it is not surprising that the wasp parasitized more puparia in blue tit nests (6–8 per nest) than in pied flycatcher nests (approximately 5 puparia per nest), simply because the former nests harboured more puparia.

In southern Spain, the probability that a nest or a puparium was parasitized by *N. vitripennis* tended to be greater in the moist zone than in the dry zone, but similar habitat-dependent variation was not found between the two habitats (pine vs. oak forests) in central Spain. One explanation is that climatic conditions in the moist zone were more suitable for adult survival of the wasp. In Canada, *N. vitripennis* adults under laboratory conditions do not prefer damp environments (Wylie 1958), but in Mediterranean environments, dry conditions are probably more limiting, with moist, riverside zones being more benign for these wasps. Indeed, in Mediterranean ecosystems, moisture can impact on the survival and abundance of parasitoids, moderate to high relative humidity being favourable for some parasitoids (Sorribas et al. 2010). In central Spain, by contrast, moisture was very similar between the two

habitats (pers. obs.), which could explain the absence of habitat-specific differences in this region. On the other hand, the prevalence of the blowfly *P. azurea* was higher in the moist zone than in the dry zone. Therefore, as explained above, a higher host density in the moist zone may have provided a higher probability of parasitoidism by *N. vitripennis*. The moist zone also has more trees, shrubs, and herbaceous cover than the dry zone, so other factors such as food availability or shelter against predators could also account for the observed habitat differences and indirectly increase host parasitization by *N. vitripennis* (Edwards 1954; Wylie 1958).

As explained in the Introduction, some parasitoid wasps may regulate the populations of their hosts by a top-down dynamic and, hence, have a pervasive influence on the ecosystem. The high prevalence of *N. vitripennis* in the two Mediterranean mountain ecosystems studied here suggests that this wasp might regulate the blowfly population by a top-down effect. Our study reports rates of parasitoidism of 44–88% of bird nests, in which 47–79% of blowfly puparia were parasitized. These rates imply that 21–69% of blowfly puparia would die as a consequence of *N. vitripennis* parasitoidism. This represents an important reduction of blowfly success, and, in this way, the incidence of parasitism by blowflies in bird populations could be reduced. Blowflies have reportedly detrimental effects on the condition and survival of nestling birds (see Introduction), decreasing the host's body condition, growth rate, and survival both in blue tits (Hurtrez-Boussès et al. 1997) and in pied flycatcher nestlings (Merino & Potti 1995), and may even have long-term implications for future reproduction (Potti 2008). Therefore, a depressed blowfly population by wasp parasitoidism could boost the breeding success of the two bird species in these habitats.

In conclusion, our study shows a high prevalence of parasitoidism of *N. vitripennis* on *P. azurea* in four well-differentiated forest habitats from two geographically widely separated populations. These results, in addition to those reported in the literature, suggest that *N. vitripennis* has a major impact on *P. azurea*. The parasitoid appears to have more success in blue tit nests, which harbour higher quantities of blowfly puparia. A trend for higher wasp prevalence in moist environments was also found, suggesting that, in Mediterranean ecosystems, moisture enhances the wasp abundance.

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Chapter 2

Habitat-dependent *Culicoides* species composition and abundance in blue tit (*Cyanistes caeruleus*) nests

ABSTRACT Wild birds are hosts of *Culicoides* from as early on as the nesting stage when constrained to their nests. However, the environmental factors which determine the abundance and composition of *Culicoides* species within each bird nest are still understudied. We sampled *Culicoides* from Eurasian blue tit (*Cyanistes caeruleus*) nests found in 2 types of forests located in southern Spain. Firstly, we monitored the abundance of *Culicoides* species in bird nests from a dry Pyrenean oak deciduous forest and a humid mixed forest comprising Pyrenean and Holm oaks throughout 2 consecutive years. During the 3rd year, we performed a cross-fostering experiment between synchronous nests to differentiate the role of rearing environment conditions from that of the genetically determined or maternally transmitted cues released by nestlings from each forest. We found 147 female *Culicoides* from 5 different species in the birds' nests. The abundance of *Culicoides* was higher in the dry forest than in the humid forest. *Culicoides* abundance, species richness and prevalence were greater when the nestlings were hatched later in the season. The same pattern was observed in the cross-fostering experiment, but we did not find evidence that nestling's features determined by the forest of origin had any effect on the *Culicoides* collected. These results support the notion that habitat type has a strong influence on the *Culicoides* affecting birds in their nests, while some life history traits of birds, such as the timing of reproduction, also influence *Culicoides* abundance and species composition.

Keywords: Avian malaria, avian nests, biting midges, blood-feeding insects, *Haemoproteus*, host selection, vectors

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Introduction

Culicoides biting midges (Diptera: Ceratopogonidae) are one of the world's smallest and most abundant blood-sucking flies (Mellor et al. 2000). *Culicoides* have a widespread distribution with haematophagous females acting as vectors of various pathogens. Most research on this group focuses on its role in the transmission of viruses (e.g. African horse sickness virus, bluetongue virus or Schmallenberg virus, among others) to livestock, because of its economic impact on the industry (Mellor et al. 2000; Carpenter et al. 2013; Sick et al. 2019; Martínez-de la Puente et al. 2021). However, *Culicoides* are well-known vectors of other parasites including avian trypanosomes (Svobodová et al. 2017) and the avian malaria-like *Haemoproteus* (Valkiūnas 2005; Martínez-de la Puente et al. 2011a), also supporting their role in the transmission of parasites to wildlife. In spite of this, there are still relatively few studies into the ecological interactions between biting midges and wild birds. This is especially relevant considering the impact of *Culicoides* on the body condition of nestlings (Tomás et al. 2008a), together with the deleterious effects of the *Culicoides*-borne parasites on bird health (Merino et al. 2000; Tomás et al. 2008a; Martínez-de la Puente et al. 2010a).

The development of traps inside nest boxes (Tomás et al. 2008b; Votýpka et al. 2009) allowed researchers to identify the diversity of *Culicoides* species attracted to bird nests in different European regions (Table 1). For instance, Martínez-de la Puente et al. (2009a) identified the presence of 7 different species of *Culicoides* in blue tit (*Cyanistes caeruleus*) nests found in central Spain. Additional studies have also identified which species of *Culicoides* are attacking birds in their nests, including studies on different avian species conducted in several countries (Czech Republic: Votýpka et al. 2009; Spain: Veiga et al. 2018; Lithuania and Russia: Žiegytė et al. 2021). Moreover, the development of molecular techniques to identify the origin of blood meals from engorged biting midge females has confirmed the ornithophilic feeding preference of most *Culicoides* species collected from bird nests (Bobeva et al. 2015; Martínez-de la Puente et al. 2015). Thus, the results from these articles support the fact that different species of biting midges are attracted to bird nests, but there is still a lack of studies into what determines *Culicoides* species composition and abundance.

Table 1. Summary of *Culicoides* species found in nest boxes of different European bird species, namely blue tit (*Cyanistes caeruleus*), great tit (*Parus major*), pied flycatcher (*Ficedula hypoleuca*) and European roller (*Coracias garrulus*).

Culicoides species	Bird host [a]	Region	Country	*Haemoproteus* positive	Reference
Culicoides circumscriptus	Blue tit	Granada	Spain	Not examined	This study
		Segovia	Spain	Not examined	Martínez-de la Puente et al. (2009a)
		Segovia	Spain	Yes	Martínez-de la Puente et al. (2011a)
	European roller	Almería	Spain	Yes	Veiga et al. (2018)
	Not reported	Mikulov	Czech Republic	Not examined	Votýpka et al. (2009)
Culicoides duddingstoni	Not reported	Mikulov	Czech Republic	Not examined	Votýpka et al. (2009)
Culicoides festivipennis	Blue tit	Granada	Spain	Not examined	This study
		Segovia	Spain	Not examined	Martínez-de la Puente et al. (2009a)
		Segovia	Spain	Yes	Martínez-de la Puente et al. (2011a)
	Great tit	Vilnius	Lithuania	No	Žiegytė et al. (2021)
		Rybachy	Russia	No	Žiegytė et al. (2021)
	Pied flycatcher	Vilnius	Lithuania	No	Žiegytė et al. (2021)
		Rybachy	Russia	No	Žiegytė et al. (2021)
	Not reported	Mikulov	Czech Republic	Not examined	Votýpka et al. (2009)
Culicoides impunctatus	Great tit	Vilnius	Lithuania	No	Žiegytė et al. (2021)
		Rybachy	Russia	No	Žiegytė et al. (2021)
	Pied flycatcher	Vilnius	Lithuania	No	Žiegytė et al. (2021)
		Rybachy	Russia	No	Žiegytė et al. (2021)
Culicoides kibunensis	Blue tit	Granada	Spain	Not examined	This study
		Segovia	Spain	Not examined	Martínez-de la Puente et al. (2009a)
		Segovia	Spain	Yes	Martínez-de la Puente et al. (2011a)
	Great tit	Vilnius	Lithuania	No	Žiegytė et al. (2021)
		Rybachy	Russia	Yes	Žiegytė et al. (2021)
	Pied flycatcher	Vilnius	Lithuania	No	Žiegytė et al. (2021)
		Rybachy	Russia	Yes	Žiegytė et al. (2021)
	Not reported	Mikulov	Czech Republic	Not examined	Votýpka et al. (2009)

Table 1. (Continued)

Culicoides minutissimus	Not reported	Mikulov	Czech Republic	Not examined	Votýpka et al. (2009)
Culicoides obsoletus complex	Great tit	Vilnius	Lithuania	No	Žiegytė et al. (2021)
		Rybachy	Russia	No	Žiegytė et al. (2021)
	Pied flycatcher	Vilnius	Lithuania	No	Žiegytė et al. (2021)
		Rybachy	Russia	No	Žiegytė et al. (2021)
Culicoides pallidicornis	Great tit	Rybachy	Russia	No	Žiegytė et al. (2021)
	Pied flycatcher	Rybachy	Russia	No	Žiegytė et al. (2021)
Culicoides paolae	European roller	Almería	Spain	Yes	Veiga et al. (2018)
Culicoides pictipennis	Blue tit	Segovia	Spain	Not examined	Martínez-de la Puente et al. (2009a)
		Segovia	Spain	No	Martínez-de la Puente et al. (2011a)
	Great tit	Vilnius	Lithuania	No	Žiegytė et al. (2021)
		Rybachy	Russia	Yes	Žiegytė et al. (2021)
	Pied flycatcher	Vilnius	Lithuania	No	Žiegytė et al. (2021)
		Rybachy	Russia	Yes	Žiegytė et al. (2021)
	Not reported	Mikulov	Russia	Not examined	Votýpka et al. (2009)
Culicoides punctatus	Blue tit	Rybachy	Russia	Yes	Žiegytė et al. (2021)
	Great tit	Rybachy	Russia	Yes	Žiegytė et al. (2021)
Culicoides reconditus	Blue tit	Granada	Spain	Not examined	This study
	Great tit	Vilnius	Lithuania	No	Žiegytė et al. (2021)
		Rybachy	Russia	No	Žiegytė et al. (2021)
	Pied flycatcher	Vilnius	Lithuania	No	Žiegytė et al. (2021)
		Rybachy	Russia	No	Žiegytė et al. (2021)
Culicoides segnis	Blue tit	Segovia	Spain	Not examined	Martínez-de la Puente et al. (2009a)
		Segovia	Spain	Yes	Martínez-de la Puente et al. (2011a)
	Great tit	Vilnius	Lithuania	Yes	Žiegytė et al. (2021)
		Rybachy	Russia	Yes	Žiegytė et al. (2021)
	Pied flycatcher	Vilnius	Lithuania	Yes	Žiegytė et al. (2021)
		Rybachy	Russia	Yes	Žiegytė et al. (2021)
Culicoides simulator	Blue tit	Segovia	Spain	Not examined	Martínez-de la Puente et al. (2009a)
		Segovia	Spain	Yes	Martínez-de la Puente et al. (2011a)

Table 1. (Continued)

Culicoides simulator	Not reported	Mikulov	Czech Republic	Not examined	Votýpka et al. (2009)
Culicoides subfascipennis	Great tit	Vilnius	Lithuania	No	Žiegytė et al. (2021)
	Pied flycatcher	Vilnius	Lithuania	No	Žiegytė et al. (2021)
Culicoides sphagnumensis	Great tit	Rybachy	Russia	No	Žiegytė et al. (2021)
	Pied flycatcher	Rybachy	Russia	No	Žiegytė et al. (2021)
Culicoides truncorum	Blue tit	Granada	Spain	Not examined	This study
		Segovia	Spain	Not examined	Martínez-de la Puente et al. (2009a)
		Segovia	Spain	Yes	Martínez-de la Puente et al. (2011a)
	Not reported	Mikulov	Czech Republic	Not examined	Votýpka et al. (2009)

[a] Votýpka et al. (2009) sampled biting midges from the nests of tree sparrow (*Passer montanus*), great tit (*P. major*), blue tit (*C. caeruleus*), spotted flycatcher (*Muscicapa striata*), nuthatch (*Sitta europaea*) and wryneck (*Jynx torquilla*).

The type of habitat may have a significant effect on the *Culicoides* species breeding in the area, ultimately determining the abundance and composition of *Culicoides* inside the bird nests. The type of substrate and water sources are essential for the development of *Culicoides* larvae (Uslu & Dik 2010; Erram et al. 2019), but the distance to water sources from avian nests does not seem to affect the abundance of adult biting midges in the nests (Tomás et al. 2008b). In addition, weather conditions, which are partially modulated by habitat characteristics, may also influence the abundance of biting midges in avian nests. For example, higher winds early in the morning may negatively affect *Culicoides* flight performance and, consequently, reduce their abundance in nests (Martínez-de la Puente et al. 2009b). Nevertheless, there is scant information about how habitat-dependent variation in environmental factors may affect *Culicoides* abundance and species composition inside nests. In fact, only a few studies have examined the spatial variation of biting midge abundance in natural habitats. For instance, the management and structure of the habitat may affect the abundance of *Culicoides* in the area (van Hoesel et al. 2019), with more *Culicoides* females captured in the canopy compared to the ground level in a vertical axis (Černý et al. 2011). *Culicoides* abundance may also vary between habitats, but this depends on the host breeding period (Tomás et al. 2020) and patch extension (Rivero de

Aguilar et al. 2018). Several factors related to avian host physiology, behaviour and breeding performance may also affect the number of biting midges entering nests. Haematophagous vectors such as *Culicoides* use different cues (e.g. odourant molecules) to locate their hosts, such as 1-octen-3-ol, carbon dioxide (CO_2) and kairomones (Bhasin et al. 2000a, 2000b; Castaño-Vázquez et al. 2020). This may at least partly explain the positive correlation between *Culicoides* abundance and brood size in different bird species (Martínez-de la Puente et al. 2009a, 2009b; Martínez-de la Puente et al. 2010b; Castaño-Vázquez & Merino 2022), likely due to a greater release of attractive molecules from nests with larger broods.

The aim of this study was to identify the effect of habitat type on the abundance and species composition of biting midge *Culicoides* attacking avian hosts in their nests. To this end, we sampled *Culicoides* in nest boxes occupied by blue tits in 2 neighbouring forests in southern Spain with different environmental characteristics during the bird's breeding season for 3 consecutive years. We first compared the prevalence, abundance and species richness in nests situated in the 2 habitat types over the first 2 years. Secondly, we developed a cross-fostering experiment based on the results obtained and considering that the observed differences between the 2 forests could be due to the effects of habitat type on the emission of nestling cues (e.g. microbiota, Ruiz-López 2020; or the composition of uropygial secretions, Tomás et al. 2020), rather than a direct association between habitat type and *Culicoides*. This study design meant we could examine the role of rearing environment conditions (i.e. forest type) independently from the genetically determined attractants released by birds in each forest.

Materials and Methods

Study area

The study was carried out during the spring of 2017, 2018 and 2019 using blue tits breeding in nest boxes in the Sierra Nevada National Park (southeast Spain, 36°57′N, 3°24′W, 1700–1800 m a.s.l.). Nest boxes were placed in 2 different, but adjacent forests separated by approximately 1.5 km. One site was a dry Pyrenean oak (*Quercus pyrenaica*) deciduous forest (hereinafter, ‘dry forest’), while the other was a mixed forest consisting mainly of Pyrenean oaks along with some Holm oaks (*Quercus ilex*)

and which was crossed by a stream (Acequia Almiar), conferring it a moister ambient (hereinafter, 'humid forest'). The humid forest had a higher relative humidity, lower mean temperature, higher solar irradiation and lower insolation time compared to the dry forest (see supplementary material; Garrido-Bautista et al. 2021).

Blue tit sampling and cross-fostering experiment

Overall, 199 nest boxes were occupied by blue tits over the 3 years (69 in 2017, 56 in 2018 and 74 in 2019), of which 95 were randomly selected and followed to collect the data included in this study. Biting midges were monitored in 45 nest boxes in the dry forest and 50 in the humid forest. All the nest boxes were ICONA C model (details in Moreno-Rueda 2003) and were cleaned every year before the breeding season. They were hung from an oak tree's branch at a height of 3–4 m. We monitored the nest boxes each year to determine the hatching date (day the first egg hatched each year = day 0) and brood size at day 13.

During the spring of 2019, we conducted a cross-fostering study to identify the potential effect of nestling characteristics in the 2 habitats on *Culicoides* abundance in their nests. Hence, we designed a cross-fostering experiment in which whole broods were exchanged between the dry and humid forests or within each forest, according to the treatment. When nestlings were 3 days old, broods of the same age were exchanged according to their size (± 2 nestlings) using warm, breathable bags. Nestling broods exchanged and reared in the same forest served as manipulation controls. All nestlings in the nest boxes were exchanged, but the procedure was performed in 2 steps to ensure the nests always contained at least 3 nestlings and therefore prevent parent desertion. In total, the cross-fostering experiment included broods from 35 nests: 11 broods were exchanged within the humid forest (humid–humid treatment), 6 within the dry forest (dry–dry treatment), 9 were moved from the dry to the humid forest (dry–humid treatment) and 9 were moved from the humid to the dry forest (humid–dry treatment).

Culicoides *collection and identification*

Biting midges were captured in blue tit nest boxes following the method described by Tomás et al. (2008b) with minor modifications. A Petri dish (60 mm diameter) layered with body gel-oil (Johnson's ® Baby Oil Gel with chamomile, Johnson &

Johnson, Dusseldorf, Germany) was placed in the inner roof of the nest boxes when nestlings were 12 days old. The gel-oil was made up of paraffinum liquidum, hexyl laurate, ethylene/propylene/styrene copolymer, cyclopentasiloxane, butylene/ethylene/styrene copolymer, chamomilla recutita, bisabolol and perfume (FPT1353). The Petri dishes were collected the next day, when nestlings were 13 days old, and stored in a freezer until further analysis.

The biting midges were removed from the Petri dishes by applying xylene for a few seconds, then passed to absolute ethanol. After approximately 5 min, the biting midges were transferred to Eppendorf tubes with 70% ethanol and maintained at −20 °C. The *Culicoides* specimens were sexed and identified to the species level according to their morphological characteristics (e.g. wing spot patterns and the presence of coeloconic sensilla on the antennae) and based on available keys (Rawlings 1996; González & Goldarazena 2011), including the IIKC website (Mathieu et al. 2012). Species identification was further confirmed by mounting between 4 and 10 individuals of each species. The parity of *Culicoides* females was determined visually: (1) those that had never fed on blood (nulliparous females), (2) those showing a burgundy pigment in the subcutaneous cells of the abdomen indicating a previously digested blood meal (parous females; Dyce 1969) and (3) those with a recent blood meal in their abdomen (engorged females). We calculated the species richness for each nest box as the sum of the different *Culicoides* species collected. The prevalence of biting midges was calculated as the percentage of infested nests with respect to the total number of nests analysed. We estimated the abundance of *Culicoides* as the number of specimens captured for all the species in each nest. The total abundance of *Culicoides* was calculated as the sum of nulliparous, parous and engorged females per nest, while also considering any unidentified individuals.

Statistical analyses

We used Cleveland plots to check for outliers in the abundance of biting midges and tested the normality of the abundances of *Culicoides* and species richness graphically (Zuur et al. 2010). An outlier was detected in a nest box from the humid forest in 2019, which far exceeded the standard deviation (s.d.) of the mean biting midge abundance (mean ± s.d. = 1.04 ± 1.73; n = 94; outlier: 49 individuals). This outlier probably reflected a close breeding area of *Culicoides reconditus*, as 39 out of 49 of the

individuals collected corresponded to this species, and 32 of the 39 were nulliparous females. Thus, we performed the analyses using both the original dataset and one that excluded the outlier. Models including the outlier gave qualitatively the same results as those without it. Here, we report the statistical analyses without the outlier, although we included it in the descriptive statistics. The total abundance of *Culicoides* females and species richness followed a Poisson distribution. Analyses on *Culicoides* species were restricted to the 2 most common species captured, namely *C. reconditus* and *Culicoides circumscriptus* (see Table 2).

Generalized linear mixed models (GLMMs) with a Poisson distribution and a logit-link function were used to examine the variation in the abundance of biting midges and species richness with forest type. For data collected in 2017 and 2018, the full models had the following structure: total abundance, abundance of *C. reconditus*, abundance of *C. circumscriptus* and species richness were included as dependent variables in separate models; forest type, year and their interaction were fixed factors; hatching date and day 13 brood size were covariates and nest identity was a random factor. We decided to incorporate hatching date and brood size in the models as previous studies highlighted their importance when interpreting the abundance of biting midges in blue tit nests (Tomás et al. 2008a; Martínez-de la Puente et al. 2009a, 2009b; Castaño-Vázquez & Merino 2022). We did not consider the impact of the forest–year interaction on the abundance of *C. circumscriptus* because no specimens were collected in the humid forest in 2017. GLMM with a binomial distribution and a logit-link function was used to test the relationship between the presence/absence of *Culicoides* in nests and the type of forest. The presence/absence of *Culicoides* was used as a dependent variable, and forest, year and forest–year interaction as fixed factors. Hatching date and brood size were introduced as covariates and nest identity as random factor.

Generalized linear model (GLM) with a Poisson distribution and a logit-link function was used to test the effect of the cross-fostering experiment. In this case, total *Culicoides* abundance and species richness were included as the dependent variables in each full model and forest of origin, forest of fostering and their interaction were included as fixed factors, with hatching date and brood size as covariates. A full GLM, with a binomial distribution and a logit-link function, and the same structure as the

cross-fostering experiment, was also used to check the relationship between the presence/absence of biting midges and treatments.

In all cases, we applied a model–selection approach to choose the best models of all possibilities derived from the aforementioned full models. To do so, we used Akaike's information criterion (AIC) and selected models within a ΔAIC <2 units (Quinn & Keough 2002). The parameters were estimated by model averaging all models with a ΔAIC under 2 units (Symonds & Moussalli 2011). We tested the normality of the residuals from the models graphically following Zuur et al. (2010). For the descriptive analyses, we used the Pearson product–moment correlation to examine the correlations between continuous variables. A *t*-test was used to analyse the differences in hatching dates between infected and uninfected nests. The basic statistics are given as mean ± standard error (s.e.). All analyses were performed in software R v 4.0.0 (R Development Core Team 2020), using the packages 'lme4' (Bates et al. 2020) and 'MuMIn' (Bartoń 2020).

Results

A total of 147 female biting midges were captured in 42 of the 95 nests monitored during the 3 years (prevalence 44.21%), corresponding to 5 different species (Table 2). Four biting midges (2.72% of the total captured) were not identified to the species level because they lacked wings or other distinctive structures. A mean of 1.55 ± 0.54 (range: 0–49) biting midges were captured per nest. In nests with biting midges, there was a mean of 1.57 ± 0.13 different species (range: 1–4) per nest. *Culicoides reconditus* (40.14%) and *C. circumscriptus* (22.45%) were the most common species found in blue tit nests (Table 2). Most of the 147 *Culicoides* females captured were nulliparous (75.55%), while we captured 34 parous females (23.13%) and only 2 engorged females (1.36%). Parous females corresponded to the species *C. circumscriptus* (n = 14), *C. reconditus* (n = 11), *Culicoides truncorum* (n = 4), *Culicoides kibunensis* (n = 2), *Culicoides festivipennis* (n = 1) and 2 parous individuals were not identified to the species level. The engorged females belonged to the species *C. truncorum* and *C. kibunensis*. No males were found in the nests.

Table 2. Abundance of *Culicoides* species captured in blue tit nests from 2 different types of forests during the breeding seasons of 2017, 2018 and 2019. The percentage of infected nests is shown in parentheses.

	2017		2018		2019		
	Dry forest (n = 12)	Humid forest (n = 11)	Dry forest (n = 18)	Humid forest (n = 19)	Dry forest (n = 15)	Humid forest (n = 20)	Total (n = 95)
C. reconditus	1 (8.33)	2 (18.18)	6 (22.22)	2 (5.26)	7 (33.33)	41 (15.00)	59 (16.84)
C. circumscriptus	2 (16.67)	0 (0)	23 (50.00)	8 (26.32)	0 (0)	0 (0)	33 (16.84)
C. kibunensis	1 (8.33)	1 (9.09)	1 (5.55)	2 (10.52)	8 (20.00)	11 (25.00)	24 (13.68)
C. truncorum	3 (25.00)	2 (18.18)	9 (11.11)	1 (5.26)	2 (13.33)	5 (15.00)	22 (13.68)
C. festivipennis	0 (0)	0 (0)	0 (0)	1 (5.26)	2 (13.33)	2 (5.00)	5 (4.21)
Unidentified *Culicoides*	1 (8.33)	0(0)	1 (5.55)	0 (0)	1 (6.67)	1 (5.00)	4 (4.21)
Total	8 (50.00)	5 (27.27)	40 (55.56)	14 (36.84)	20 (46.67)	60 (45.00)	147 (44.21)

Correlative analyses

Table 3 provides a summary of the results of the model selection for the correlative study. The best model for *Culicoides* abundance included forest and hatching date as predictor variables, while the second-best model also included the year (although it was not significant). The abundance of *Culicoides* captured in blue tit nests was higher

in the dry (1.60 ± 0.41) than in the humid forest (0.63 ± 0.21; estimate = 0.99, z = 2.31, p = 0.021; Fig. 1A) and correlated positively with hatching date (estimate = 0.12, z = 3.50, p < 0.001; r = 0.32, p = 0.014; Fig. 2A).

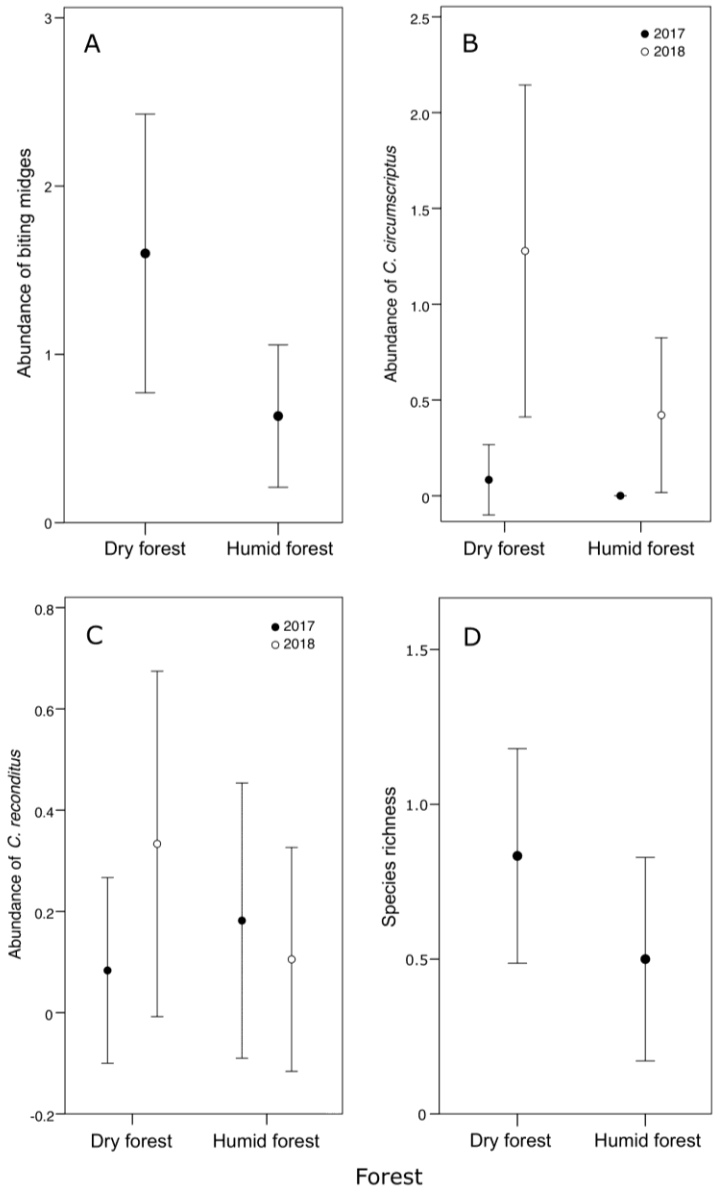

Figure 1. Abundance of biting midges (*Culicoides*) (A), abundance of *Culicoides circumscriptus* (B), abundance of *Culicoides reconditus* (C) and species richness (D) in blue tit nests located in humid and dry forests during the breeding seasons of 2017 and 2018. Means were calculated without the outlier. Bars represent s.e.

The best model for the abundance of *C. circumscriptus* included forest type, year and hatching date as predictors, and the second best also included brood size as a predictor, although it was not significant. The same forest-dependent variation was found for the abundance of *C. circumscriptus* (estimate = 1.25, z = 2.07, p = 0.039; Fig. 1B), which was higher in 2018 than in 2017 (estimate = 1.94, z = 2.37, p = 0.018; Fig. 1B). In addition, the abundance of *C. circumscriptus* increased when the nestlings were hatched later in the season (estimate = 0.10, z = 1.96, p = 0.049; r = 0.27, p = 0.039; Fig. 2B). The model selected for the abundance of *C. reconditus* included brood size and hatching date as predictors. We found a negative and significant relationship between the abundance of *C. reconditus* and brood size (χ^2 = 8.94, p = 0.003; r = −0.29, p = 0.024). The abundance of *C. reconditus* was positive and significantly related to hatching date (χ^2 = 6.01, p = 0.014; Fig. 2B). With respect to species richness, the best model included hatching date and brood size as predictors, but only hatching date was significant. Three additional models had a ΔAIC <2, but only hatching date had a significant effect on species richness in all these models. Specifically, species richness was positively associated with hatching date (estimate = 0.09, z = 3.02, p = 0.002; r = 0.31, p = 0.016; Fig. 2C).

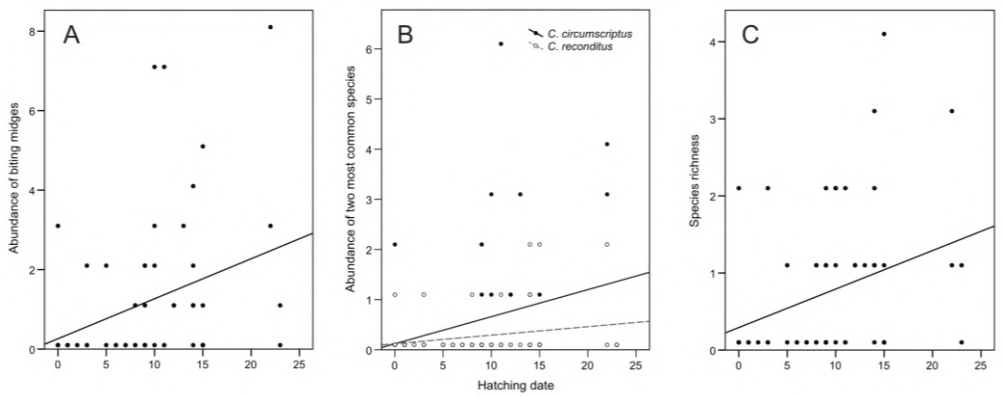

Figure 2. Relationships between hatching date and the abundance of biting midges (*Culicoides*) (A), the abundance of the 2 most common species (*C. reconditus* and *C. circumscriptus*) (B) and species richness (C) in blue tit nests during the breeding seasons of 2017 and 2018. The regression lines were calculated without the outlier (total abundance: adjusted R^2 = 0.084, p = 0.014; abundance of *C. reconditus*: R^2 = 0.021, p = 0.137; abundance of *C. circumscriptus*: R^2 = 0.055, p = 0.039; species richness: adjusted R^2 = 0.081, p = 0.016). The hatching date is standardized (0 = day the first egg hatched each year).

Table 3. Models (within ΔAIC < 2 units) describing the total abundance of *Culicoides*, species richness, biting midge prevalence and the abundance of the 2 most common *Culicoides* species (*C. reconditus* and *C. circumscriptus*) in blue tit nests during the breeding seasons of 2017 and 2018. The significant predictors ($p < 0.05$) are marked in bold.

Variable	AIC	ΔAIC
Total abundance		
Forest, **hatching date**	166.8	0.00
Forest, **hatching date**, year	168.2	1.43
Species richness		
Hatching date, brood size	130.3	0.00
Hatching date, forest	130.5	0.23
Hatching date	131.1	0.77
Hatching date, forest, brood size	131.3	0.96
Presence/absence		
Hatching date, forest	77.0	0.00
Hatching date	77.5	0.43
Hatching date, year	78.9	1.83
Hatching date, year, forest	78.9	1.83
Abundance of *C. reconditus*		
Hatching date, **brood size**	55.0	0.00
Abundance of *C. circumscriptus*		
Forest, **hatching date**, **year**	106.0	0.00
Hatching date, **year**, forest, brood size	107.7	1.75

Finally, the best models for the presence/absence of biting midges included hatching date, forest and year as predictors, but only hatching date significantly affected the presence of *Culicoides* in all these models. The presence of *Culicoides* correlated positively with hatching date (estimate = 0.17, $z = 2.65$, $p = 0.008$), suggesting that nests with late-hatching nestlings were more likely to be infected by biting midges than those which hatch earlier in the breeding season (*t*-test: $t_{58} = -2.75$, $p = 0.008$).

Cross-fostering experiment

Table 4 shows the model selection results for the cross-fostering experiment conducted in 2019. The results reflect that the independent variables did not have a significant effect on either species richness or biting midge prevalence as none of the models differed significantly from the null models (Table 4). However, 2 different models for *Culicoides* abundance had a ΔAIC < 2 each including hatching date and foster forest (model 1) and hatching date, foster forest and origin forests (model 2) as predictors. However, only hatching date and foster forest had a significant impact on *Culicoides* abundance. As for the correlative study, the abundance of *Culicoides* was higher in the dry (1.33 ± 0.52) than in the humid forest (0.58 ± 0.19; estimate = 0.85, $z = 2.13$, $p = 0.033$) and was positively associated with hatching date (estimate = 0.11, $z = 2.70$, $p = 0.007$).

Table 4. Models (within ΔAIC < 2 units) describing the total abundance of *Culicoides*, species richness and prevalence of biting midges in blue tit nests during the cross-fostering experiment conducted in 2019. The significant predictors ($p < 0.05$) are marked in bold.

Variable	AIC	ΔAIC
Total abundance		
Foster, **hatching date**	96.1	0.00
Foster, **hatching date**, origin	97.8	1.69
Species richness		
Null model [a]	75.8	0.00
Presence/absence		
Brood size	48.8	0.00
Null model [a]	48.8	0.03
Hatching date	50.1	1.36

[a] Null models only include the intercept.

Discussion

We identified *Culicoides* species composition and examined different factors that influence their abundance in blue tit nests. We found 5 *Culicoides* species, all previously captured in bird nest boxes in other regions (Table 1). In addition, 3 out of

5 *Culicoides* species found here were previously recorded in nests from the same bird species in central Spain, although the dominant species clearly differed between locations (Martínez-de la Puente et al. 2009a; this study). Specifically, *Culicoides simulator* was the most common species in central Spain accounting for 56.89% of all *Culicoides* captured in blue tit nests, while the most common species observed in the present study was *C. reconditus* (40.14%). Interestingly, to the best of our knowledge, this is the first account of *C. reconditus* in the Iberian Peninsula (Delécolle 2002; Alarcón-Elbal & Lucientes 2012). In a previous study in central Spain, Martínez-de la Puente et al. (2011a) found a high intraspecific genetic variance of a fragment of the cytochrome *c* oxidase subunit 1 gene in specimens morphologically identified as *Culicoides segnis*, which suggests the sequences could correspond to 2 different species. The authors argued that the results could be due to the presence of *C. reconditus* in the area, because it is closely related to *C. segnis* only differing in the distribution of their coeloconic sensilla on the antennae and shape of abdominal sclerites (Mathieu et al. 2012). These characteristics were clearly identified in 10 specimens mounted in this study, 2 of which were deposited in the National Museum of Natural Sciences (MNCN-CSIC), Madrid, Spain, mounted on 4 slides (2 per individual) under the accession numbers MNCN_Ent_319173, MNCN_Ent_267740, MNCN_Ent_267741 and MNCN_Ent_267742.

Correlative analyses

We found habitat influenced on the abundance of biting midges with a higher abundance in nests located in the dry forest compared to the humid forest over the 2 years of the correlative study. Some of the possible causes of this difference include the interhabitat variation in the total abundance of *Culicoides* and/or because the *Culicoides* have a different capacity to reach avian nests in each habitat. The availability and extension of water sources in a habitat, together with abiotic soil characteristics, are strong determinants of *Culicoides* abundance because of their importance for larval development (Uslu & Dik 2010; Erram et al. 2019). For instance, *C. festivipennis* preferably breeds in nutrient-rich muds found in streams, while other species, such as *C. circumscriptus*, are more generalist when breeding (Uslu & Dik 2010, and references therein). On the other hand, the weather conditions in the 2 habitats are evidently different; the dry forest had a higher temperature and a lower humidity than the humid forest (supplementary material; Garrido-Bautista et al.

2021). The relative humidity may negatively affect the large-scale abundance of biting midges in the area (van Hoesel et al. 2019), which could partially explain the lower *Culicoides* abundance in the humid forest. In addition, in an experimental study affecting the humidity inside nest boxes occupied by European roller (*Coracias garrulus*), Castaño-Vázquez et al. (2022) found a lower abundance of *Culicoides* in nests with a higher humidity. Nevertheless, humidity seems to be less determinant for *Culicoides* abundance and flight performance than temperature. During the breeding season of the blue tits, ambient temperature correlates with nest temperature (Ardia et al. 2006), while *Culicoides* abundance increases with ambient temperature (Bernotienė et al. 2021; Castaño-Vázquez & Merino 2022) and temperature inside the nest (Martínez-de la Puente et al. 2010b). In fact, heat gradients are important cues which biting midges, and other vectors, use to locate their hosts (Lehane 2005).

On the other hand, other variables, such as early morning wind speed – when biting midges are more active – (Lehane 2005), could affect the number of vectors visiting nest boxes. The dry and humid forests were located opposite each other on south-west and south-east mountain slopes, respectively. Since the prevailing wind in this region is westerly during spring (Viedma-Muñoz 1998), the humid forest was expected to receive higher wind speeds, ultimately reducing *Culicoides* flight activity and, consequently, decreasing their abundance in bird nests (Martínez-de la Puente et al. 2009b). Variations in forest leaf density between large areas may also impact *Culicoides* population numbers, with some species favouring sparsely vegetated areas, while others prefer habitats with a higher leaf density (Conte et al. 2007). This variation in forest cover goes some way to explaining the different abundances of some *Culicoides* species between habitats. Lastly, we should not ignore the fact that the interforest variation in *Culicoides* abundance could be due to a geographical singularity of the 2 sampled localities independent of the habitat differences; however, unfortunately, we cannot study this premise as we do not have any spatial replicates for this system.

Differences in the weather conditions may also explain the positive association between hatching date and all the variables analysed. This implies that nestlings from nests breeding later in the season were affected by more *Culicoides* and from more species. As the ambient temperature increases throughout the breeding season, we would normally expect more biting midges to visit more nests (e.g. Bernotienė et al.

2021). Several studies have reported effects of seasonality on the abundance of different vector groups, including *Culicoides*, and found that their abundance generally augmented as the spring progressed (Sarto i Monteys & Saiz-Ardanaz 2003; Ferraguti et al. 2013; Lalubin et al. 2013; Bernotienė et al. 2021). This was also true of cavity-nesting birds, as *Culicoides* abundance in their nests increased as the breeding season advanced (Tomás et al. 2008a; Martínez-de la Puente et al. 2009a, 2009b; Castaño-Vázquez & Merino 2022; this study). In addition to the detrimental effect of the blood-sucking activity of *Culicoides*, these results support the fact that nestlings which hatch later in the season may be subject to a greater susceptibility to blood parasite infections (Martínez-de la Puente et al. 2013). At least, 4 out of 5 species of *Culicoides* captured here may act as vectors for *Haemoproteus* parasites, and parous females of all of these species have been found in avian nests (*C. circumscriptus*: Martínez-de la Puente et al. 2011a; Veiga et al. 2018; *C. festivipennis*: Martínez-de la Puente et al. 2011a; *C. kibunensis*: Martínez-de la Puente et al. 2011a; Bernotienė et al. 2019; Žiegytė et al. 2021; *C. truncorum*: Martínez-de la Puente et al. 2011a). Thus, nestlings from late-nesting parents could be impaired in terms of future reproduction success (Merino et al. 2000) or even long-term survival (Martínez-de la Puente et al. 2010a). Further studies should be conducted in order to identify the parasites potentially transmitted by *Culicoides* species in the area.

Furthermore, there was a negative relationship between insect abundance and brood size in the case of *C. reconditus*, while the other variables analysed returned statistically non-significant associations. If more nestlings release a greater concentration of attractive molecules (e.g. CO_2, kairomones), then one would expect higher *Culicoides* abundances in nests with larger broods (Martínez-de la Puente et al. 2009a, 2010b; Castaño-Vázquez & Merino 2022). Nevertheless, contrasting results have previously been reported in blue tits, with some studies showing a positive relationship between *Culicoides* abundance and brood size (Martínez-de la Puente et al. 2009a, 2009b), yet another study also reported non-significant associations (Tomás et al. 2008a). Given the correlative nature of these results, further experimental research into different brood sizes is required to clarify the influence of brood size on the birds' susceptibility to *Culicoides* attacks.

Cross-fostering experiment

Based on the results from the previous 2 years, we developed an experimental approach to identify the role of habitat vs nestling traits in determining the birds' susceptibility to *Culicoides* attacks. Nestlings from different habitats could produce different attractants to insect vectors. For example, different studies have proposed that odours derived from avian uropygial gland secretions are involved in the attraction of different vectors (Russell & Hunter 2005; Garvin et al. 2018; review in Moreno-Rueda 2017; Tomás et al. 2020). This secretion, together with other scent-producing body sources, such as the skin or feathers (Menon & Menon 2000; Campagna et al. 2012), may determine bird odour (Campagna et al. 2012) and probably has a genetic origin (Krause et al. 2018). For the case of biting midges, Tomás et al. (2020) found that uropygial secretions from hoopoe (*Upupa epops*) nestlings may repel some insect vectors, depending on the habitat. However, other studies failed to identify any similar associations in biting midges (Martínez-de la Puente et al. 2011b) and mosquitos (Díez-Fernández et al. 2019). In addition, uropygial secretions can harbour symbiotic bacteria that may release several chemical cues (Maraci et al. 2018), which, together with the skin microbiota, may affect vector attraction (review in Ruiz-López 2020). However, in this work we failed to identify any significant association supporting this scenario, which suggests that habitat may have a major significant impact on nestling exposure to biting midges. Our results indicate that the habitat of origin of blue tit nestlings did not affect the degree to which they attracted biting midges to their nests, suggesting that nestlings did not exhibit repellent or attractant chemical properties to these vectors unrelated to rearing habitat (due to genetic differentiation or maternal effects). On the other hand, the abundance of biting midges did differ between habitats, lending further support to the influence of this variable on *Culicoides* abundance in avian nests.

Concluding remarks

The abundance of biting midges in blue tit nests is mainly determined by habitat type, which may explain the different patterns of blood–parasite transmission observed in birds from different habitats (e.g. Ferraguti et al. 2018). It is important to take these results into account when trying to understand local variations in bird species' susceptibility to vector attacks. These attacks may adversely affect the health and

fitness of wild birds due to the detrimental effects of their bites and the parasites they can transmit. This should be especially relevant due to the impact of global change on the distribution and abundance of vectors of pathogens, including those attacking birds in their nests (Castaño-Vázquez & Merino 2022).

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Supplementary material

Table S1. Temperature, relative humidity, solar radiation, insolation and normalized difference vegetation index (NDVI) for the 2 forest types of the study area. Table shows the mean values, standard deviation (s.d.), statistic and *p*-value.

Factor	Forest type		Statistic	p-value
	Dry forest	Humid forest		
Temperature (ºC) [1]	17.81 ± 6.47	16.80 ± 6.19	$t_{1338} = 14.11$	< 0.001
Relative humidity (%) [1]	41.37 ± 18.02	42.99 ± 17.90	$t_{1338} = 9.21$	< 0.001
Solar radiation ($kWh/m^2/day$) [2]	13.46 ± 0.34	11.98 ± 0.21	U = 0.00	< 0.001
Insolation (h) [3]	6.63 ± 0.20	6.45 ± 0.19	U = 530.00	< 0.001
NDVI [4]	0.54 ± 0.08	0.59 ± 0.04	U = 17.00	0.112

[1] Data obtained from dataloggers iButton® installed in outer walls of nest-boxes. Year: 2019. Statistic: *t*-test.

[2] Data obtained from software GRASS SIG 7.8.2, integrated in QGIS 3.10.5, using the "r.sun.insoltime" algorithm. Insolation obtained every five days and covering breeding seasons of blue tits from 2017 to 2019. Statistic: U Mann-Whitney test.

[3] Data obtained from software ArGis Desktop 10.3.1, using the "Area solar radiation" algorithm. Solar irradiation obtained every five days for breeding seasons of each year (2017, 2018 and 2019). Statistic: U Mann-Whitney test.

[4] Data obtained from software QGIS 3.10.5 using Landsat-8 satellite images covering the breeding seasons of each year (2017, 2018 and 2019). NDVI calculated based on Landsat-8 bands B4 (red) and B5 (near infrared) as: (B5 – B4) / (B5 + B4). Statistic: U Mann-Whitney test.

Chapter 3

Prevalence, molecular characterization, and ecological associations of filarioid helminths in a wild population of blue tits (*Cyanistes caeruleus*)

ABSTRACT Filarioid nematodes (commonly known as filarial worms) are known to impact human and domestic animal health, but studies examining their ecological relevance and impacts on wildlife are still underrepresented. In the case of birds, microfilariae are typically found at low prevalence, but they may negatively affect some fitness-related traits. Here, we study the prevalence and associations of microfilariae in a wild population of blue tits (*Cyanistes caeruleus*) inhabiting a woodland comprising different forestry formations. In addition, we characterize the filarioid lineages through the cytochrome *c* oxidase subunit I (*COI*) gene sequence. We found a moderate prevalence of microfilariae in the blue tit population (9.4%) and that the presence of such parasites was negatively associated with host body mass. Neither forest type nor host sex influenced microfilariae presence. Phylogenetic analyses revealed the presence of five filarioid lineages clustered in the Onchocercidae family—four out of five lineages clustered in the *Splendidofilaria* clade, while the remaining lineage could not be clearly assigned to a genus. In addition, this is the first study examining the filarioid lineages infecting the blue tit. Our results suggest that hosts in poorer body condition, in terms of lower body mass, are more susceptible to be parasitized by filarioid nematodes and call for further genetic studies of these parasites.

Keywords: Bird hosts, filarial nematodes, microfilariae, parasite-host ecology, PCR, wildlife diseases

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Introduction

Helminth infections are widespread (McCarthy & Moore 2000) and part of a global health concern affecting over a billion people, especially in tropical and subtropical regions (de Silva et al. 2003). Lymphatic filariasis and onchocerciasis are vector-borne helminth diseases caused by filarioid nematodes, which affect millions of humans worldwide (Taylor et al. 2010). During the last few decades, new advances in vaccine development (Kalyanasundaram et al. 2020), bacteria-based treatments (Allen et al. 2008), and diagnostic methods (Molyneux 2009; Dieki et al. 2022) have been developed because of the epidemiological and public health relevance of filariasis. Moreover, diseases caused by some filarioid species represent emerging zoonosis for humans and domestic animals nowadays (Simón et al. 2012; Otranto et al. 2013). Adult filarioids dwell in specific vertebrate–host tissues and cavities (depending on the filarioid group). They produce microfilariae as the first-stage larvae, typically present in the host bloodstream, which then develop into the infective third-stage larvae in the blood-sucking arthropod vectors (e.g. mosquitoes, biting midges, blackflies) (Orihel & Eberhard 1998; Bain & Babayan 2003; Bartlett 2008). Thus, most ecological studies using host populations utilize microfilariae as a non-lethal filaremia indicator, as they are the most readily accessible stages of filarioids in wild animals.

Although most research on filarioid nematodes focuses on humans and domestic animals, studies examining their ecological relevance and impacts on wildlife are still underrepresented. Filarioid nematodes can infect a wide variety of wild vertebrates, including mammals (Laaksonen et al. 2009; Mutinda et al. 2012), reptiles (Bain 2002), amphibians (Readel & Goldberg 2010), and birds (Bartlett 2008). In wild populations of birds, the prevalence of filarioids across the globe is usually low, although some geographical and species-dependent variation exists. For example, studies examining bird communities report the presence of microfilariae in 3.6–11.0% of birds from African (Sehgal et al. 2005a, 2005b; Savage et al. 2009), 0.3–0.6% from Asian (Valkiūnas & Iezhova 2001; Elahi et al. 2014), 1.0–6.6% from South American (Bennett et al. 1991; Matta et al. 2004; Londoño et al. 2007; Silveira et al. 2010; Sebaio et al. 2012; de la Torre & Campião 2021), 1.0–8.1% from Central American (Young et al. 1993; Valkiūnas et al. 2004; Benedikt et al. 2009; Villalva-Pasillas et al. 2020), and 0.4–3.2% from European (Merino et al. 1997; Haas et al. 2011) regions, but the

prevalence may reach up to 30–40% in bird populations inhabiting insular habitats (Travis et al. 2006a, 2006b; Merkel et al. 2007; Clark et al. 2016). In addition, some bird species appear to be more susceptible to be infected by filarioids than others, such as the song thrush (*Turdus philomenos*) in Europe (Cardells-Peris et al. 2020), the rufous-crowned sparrow (*Aimophila ruficeps*) in North America (Deviche et al. 2005), or alethes (*Alethe* spp.) in Africa (Sehgal et al. 2005a, 2005b). Additionally, the prevalence of filarioids and their impacts on bird hosts (see below) may be driven by environmental factors, such as habitat characteristics, that can limit vector abundance or distribution (Sehgal 2015; Ferraguti et al. 2018). This habitat-dependent vector abundance may be patent even at smaller spatial scales (e.g. biting midges; Garrido-Bautista et al. 2022a), which could explain the local variations in susceptibility to filarial infection among bird species (Clark et al. 2016). In fact, some studies report that filarioid nematodes infecting wild birds are less frequent in highlands compared to lowlands in mountain systems (Siers et al. 2010; González et al. 2014) and also that the abiotic conditions of a particular habitat, such as the temperature, precipitation, or insular environmental particularities, may affect the prevalence of these blood parasites in wild birds (Siers et al. 2010; Clark et al. 2016).

However, most of the aforementioned studies conducted at the community level typically reported a low sample size per bird species or population, making the analysis of filarial effects on the health or condition of birds unfeasible. Although infections by filarioids are traditionally considered non-pathogenic with negligible impacts on wild bird hosts (Campbell & Ellis 2007; Bartlett 2008), recent evidence suggests that these parasites may affect body condition, blood physiology, or even some life-history traits, with potential negative impacts on host fitness. For instance, in several bird species, infection by microfilariae is associated with a decrease in body mass (Atawal et al. 2019; de la Torre et al. 2020), reduced feather growth (Höglund et al. 1992), or multiple impacts on blood protein and immune-cell physiology (Travis et al. 2006b; Valera et al. 2006; Clark et al. 2016). Altogether, filarioids may alter some life-history traits of birds, such as diminishing the migration return rate (Davidar & Morton 2006), thus potentially decreasing survival prospects, especially when the host is heavily parasitized (Larrat et al. 2012, but see Höglund et al. 1992).

On the other hand, the diversity of Filarioidea species infecting wildlife remains insufficiently explored. Most ecological studies examining blood smears have

reported on avian filarioid species or genera exhibiting microfilaria's morphological characters (e.g. *Splendidofilaria* spp., *Eufilaria delicata* or *Paronchocerca* spp.; Merino et al. 1997; Valera et al. 2006; Haas et al. 2011; Elahi et al. 2014), but this methodology is limited due to the similarities in the morphology of microfilaria parasites or differences in blood film preparations (Binkienė et al. 2021). In this sense, molecular-based techniques have proven to be useful in the detection of filarioids, species identification (Bain et al. 2008; Ferri et al. 2009), and for making inferences regarding their phylogenetic relationships (Bain et al. 2008; Eamsobhana et al. 2013). Yet, few DNA sequences have been published from avian filarioids (Binkienė et al. 2021, and references therein), but recent studies have developed suitable techniques and methods to molecularly characterize avian filarioids, which mainly target the mitochondrial cytochrome *c* oxidase subunit I (*COI*) and the nuclear *18S* rRNA gene (Hamer et al. 2013; Binkienė et al. 2021; Chagas et al. 2021).

The aim of this study is to examine the prevalence of microfilariae and the effects of these blood parasites in a wild population of blue tits (*Cyanistes caeruleus*), examining the variation with laying date, host sex and habitat, but also the relationship with host body condition. Moreover, we molecularly characterize the filarioid species infecting this host species to better understand the diversity and the ecological role of these parasites infecting wildlife.

Materials and methods

Study area and blue tit sampling

The study was conducted during the springs of 2017, 2018, and 2019 in the Sierra Nevada National Park (southeastern Spain, 36°57′N, 3°24′W, 1700–1800 m a.s.l.), in a continuum woodland separated by a river (Río Chico). Because of the different levels of exposure to the sun, the western part of the woodland, which is composed of Holm oak (*Quercus ilex*) forest and Pyrenean oak (*Q. pyrenaica*) forest, was drier than the eastern part, which is composed by a Scots pine (*Pinus sylvestris*) forest and a mixed forest of Holm oaks and Pyrenean oaks. These forests differed in several other abiotic environmental factors, as well as ectoparasites (blowflies, fleas, etc.) and vectors (biting midges, blackflies, etc.) prevalence. A detailed description of the study area can be found in (Garrido-Bautista et al. 2021, 2022b).

The blue tit population bred in nest boxes, all of the same type (ICONA C model; Moreno-Rueda 2003), which were hung from tree branches using metal hooks. The nest boxes were placed at a 3–4 m height in 100 m intervals and inspected regularly during all breeding seasons to determine laying date (standardized as Julian date) and hatching date. When the nestlings were between 8 and 11 days old (day 0 = hatching date), we captured adult blue tits in their nest boxes using scuttles which closed the nest box entry when entered to feed the nestlings. This age range was chosen in order to ensure that nestlings were not harmed because the capture of parents provokes a delay in their return to the nest box (Schlicht & Kempenaers 2015), but blue tit nestlings can self-thermoregulate from day 8 (Perrins 1979). Once captured, adults were banded with aluminum rings and sexed, checking for the presence of brood patches in females. Tarsus length was measured with a digital caliper (accuracy: 0.01 mm; always by the same researcher, G.M.R.) and body mass with a digital portable scale (accuracy: 0.1 g).

Before releasing blue tits (at maximum 10 m away from their nest boxes), we took a 100 μL blood sample from their jugular vein using heparinized insulin syringes in sterile conditions, following the actions taken in Owen (2011). This procedure was performed by the same researcher (G.M.R.). When collecting the morphometric measurements and blood samples, the handling time was kept at minimum to reduce bird stress (de Jong 2019). Microfilariae are present in the bird bloodstream permanently, normally showing a circadian rhythm with peaks of intensity in the evening and night (Chagas et al. 2021). To avoid bias in microfilariae detection, the blue tits were typically sampled at afternoon (16:00 to 18:00 h GMT +2). Blood was preserved in 1.5 mL tubes with absolute ethanol and then transported to the laboratory, where they were stored at −20 °C until further genetic analyses. In total, we sampled 171 adult blue tits (sample size per year: 2017—48, 2018—64, 2019—59).

DNA extraction, filarioid PCR screening and sequencing

Approximately 10 μL of blood per blue tit was used for the DNA isolation procedure. Genomic DNA was extracted with the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, except for the sample heating time, which was increased to 1 h. DNA was stored at −20 °C until further procedure.

The DNA was screened for the presence of filarioid nematodes targeting sections of both the nuclear *18S* rRNA gene and the mitochondrial *COI* gene. All samples were screened with the primers ChandFO (5′–GAG ACC GTT CTC TTT GAG GCC–3′) and ChandRO (5′–GTC AAG GCG TAN NTT TAC CGC CGA–3′) (Hamer et al. 2013) to obtain a 560 pb fragment of the *18S* rRNA gene. All positive samples were additionally screened with the primers COIint-F (5′–TGA TTG GTG GTT TTG GTA A–3′) and COIint-R (5′–ATA AGT ACG AGT ATC AAT ATC–3′) (Casiraghi et al. 2001) to obtain a 689 pb fragment of the *COI* gene. Since all PCRs using the latter primer set were negative, we designed primers based on all complete *COI* sequences of Onchocercidae available on NCBI Genbank. The new primers OnchoCOI_F1 (5′–TTG TGG AAT GAC TTT TGG YAA T–3′)/OnchoCOI_R1 (5′–AAT CTT AAC AGC TCT AGG AAT AGC–3′) and OnchoCOI_F2 (5′–CTG TTA ATC ATA AGA CTA TTG GTA CT–3′)/OnchoCOI_R2 (5′–CAG CAC TAA AAT AAG TAC GAG TAT C–3′) allowed for the amplification of a 900 bp section of the *COI* in a nested PCR. The PCR protocol for each nested step was as follows: initial denaturation (95 °C for 2 min), 35 cycles of denaturation (95 °C for 1 min), annealing (nested step 1: 53 °C for 1 min; nested step 2: 50 °C for 1 min) and extension (72 °C for 1 min), before a final extension at 72 °C for 5 min. Each 1 µL of the first PCRs was used as a template for the nested PCRs.

The PCRs were performed in 25 µL volumes using the GoTaq® G2 DNA polymerase (Promega Biotech, Madison, WI, USA). The master mixes contained 14.375 µL nuclease-free water, 5 µL 5× Green Reaction Buffer, 2 µL $MgCl_2$ (25 mM), 0.5 dNTPs (10 mM), 0.125 µL GoTaq G2 Polymerase (5 u/µL), 1 µL primer (10 pmol/µL) each, and 1 µL DNA. In all PCR runs, a negative control (nuclease-free water) and a known positive control were included. PCR products were separated by electrophoresis on 2% agarose gels stained with Midori Green Advance DNA stain (Nippon Genetics Europe, Düren, Germany). When conducting the PCRs, we followed the recommendations outlined in Bensch et al. (2021) to avoid potential cases of cross-contamination.

All PCR-positive samples were sent for purification and sequencing (in both directions) to LGC Genomics (Berlin, Germany). The raw sequences were analyzed and aligned using BioEdit v.7.0.5.3 (Hall 1999). The *18S* and *COI* sequences obtained in the present study were deposited in NCBI GenBank under the accession numbers: OQ859189 to OQ859204 (*18S* gene), and OQ848453 to OQ848460 (*COI* gene).

Filarioid phylogenetic analysis

Phylogenetic trees were calculated based on a 600 bp section of the *COI* gene. The *COI* sequence was used for calculating the phylogenetic tree because the 560 bp section of the *18S* gene was too conserved and sequences were available from few Onchocercidae taxa only. GenBank sequences were retrieved by performing a BLAST search targeting Onchocercidae. The BLAST search retrieved 466 sequences, which covered the entire 600 bp section. The sequences were aligned with MAFFT v.7.311 (Kumar et al. 2016) with the default options applied and collapsed to haplotypes with DAMBE v.7.0.51 (Xia 2018), resulting in 296 unique sequences (including sequences of the 5 lineages found in the present study). Based on this alignment, one sequence per species/lineage (in the case of *Splendidofilaria* and *Eufilaria*, for all sequences) was selected, resulting in a final alignment of 114 sequences. A sequence of *Oswaldofilaria chabaudi* (KP760204), taking a basal position in the Onchocercidae phylogeny, was used as outgroup. The best-fit substitution model, according to the corrected Akaike Information Criterion (AICc), was evaluated using IQ-TREE v.1.6.12 (Nguyen et al. 2015), resulting in the model GTR+I+G. A Maximum Likelihood 'majority rule consensus' tree was calculated using IQ-TREE v.1.6.12 (Nguyen et al. 2015) by performing 1000 bootstrap replicates each. A Bayesian Inference tree was calculated using MrBayes v.3.2 (Ronquist et al. 2012); the analyses were run for 5 million generations (2 runs each with 4 chains, one of which was heated) and every thousandth tree was sampled. The first 25% of trees were discarded as burn-in and 50% majority rule consensus trees were calculated from the remaining 37,500 trees each.

Statistical analysis

Before the calculation of body condition, body mass and tarsus length of the blue tits were log-transformed. The log body mass of male and female blue tits was compared in a linear model using log tarsus length as a covariate, sex as a fixed factor, and the interaction between both variables. There was a significant positive correlation between body mass and tarsus length ($F_{1, 165} = 15.24$, $p < 0.001$), but neither sex ($F_{1, 165} = 0.60$, $p = 0.44$) nor the interaction between tarsus length and sex ($F_{1, 165} = 0.57$, $p = 0.45$) were significant. Thereby, males and females were pooled when estimating the body condition. We calculated the body condition index as the residual of the ordinary

least squares (OLS) regression of log body mass on log tarsus length (Labocha & Hayes 2012). For the subsequent statistical analyses, we considered both residuals of such regression and body mass itself as proxies of body condition in separate models as they has been proved to adequately reflect the fat content in birds (Labocha & Hayes 2012).

During the spring of 2019, we performed a cross-fostering experiment which involved the exchange of whole broods between two out of the four forest types (i.e. between the western and eastern part of the woodland). This cross-fostering study performed in our study population was previously described in (Garrido-Bautista et al. 2022a). Although the experiment was developed to identify the potential genetic (or maternal) and environmental components of nestling physiology variance, rearing a non-own brood could affect the probability of infection by filarioids in blue tit parents. Because of this, we first explored whether the cross-fostering experiment influenced the probability of infection using a generalized linear model (GLM) with a binomial distribution and linked to a logit function. The prevalence of filarioids was the dependent variable and forest of origin, forest of fostering, and their interaction were included as fixed factors. The probability of infection did not show significant variations with any of these factors (forest of origin: $z = -0.004$, $p = 0.99$; forest of fostering: $z = 1.78$, $p = 0.074$; interaction forest of origin*forest of fostering: $z = 0.00$, $p = 0.99$); thus, adults sampled in 2019 were pooled with those from 2017 and 2018 for further statistical analyses.

To test for the variation in probability of infection by filarioids, we constructed two different full GLM with binomial distribution and linked to a logit function. The two full GLM included sex (two levels), forest (two levels: western and eastern), year (three levels), and laying date as independent variables, but the first one also included body condition index as independent variable, while the second one included body mass in the place of body condition index. Interactions between independent variables were removed from the two full GLM because none proved significant. To choose the best models of all possibilities, we applied a model-selection approach independently for each aforementioned full GLM. We used the Akaike's information criterion (AIC) and selected those models with a ΔAIC under 2 units (Quinn & Keough 2002). The parameters were estimated by model averaging all models with a ΔAIC under 2 units (Symonds & Moussalli 2011). We tested the normality and homoscedasticity of model

residuals by following the methodology in (Zuur et al. 2010). As sequencing was more accurate than PCR screening when describing the prevalence of filarioids (see Results), we used the prevalence data obtained by sequences for the aforementioned models. All analyses were performed using the software R 4.0.0. (R Development Core Team 2020), with the package 'MuMIn' (Bartoń 2020).

Results

Prevalence and probability of infection by filarioids

Overall, using the *18S* PCR screening, 18 out of 171 (10.53%) blue tits were positive for filarioid helminths. However, only 16 of the 18 PCR-positive samples were confirmed by sequencing, slightly reducing the prevalence to 9.36%.

The results of the model selection from the first full GLM showed that the best model for explaining the probability of infection by filarioids included only the body condition index as the predictor variable (Table 1). Blue tits in a poorer condition were more susceptible to be infected by filarial nematodes than those in a better condition, although the relationship was marginally non-significant (estimate = −24.24, z = −1.75, p = 0.078). However, none of the selected models differed significantly from the null model (Table 1); thus, the independent variables did not have any significant effect on the probability of infection by filarioids.

The results of the model selection from the second full GLM revealed that the best model explaining the probability of infection included only body mass as the predictor variable (Table 1). The probability of infection by filarioids increased significantly with decreasing body mass of adult blue tits (estimate = −1.11, z = −1.96, p = 0.049; Fig. 1). The second and third best models included also laying date and forest, respectively, as predictors, but these variables did not significantly affect the probability of infection (laying date: estimate = 0.04, z = 1.16, p = 0.24; forest: estimate = −0.32, z = 0.58, p = 0.56). The best model significantly differed from the null model (null model: AIC = 107.9, ΔAIC = 2.04).

Table 1. AIC values and ΔAIC of the models for probability of infection by filarioids with the variables included in the models indicated. The variables that were significant at $p < 0.05$ are shown in bold. See the section of statistical analysis for the structure of the full models relative to body condition index and body mass.

Variable	AIC	ΔAIC
Body condition index models		
Body condition index	106.7	0.00
Body condition index, laying date	107.5	0.87
Null model [a]	107.9	1.23
Body condition index, forest	108.4	1.75
Body condition index, sex	108.6	1.94
Body mass models		
Body mass	105.9	0.00
Body mass, laying date	106.6	0.76
Body mass, forest	107.6	1.73

[a] Null model included only the intercept.

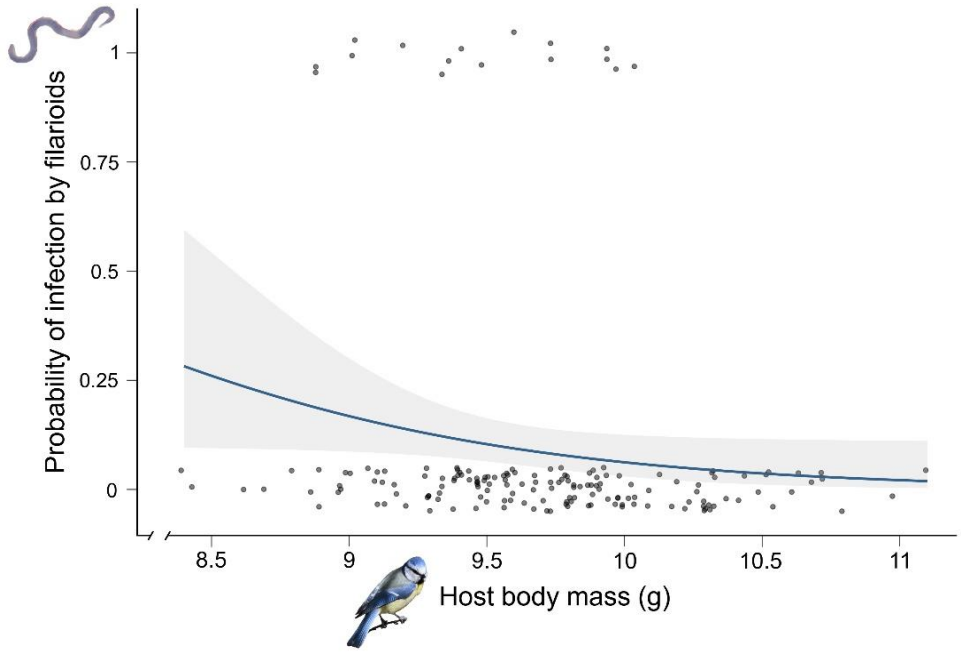

Figure 1. Probability of infection by filarioid nematodes with fitted values of blue tit (*Cyanistes caeruleus*) body mass, obtained by the binomial GLM. The shade corresponds to the 95% confidence interval.

Filarioid sequences and phylogenetic tree

In total, sixteen individuals were confirmed positive for Onchocercidae by PCR and sequencing the 560 pb section of the *18S* gene. Five samples (Cc017, Cc056, Cc095, Cc139, and Cc150) featured two lineages differing by few bp from those of Onchocercidae detected in the American robin (*Turdus migratorius*) (JQ867037, JQ867035, JQ867026) and the common grackle (*Quiscalus quiscula*) (JQ867040). Eleven samples (Cc023, Cc030, Cc038, Cc039, Cc053, Cc058, Cc070, Cc106, Cc115, Cc166, Cc169) featured a new lineage separated by at least five bp from other Onchocercidae.

The 900 pb *COI* section was successfully sequenced in eight samples only. A phylogenetic tree was calculated based on a 600 pb section of the *COI*, including representatives of all Onchocercidae species (supplementary material, Fig. S1). The two clades featuring lineages detected in the present study are shown in Fig. 2. The lineage detected in samples Cc39 and Cc139 differed by 0.5% to 0.7% from *Splendidofilaria mavis* isolated from the blackbird (*Turdus merula*) (OK631737, OK631738) and song thrush (OK631739, OK631740) in Lithuania (Chagas et al. 2021) and likely belongs to the same parasite species. The samples Cc56 and Cc115, Cc95, and Cc17 featured three additional lineages, which also clustered in the *Splendidofilaria* clade and likely belong to three separate *Splendidofilaria* species. The lineage of samples Cc56 and Cc115 differed by 7.7% to 8.3% from already known Splendidofilaria lineages, Cc95 by 7.2% to 7.8%, and Cc17 by 6.7% to 7.8%. The samples Cc53 and Cc106 featured an Onchocercidae lineage that could not be clearly assigned to any genus of filarioid parasites. It clustered in a well-supported clade with representatives of four Onchocercidae genera, forming the sister clade to *Micipsella iberica* from the Iberian hare (*Lepus granatensis*) (MW934617) and *Rumenfilaria andersoni* from the reindeer (*Rangifer tarandus*) (Q888273). Moreover, the clade also contained sequences of *Chandlerella quiscali* from the common grackle (HM773029) and *Madathamugadia hiepei* from the gecko *Pachydactylus turneri* (JQ888262). The sequences of samples Cc53 and Cc106 differed from the latter four lineages by 10.5% to 14.5%.

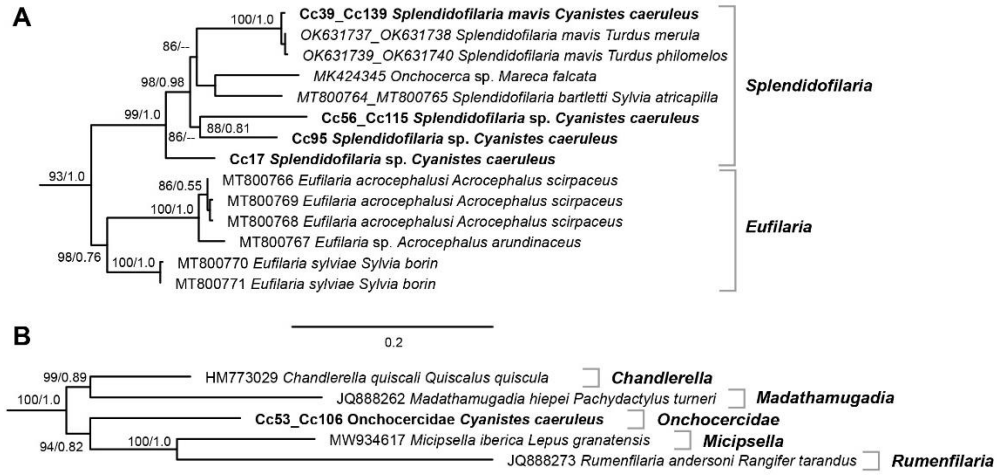

Figure 2. Clades of Maximum Likelihood tree (supplementary material, Fig. S1) calculated based on 600 bp sections of the *COI* gene. Maximum likelihood bootstrap values and Bayesian posterior probabilities are indicated at all nodes. The scale bar indicates the expected mean number of substitutions per site according to the model of sequence evolution applied. (A) Clade containing *Splendidofilaria* and *Eufilaria* sequences; (B) Clade containing unknown Onchocercidae lineage.

Discussion

Our results showed that blue tits in a poorer body condition, in terms of lower body mass, are more susceptible to be parasitized by filarioid nematodes. Although a strong relationship was not apparent, the findings of the present study are in accordance with other studies which found a negative association between microfilarial infection status and bird body mass (Atawal et al. 2019; de la Torre et al. 2020). Furthermore, we molecularly characterized the microfilariae infecting such bird hosts, revealing that blue tits are parasitized by several lineages of Onchocercidae in our study area. Our molecular data thus added new useful information to better understand the diversity of avian filarioids, calling for further genetic studies of this understudied nematode group (Binkienė et al. 2021; Chagas et al. 2021). In contrast, although habitat type has been shown to affect filarioid prevalence or distribution in other bird populations (Savage et al. 2009; Siers et al. 2010; Clark et al. 2016), we did not find evidence to suggest that forest type at smaller scales can alter the prevalence of these blood parasites infecting blue tits. Lastly, host sex did not alter the probability of infection by filarioids, suggesting the absence of sex differences in the infection susceptibility

to these parasites in avian hosts. Other studies have observed the same pattern in other bird species (Davidar & Morton 2006; Astudillo et al. 2013). These aspects are discussed in more detail below.

Host body condition and infection by filarioid nematodes

In our study area, blue tits with a lower body mass appear to be more likely to be parasitized by filarioid nematodes. We found a negative association between microfilariae infection status and host body mass — a proxy for body condition (Labocha & Hayes 2012) —although the relationship was not too pronounced (the body condition index models did not significantly differ from the null model, whereas body mass models did; Table 1). This same pattern has been observed in some wild bird populations, but not in others. For example, the intensity of infection by microfilaria is negatively correlated with host body mass in the white-necked thrush (*Turdus albicollis*) (de la Torre et al. 2020), while village weavers (*Ploceus cucullatus*) infected by microfilariae show a lower body mass compared to uninfected individuals (Atawal et al. 2019). In contrast, no association between host body mass or condition index and microfilarial infection status has been reported in *Aimophila* sparrows (Deviche et al. 2005), nor in some insular birds, such as Galápagos penguins (*Spheniscus mendiculus*) or flightless cormorants (*Phalacrocorax harrisi*) (Merkel et al. 2007). Even a positive relationship between microfilariae infection status and host body mass has been observed in the fire-crested alethe (*Alethe diademata*) (Sehgal et al. 2005b). Moreover, other studies have revealed complex relationships between filarial infections and host immune system and blood physiology. For example, New Caledonian *Zosterops* spp. individuals exhibit elevated heterophil to lymphocyte ratios when infected by microfilariae, mainly because these parasites provoked a proliferation of heterophils in the bloodstream (Clark et al. 2016), but no alterations of the heterophil to lymphocyte ratio have been observed in the white-necked thrush (de la Torre et al. 2020). Furthermore, microfilariae may diminish the erythrocytic sedimentation rate (an indirect indicator of immunocompetence; Valera et al. 2006) and increase the blood packed cell volume (Travis et al. 2006b), but by contrast, these parasites may not alter other measures of blood physiology, such as hematocrit (Valera et al. 2006; Atawal et al. 2019), serum biochemistry (Travis et al. 2006a), or polychromasia levels (Astudillo et al. 2013). Thus, the potential alteration of the blood homeostasis could enhance a mobilization of host nutrient stores, causing

microfilariae to parasitize and successfully develop in birds with a lower body mass or poorer condition. However, further experimental studies are necessary to clarify the observed associations between filarial infection and variation in host body mass, as positive and negative correlations have been reported in several filarioid–bird systems (see above).

Altogether, the aforementioned studies suggest that filarioid nematodes commonly establish complex relationships with their hosts, not always negatively affecting host energy stores or blood physiology. Nevertheless, these parasites may sometimes exert a cost to wild birds in the same way that other well-studied blood parasites do (e.g. avian malaria and avian malaria-like parasites; Merino et al. 2000; Marzal et al. 2005; Christe et al. 2012), especially to those individuals with a poorer body condition, which may be more likely to be parasitized by filarioids (Atawal et al. 2019; this study) or harbor a greater number of microfilariae in their bloodstream (de la Torre et al. 2020). Thereby, parasitized birds in poor body condition could be impaired, not only because of the direct impacts of filarioids, but also in terms of the increased risk of co-infection, as nematode-induced immune modulation may facilitate malaria co-infections (Druilhe et al. 2005; Clark et al. 2016). This could have negative consequences for such birds relative to blood-protein physiology (Merino et al. 2002) or future survival prospects (Davidar & Morton 2006) when being co-infected with avian malaria or avian malaria-like parasites, but further studies are necessary to further understand this possibility.

Molecular characterization of filarioids infecting blue tits

The sequencing of a 900 bp *COI* section of eight individuals revealed the presence of five different Onchocercidae lineages. Four of the lineages clustered in the *Splendidofilaria* clade, but only one lineage could be attributed to a known species, *Splendidofilaria mavis*. The remaining lineage clustered in an Onchocercidae lineage that could not be clearly assigned to any genus. Although, to date, few studies have addressed the molecular characterization of filarioid nematodes infecting wild birds, as the lineages they found (based on *18S* rRNA, *28S* rRNA, and *COI* fragments) are in line with the results of the present study. For example, Hamer et al. (2013) found two major clades of filarioids, one belonging to the putative *Chandlerella quiscali* and the second to *Splendidofilaria* spp., infecting several passerine species. In fact, four out

of five lineages found here clustered in the *Splendidofilaria* clade (Figure 2A), while the remaining lineage was genetically close to *C. quiscali* (Figure 2B). In addition, *S. mavis* has been detected in blackbirds and song thrushes from Lithuania (Chagas et al. 2021), and indeed we found *S. mavis* in two of the infected blue tits. Apart from *S. mavis*, the only other molecularly known species from this genus is *S. bartletti*, which was detected in Eurasian blackcaps (*Sylvia atricapilla*) (Binkienė et al. 2021). This study also identified and discovered new filarioid species (*Eufilaria acrocephalusi*, *E. sylviae* and *S. bartletti*) infecting common reed warblers (*Acrocephalus scirpaceus*), garden warblers (*Sylvia borin*), and Eurasian blackcaps (Binkienė et al. 2021). Overall, the results suggest that blue tits harbor a relatively large diversity of filarioid nematode lineages, and, to our knowledge, this is the first study examining and identifying the filarioid nematodes infecting blue tits. Still, lineages need to be linked to morphospecies, and our study call for further genetic studies of this group of parasites.

Prevalence of filarioids unaffected by habitat type and host sex

We also found that the probability of infection by microfilariae did not vary with the forest type. Other studies have shown that habitat characteristics may have important implications for the prevalence of filarioids in bird populations. For example, the occurrence of microfilariae is greater in dry forests than in humid forests (Savage et al. 2009), and in insular systems, the presence of microfilaria typically vary according to several environmental factors, such as ambient temperature, precipitation, or vegetation quality (Siers et al. 2010), leading to an island-dependent mosaic distribution (Clark et al. 2016). A forest-dependent probability of infection by microfilaria was expected in our study area, as blood parasites (especially vector-borne parasites) are strongly driven by environmental factors, which can limit vector distribution (Sehgal 2015; Ferraguti et al. 2018). Both parts of the woodland (eastern and western) from our study area differed in several abiotic and biotic factors (Garrido-Bautista et al. 2021, 2022b), and in fact, we have previously shown that biting midge (*Culicoides* spp.) abundance within blue tit nest boxes was higher in the dry, western woodland, than in the humid, eastern woodland (Garrido-Bautista et al. 2022a). Additionally, the prevalence of black flies (*Simulium* spp.) in our study area tended to be higher in nest boxes from the humid woodland than in the dry woodland (unpublished data). As both haematophagous arthropod groups are competent

vectors for avian filarioids (Bartlett 2008), we expected some forest-dependent variation in the occurrence of microfilariae infecting adult blue tits. The obtained results are attributable to two possibilities: (1) filarioid occurrence developing in each vector group (biting midges and black flies) did not show any forest-dependent variation, or (2) the prevalence of filarioids was similar in biting midges and in black flies, leading to the observed prevalence in the present study, as biting midges were more abundant in the dry forests, while black flies tended to be more abundant in the humid forests (see above). However, both possibilities assume a similar vector competence or transmission efficiency across the woodland. Further studies should be conducted in order to identify the presence of filarioid nematodes developing in both vector groups and whether biting midges and black flies could potentially transmit nematode immature stages to blue tits in a similar manner.

On the other hand, several studies have reported a clear altitudinal pattern in the presence of microfilaria infecting wild birds across the globe, with birds from highland zones having the lowest prevalences. For example, in the Neotropical region, the probability of filarioid infection is higher in birds from lowland humid forests than in highland forests (de la Torre & Campião 2021), with microfilariae being absent from elevations of 3.000 m (González et al. 2014). In Central Africa and Madagascar, the filarioid prevalence is also negatively correlated with elevation, with microfilariae not being present in highland mountain forests (Sehgal et al. 2005b; Savage et al. 2009). Lastly, in the Galápagos islands, a negative association between microfilariae prevalence and elevation has also been reported for some avian species (Siers et al. 2010). These studies are in accordance with the general assumption that haemoparasite prevalence decreases with elevation (Zamora-Vilchis et al. 2012), but interestingly, we found a relatively high microfilariae prevalence (9.4%) in a Mediterranean mountain woodland located at ca. 1.800 m a.s.l. — an altitude point in which the prevalence of filarioids is typically less than 3% or even zero (Savage et al. 2009; González et al. 2014). However, elevational patterns of parasites infecting birds may be complex depending on the parasite group (Rooyen et al. 2013) or the mountain system studied (Illera et al. 2017). Concretely, in the Sierra Nevada mountain range, the abundance and prevalence of ectoparasites, vectors, and haemoparasites may decrease, increase, or be stable with elevation depending on the taxa (Illera et al. 2017; Álvarez-Ruiz et al. 2018; Moreno-Rueda 2021). Thus, in Sierra Nevada, the relatively

high microfilariae prevalence is likely brought about by Mediterranean climatic particularities.

Finally, we did not find evidence to suggest that host sex affected the probability of infection by microfilariae, contrasting with the general acceptance that males are more susceptible to parasites and diseases than females, mainly due to physiological causes (e.g. interaction between immune system and testosterone; reviewed in Zuk & McKean 1996; Møller et al. 1999). However, in parasite–host systems formed by filarioids and birds, no differences in the parasite's susceptibility between sexes have been reported in a wide variety of bird species, such as the purple martin (*Progne subis*) (Davidar & Morton 2006), the Northern cardinal (*Cardinalis cardinalis*) (Astudillo et al. 2013), the Galápagos cormorant (*Phalacrocorax harrisi*) (Merkel et al. 2007), the village weaver (*Ploceus cucullatus*) (Atawal et al. 2019), or the blue tit (in this study). Moreover, most of these bird species exhibit moderate to high degrees of sexual dichromatism, with males being typically brighter and more colored than females. Because the expression of secondary sexual characters is related to infection susceptibility and immune function (Møller et al. 1999), males are expected to suffer more from parasitism (Hamilton & Zuk 1982). However, this scenario has not been observed in the present study, nor in other filarioid–bird systems worldwide. The non-generalized immune costs or immune-cell overproduction associated with filarioid parasitization (see above) may explain why most of studies did not find sex differences in microfilariae infection rates.

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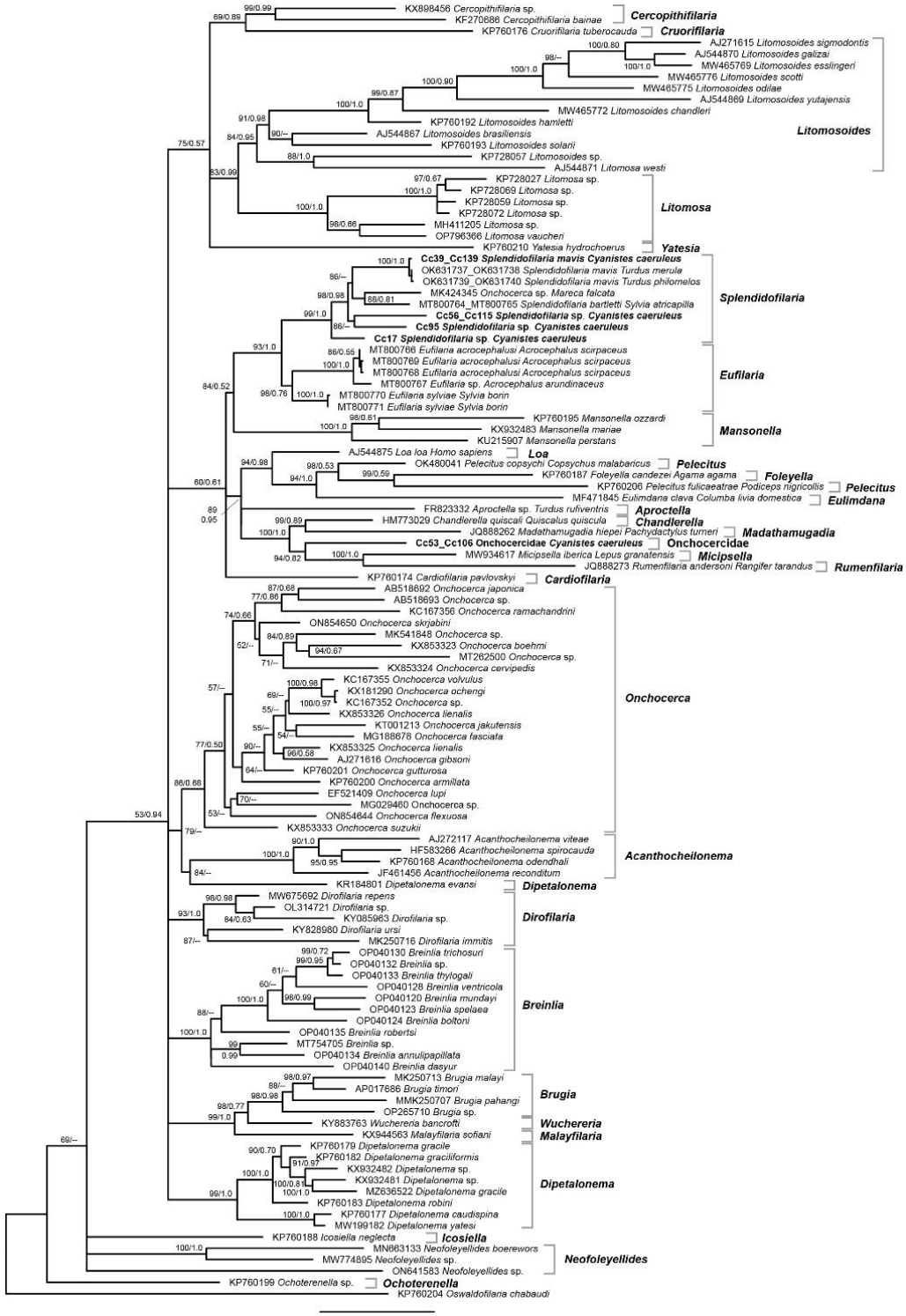
Supplementary material

Figure S1. Clades of maximum likelihood tree based on a 600 pb section of the *COI* gene, including representatives of all Onchocercidae species. Maximum likelihood bootstrap values and Bayesian posterior probabilities are indicated at all nodes. The scale bar indicates the expected mean number of substitutions per site according to the model of sequence evolution applied (continued in the next page).


KX898456 Cercopithifilaria sp.
KF270686 Cercopithifilaria bainae
Cercopithifilaria
KP760176 Cruorifilaria tuberocauda
Cruorifilaria
AJ271615 Litomosoides sigmodontis
AJ544870 Litomosoides galizai
MW465769 Litomosoides esslingeri
MW465776 Litomosoides scotti
MW465775 Litomosoides odilae
AJ544869 Litomosoides yutajensis
MW465772 Litomosoides chandleri
KP760192 Litomosoides hamletti
AJ544867 Litomosoides brasiliensis
KP760193 Litomosoides solarii
KP728057 Litomosoides sp.
AJ544871 Litomosa westi
Litomosoides
KP728027 Litomosa sp.
KP728069 Litomosa sp.
KP728059 Litomosa sp.
KP728072 Litomosa sp.
MH411205 Litomosa sp.
OP796366 Litomosa vaucheri
Litomosa
KP760210 Yatesia hydrochoerus
Yatesia
Cc39_Cc139 Splendidofilaria mavis Cyanistes caeruleus
OK631737_OK631738 Splendidofilaria mavis Turdus merula
OK631739_OK631740 Splendidofilaria mavis Turdus philomelos
MK424345 Onchocerca sp. Mareca falcata
MT800764_MT800765 Splendidofilaria bartletti Sylvia atricapilla
Cc56_Cc115 Splendidofilaria sp. Cyanistes caeruleus
Cc95 Splendidofilaria sp. Cyanistes caeruleus
Cc17 Splendidofilaria sp. Cyanistes caeruleus
Splendidofilaria
MT800766 Eufilaria acrocephalusi Acrocephalus scirpaceus
MT800769 Eufilaria acrocephalusi Acrocephalus scirpaceus
MT800768 Eufilaria acrocephalusi Acrocephalus scirpaceus
MT800767 Eufilaria sp. Acrocephalus arundinaceus
MT800770 Eufilaria sylviae Sylvia borin
MT800771 Eufilaria sylviae Sylvia borin
Eufilaria
KP760195 Mansonella ozzardi
KX932483 Mansonella mariae
KU215907 Mansonella perstans
Mansonella
AJ544875 Loa loa Homo sapiens
Loa
OK480041 Pelecitus copsychi Copsychus malabaricus
Pelecitus
KP760187 Foleyella candezei Agama agama
Foleyella
KP760206 Pelecitus fulicaeatrae Podiceps nigricollis
Pelecitus
MF471845 Eulimdana clava Columba livia domestica
Eulimdana
FR823332 Aproctella sp. Turdus rufiventris
Aproctella
HM773029 Chandlerella quiscali Quiscalus quiscula
Chandlerella
JQ888262 Madathamugadia hiepei Pachydactylus tumeri
Madathamugadia
Cc53_Cc106 Onchocercidae Cyanistes caeruleus
Onchocercidae
MW934617 Micipsella iberica Lepus granatensis
Micipsella
JQ888273 Rumenfilaria andersoni Rangifer tarandus
Rumenfilaria
KP760174 Cardiofilaria pavlovskyi
Cardiofilaria
AB518692 Onchocerca japonica
AB518693 Onchocerca sp.
KC167356 Onchocerca ramachandrini
ON854650 Onchocerca skrjabini
MK541848 Onchocerca sp.
KX853323 Onchocerca boehmi
MT262500 Onchocerca sp.
KX853324 Onchocerca cervipedis
KC167355 Onchocerca volvulus
KX181290 Onchocerca ochengi
KC167352 Onchocerca sp.
KX853326 Onchocerca lienalis
KT001213 Onchocerca jakutensis
MG188678 Onchocerca fasciata
KX853325 Onchocerca lienalis
AJ271616 Onchocerca gibsoni
KP760201 Onchocerca gutturosa
KP760200 Onchocerca armillata
EF521409 Onchocerca lupi
MG029460 Onchocerca sp.
ON854644 Onchocerca flexuosa
KX853333 Onchocerca suzukii
Onchocerca
AJ272117 Acanthocheilonema viteae
HF583266 Acanthocheilonema spirocauda
KP760168 Acanthocheilonema odendhali
JF461456 Acanthocheilonema reconditum
Acanthocheilonema
KR184801 Dipetalonema evansi
Dipetalonema
MW675692 Dirofilaria repens
OL314721 Dirofilaria sp.
KY085963 Dirofilaria sp.
KY828980 Dirofilaria ursi
MK250716 Dirofilaria immitis
Dirofilaria
OP040130 Breinlia trichosuri
OP040132 Breinlia sp.
OP040133 Breinlia thylogali
OP040128 Breinlia ventricola
OP040120 Breinlia mundayi
OP040123 Breinlia spelaea
OP040124 Breinlia boltoni
OP040135 Breinlia robertsi
MT754705 Breinlia sp.
OP040134 Breinlia annulipapillata
OP040140 Breinlia dasyur
Breinlia
MK250713 Brugia malayi
AP017686 Brugia timori
MMK250707 Brugia pahangi
OP265710 Brugia sp.
Brugia
KY883763 Wuchereria bancrofti
Wuchereria
KX944563 Malayfilaria sofiani
Malayfilaria
KP760179 Dipetalonema gracile
KP760182 Dipetalonema graciliformis
KX932482 Dipetalonema sp.
KX932481 Dipetalonema sp.
MZ636522 Dipetalonema gracile
KP760183 Dipetalonema robini
KP760177 Dipetalonema caudispina
MW199182 Dipetalonema yatesi
Dipetalonema
KP760188 Icosiella neglecta
Icosiella
MN663133 Neofoleyellides boerewors
MW774895 Neofoleyellides sp.
ON641583 Neofoleyellides sp.
Neofoleyellides
KP760199 Ochoterenella sp.
Ochoterenella
KP760204 Oswaldofilaria chabaudi
0.2

Chapter 4

Oxidative status of blue tit nestlings varies with habitat and nestling size

ABSTRACT Oxidative status has been proposed as an important ecological and evolutionary force given that pro-oxidant metabolites damage molecules, cells and tissues, with fitness consequences for organisms. Consequently, organisms usually face a trade-off between regulating their oxidative status and other physiological traits. However, environmental stressors and the availability of dietary-derived antioxidants vary according to local conditions and, thus, organisms inhabiting different habitats face different oxidative pressures. Still, there is little information on how different environmental conditions influence the oxidative status of animals inhabiting terrestrial environments. In this work, we examined the variation in oxidative status in the blue tit (*Cyanistes caeruleus*), a bird species with hatching asynchrony. Specifically, we examined the oxidative status of the largest and the smallest nestlings in the brood, inhabiting four forests differing in food availability and ectoparasite prevalence. We measured lipid peroxidation (malondialdehyde) as a marker of oxidative damage, total antioxidant capacity (Trolox-equivalent antioxidant capacity) and antioxidant enzymatic activity (catalase, glutathione S-transferase, glutathione peroxidase) in blood samples. The glutathione peroxidase activity differed among the forests, being the highest in the pine forest and the lowest in a mixed oak (*Quercus*) forest in the most humid area. Lipid peroxidation was higher in larger nestlings, suggesting higher oxidative damage with an increasing growth rate. Neither brood size, laying date, nor ectoparasites were related to the oxidative status of nestlings. These results suggest that nest rearing conditions might shape the oxidative status of birds, having consequences for habitat-dependent variation in regulation of oxidative status.

Keywords: Antioxidants, blue tit, local environment, nestlings, oxidative stress

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Introduction

Oxidative stress is defined as the imbalance between the production of pro-oxidant substances and antioxidant defences in favour of the former (Costantini 2014). Pro-oxidant molecules comprise reactive oxygen species (ROS), including both radical and non-radical species (Jones 2008), which react with different biomolecules in the cell, such as proteins, lipids, and nucleic acids, typically provoking cellular damage (Halliwell 2007). To counteract the oxidative damage produced by ROS, organisms possess several antioxidant defences that can be produced endogenously (e.g. glutathione or antioxidant enzymes) or can be dietary-derived (e.g. polyphenols, carotenoids, or vitamins C and E) (Costantini et al. 2010). These antioxidant defences balance the ROS concentration maintaining the oxidative status of the organism in equilibrium. However, when the oxidative status is altered and the antioxidant system is unable to effectively counteract the oxidative damage, the organism experiences oxidative stress (Jones 2008). To regulate the oxidative status is important for fitness (Costantini 2019), but, typically, organisms face a trade-off between combating oxidative stress and investing in other physiological traits (Monaghan et al. 2009; Metcalfe & Alonso-Álvarez 2010).

ROS are mainly produced by aerobic metabolism and the immune system, so ecological situations in which organisms should raise their metabolism or mounting an immune response typically conduce to an increased ROS production. Hence, ROS production is expected to augment when organisms deploy aggressive behaviours (Mentesana & Adreani 2021) or an immune response (Costantini & Møller 2009). The capacity of organisms to counteract ROS production depends on the endogenous defences, such as antioxidant enzymes, and the acquisition of dietary-derived antioxidants. In this way, organisms suffering a shortage of exogenous antioxidants might show a perturbed oxidative status, in the form of increased oxidative damage or of up-regulated endogenous antioxidant defences.

In nestling birds, the oxidative status may be modulated by several environmental factors. Nest-dwelling ectoparasites result in costs for nestlings in terms of diminished growth, condition, or haematocrit (e.g. Hurtrez-Boussès et al. 1997; Pitala et al. 2009; Brommer et al. 2011), but ectoparasites also may expose chicks to an oxidative challenge (Hanssen et al. 2013; López-Arrabé et al. 2015). Ectoparasites typically provoke an inflammatory immune response, with a concomitant rising in ROS

production (Sorci & Faivre 2009). Moreover, parasitism stimulates the hypothalamic–pituitary–adrenal axis, resulting in an elevated metabolism and so an increase of pro-oxidants (Beaulieu & Costantini 2014).

Brood size and nestling rank position within the nest also alter the oxidative status of chicks. In some bird species, the larger the brood size, the higher the oxidative stress suffered by nestlings, probably because of competition for food resources (Costantini et al. 2006; Bourgeon et al. 2011). In asynchronous hatched birds, size hierarchies among nestlings are commonly established, implying the existence of marginal (smaller) and core (larger) nestlings within broods (Forbes et al. 1997). In these broods, parents typically feed more frequently core nestlings than marginal ones (Moreno-Rueda et al. 2007); consequently, core nestlings grow faster. Given that accelerated growth in nestlings weakens the antioxidant capacity (Alonso-Álvarez et al. 2007) and increases the oxidative damage (Hall et al. 2010; Moreno-Rueda et al. 2012; Stier et al. 2014), core nestlings, or those nestlings growing larger body sizes, might suffer an unbalanced oxidative status.

Laying date also may affect nestling oxidative status. Environmental conditions tend to be increasingly severe as the breeding season progresses (Verhulst & Nilsson 2008). Consequently, one may expect increased oxidative stress with the advance of the laying date, mainly due to diminished food availability and so in the intake of dietary-derived antioxidants. The effect of laying date on nestling oxidative status seems complex; some studies report changes in antioxidants with laying date (Norte et al. 2009a; López-Arrabé et al. 2016), but these changes may be year-dependent (Losdat et al. 2010, Losdat et al. 2011) or even habitat-dependent (Salmón et al. 2018).

Moreover, inter-habitat variation in oxidative status is expected, especially if habitats differ in food availability, particularly in dietary antioxidants. For example, great tit (*Parus major*) nestlings present higher endogenous antioxidants in habitats with lower availability of antioxidants (Salmón et al. 2018). Similar findings have been reported in adult great tits, which show higher plasmatic concentrations of glutathione (an endogenous antioxidant) when inhabiting forests poor in dietary antioxidants (Isaksson 2013). In adult Seychelles warblers (*Acrocephalus sechellensis*), birds harbour more pro-oxidant metabolites when inhabiting territories with low food availability, presumably as a consequence of increased physical effort deploy for foraging (van de Crommenacker et al. 2011).

Hence, the local environment and nest rearing conditions alter the oxidative status of nestling birds in different ways. Indeed, cross-fostering experiments in wild birds have found that both genetics and the environment can explain the variation in ROS and antioxidant capacity in nestling birds (Costantini & Dell'Omo 2006; Norte et al. 2009b; Kim et al. 2010; Losdat et al. 2014).

In this work, we examine how oxidative damage and antioxidant enzymatic and non-enzymatic capacity vary in blue tit (*Cyanistes caeruleus*) nestlings inhabiting four different forests in southern Spain and, therefore, facing distinct environmental conditions. The four forests are grouped into two main areas consisting of: (1) a dry area where two forests are situated, one comprising holm oaks (*Quercus ilex*) and another of Pyrenean oaks (*Quercus pyrenaica*); and (2) a humid area containing the remaining two forests, one of Scots pine (*Pinus sylvestris*) and another mixed woodland with holm oaks and Pyrenean oaks. In the humid area, there is a river (Río Chico) crossing the Scots pine forest and a stream (Acequia Almiar) that cross both the Scots pine and mixed forests (Fig. 1). Compared to the dry area, the humid area has a lower mean temperature, higher humidity, lower irradiation and insolation time, more canopy cover, and a higher prevalence of certain ectoparasites (supplementary material). Moreover, some forests also differ in food availability, in terms of caterpillar abundance (supplementary material).

We predicted that: (1) If ectoparasites alter the oxidative status of nestlings (Hanssen et al. 2013; López-Arrabé et al. 2015), blue tit nestlings from nests with a higher prevalence of ectoparasites will have higher oxidative damage and/or a reduction in enzymatic and non-enzymatic antioxidant levels. (2) If sibling competition for food resources and large broods increase pro-oxidants production (Costantini et al. 2006; Bourgeon et al. 2011), blue tit nestlings from larger broods will suffer more oxidative damage or lower antioxidant defence than those from smaller broods. (3) Given that core nestlings receive more food than their marginal siblings and invest the extra food in growth (Hall et al. 2010), larger blue tit nestlings are expected to suffer more from oxidative damage, or to show reduced antioxidant defences, than smaller nestlings. (4) If environmental conditions tend to be more severe as the season progresses, a negative correlation between laying date and antioxidants in blue tit nestlings is expected (Norte et al. 2009b). (5) If the various forests involve different selective and pro-oxidant pressures for the blue tit (e.g. food

availability, ectoparasites, etc.), the oxidative status of nestlings will differ according to forest type. Nestlings inhabiting poor-quality habitats are expected to upregulate some antioxidant enzymes as dietary-derived antioxidants are scarce (Monaghan et al. 2009).

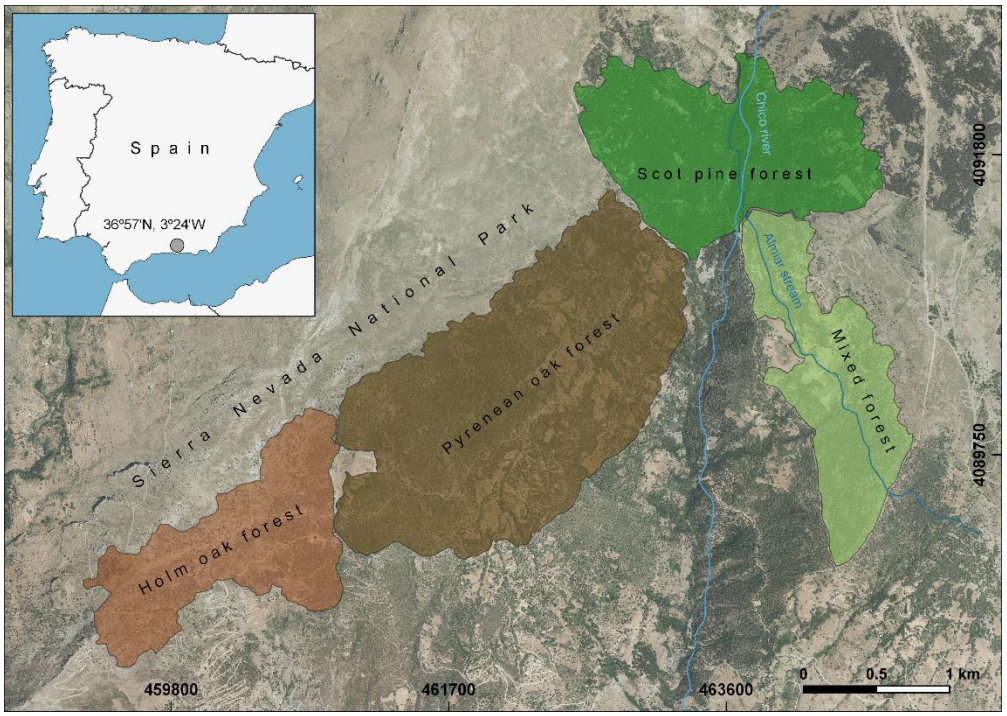

Figure 1. Location of the four forests (holm oak, Pyrenean oak, Scots pine, mixed) where sampling was performed (Sierra Nevada National Park, southern Spain). Notice the presence of a river and a stream crossing the humid area, comprising the Scots pine and mixed forests.

Because oxidative stress increases unevenly according to the characteristics of a particular ROS (Halliwell & Gutteridge 1995), and there is no single biomarker for oxidative stress (Monaghan et al. 2009; Hõrak & Cohen 2010), we measured oxidative damage, total antioxidant capacity, and the activity of various antioxidant enzymes. To do this, we quantified the oxidative damage in blood, based on the levels of malondialdehyde (MDA), the main product of lipid peroxidation (Hõrak & Cohen 2010), and we examined the Trolox-equivalent antioxidant capacity (TEAC), a parameter that assesses the cumulative action of all the antioxidants present in

plasma (Somogyi et al. 2007). Also, we examined the activity of several antioxidant enzymes: catalase (CAT), glutathione peroxidase (GPX) and glutathione S-transferase (GST). The activity of these antioxidant enzymes in the blood, and also MDA levels, reflects their level in other tissues, hence they can be considered good indicators of the oxidative status of an individual (Margaritelis et al. 2015).

Materials and methods

Study area and sampling

The blue tit is an insectivorous forest passerine inhabiting the western Palearctic, widely distributed across the Iberian Peninsula (Salvador 2016). Clutch size is, on average, 6–9 in this geographic area (Salvador 2016). The eggs typically hatch asynchronously, leading to a size hierarchy within the brood and resulting in a brood comprising core and marginal nestlings (Stenning 2018). Sampling was performed in 2017 in four different forests located between 1700 and 1800 m a.s.l. (meters above sea level) in the Sierra Nevada National Park (southern Spain, 36°57′N; 3°24′W) (see Introduction). In our study area, the blue tit breeding season begins in April, when birds start to build their nests, and ends in July, when the last nestling leave the nest. We used nest boxes (ICONA C model) with the following characteristics: basal area, 196 cm^2; height, 20 cm; hole diameter, 3 cm; material, wood with outer plastic paint layer (more details in Moreno-Rueda 2003). The nest boxes were hung from a tree branch attached to a metal hook and placed at 100 m intervals in each forest. We determined hatching day and brood size by inspecting the nest boxes.

In total, we sampled 35 nest-boxes: 7 from the holm oak forest, 15 from the Pyrenean oak forest, 8 from the Scots pine forest, and 5 from the mixed forest. For each brood, we weighed and measured the nestlings when they were 13 days old (day 0 = hatching day), the age when the body mass of blue tits reaches asymptotic growth (Björklund 1996). We used a digital portable scale (accurate to 0.1 g) and identified the largest and the smallest nestling within the brood. We also measured the tarsus length of nestlings using a digital calliper (accurate to 0.01 mm). Also when 13 days old, we took a 100 μL sample of blood (approximately 1% of nestling body mass) from the jugular vein of the nestlings within each nest, using disinfected and heparinised insulin syringes (following Owen 2011). This quantity of blood has been shown to have

a negligible effect on tit nestling survival (review in Sheldon et al. 2008). The handling time was also minimised, as far as possible (always less than 1 min), to reduce nestling stress and avoid possible artefacts in subsequent biochemical analyses (de Jong 2019). We took the blood samples in the field, preserved the samples in a portable fridge, and then transported them to the laboratory (the maximum time delay was 2 h from the time of blood collection).

Once the fledglings had left their nests, we carefully revised the nest material searching for nest-dwelling ectoparasites. We recorded the presence or absence of puparia and larvae of the blowfly *Protocalliphora azurea* in each nest, as well the presence or absence of hen flea (*Ceratophyllus gallinae*) adults and larvae. *P. azurea* larvae parasitise nestlings while in their nests (Bennett & Whitworth 1991), and their blood-sucking feeding negatively affects the growth rate and body condition of blue tit nestlings (Hurtrez-Boussès et al. 1997). The adults of *C. gallinae* take blood from both adult birds and nestlings, and this haematophagous activity reduces the haematocrit, feather growth, body condition and immune response of blue tit nestlings (Tripet & Richner 1997; Pitala et al. 2009; Brommer et al. 2011).

Oxidative stress analyses

Blood samples were mixed with a cold buffered solution (20 mM Tris-HCl, 10% glycerol and 0.1% Triton X-100 (v/v), pH 8.0) in a ratio of 1:3 (v/v), frozen for 48 h at −80 °C (to break down cell membranes), and then centrifuged at 5000 *g* for 10 min at 4 °C in a Sigma 3 K30 centrifuge. The supernatant was distributed into aliquots of 100 µL and frozen at −80 °C until analysis. All the enzymatic assays were conducted at 25 ± 0.5 °C using a PowerWavex microplate scanning spectrophotometer (Bio-Tek Instruments, USA) in duplicate in 96-well microplates (UVStar®, Greiner Bio-One, Germany). All the enzymatic reactions were started by adding the supernatant, and all biochemical assays had their controls, which consisted of all components except the blood sample. The specific assay conditions were as described below.

Lipid peroxidation levels were determined following the thiobarbituric acid assay of Buege & Aust (1978), based on the MDA levels generated as the main product of lipid peroxidation. In the presence of thiobarbituric acid, MDA reacts to produce red thiobarbituric acid reactive substances (TBARS) that can be measured spectrophotochemically at 535 nm. The net absorbance (sample reaction – sample

blank) was converted to a MDA concentration from the corresponding standard curve (0–35 μM MDA). The measures were expressed as μM MDA. Although TBARS can react with other aldehydes, most of the chromogen formed is ascribed to the complex MDA-TBARS even when the MDA concentration in the sample is low (Gutteridge & Quinlan 1983). We added 0.01% w/v butylated hydroxytoluene to the reaction mixture to neutralize the possible aldehydes that can be generated during the heating procedure.

The total antioxidant capacity, assayed as TEAC, was measured according to Erel (2004). This assay consists of oxidising ABTS (2,2′-azinobis-3-ethylbenzothiazoline-6-sulphonic acid) with hydrogen peroxide (H_2O_2) in an acidic medium, where the change to an emerald-green colour is determined through spectrophotometry at 595 nm. For this purpose, the extract was added to a reaction mixture containing 0.35 M acetate buffer (pH 5.8), 1.3 mM ABTS, and 0.25 mM H_2O_2 in 4 mM acetate buffer (pH 3.6). To eliminate the interference of the colour of haemoglobin, for each sample we used a control consisting of the blood with the buffer. Antioxidant activity refers to the equivalent of a water-soluble analogue of vitamin E (Trolox, Sigma 23001-3) dissolved in phosphate buffer, 0.1 M, pH 7.4, and which was used as a standard. The results were expressed in terms of Trolox equivalent antioxidant capacity (μM).

Catalase (CAT; EC 1.11.1.6) activity was determined spectrophotochemically by measuring the decrease in the H_2O_2 concentration over a 3 min period at 240 nm, according to Aebi (1984). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0) and freshly prepared 10.6 mM H_2O_2.

Glutathione peroxidase (GPX; EC 1.11.1.9) activity was measured according to Flohé & Günzler (1984). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.1), 1 mM EDTA, 3.9 mM GSH, 3.9 mM sodium azide, 1 IU/mL glutathione reductase, 0.2 mM NADPH, and 0.05 mM cumene hydroperoxide. After the addition of cumene hydroperoxide, the NADPH consumption rate was determined spectrophotochemically at 340 nm.

Glutathione S-transferase (GST; EC 2.5.1.1.8) activity was determined following the method of Habig et al. (1974), but adapted to a microplate. A reaction mixture consisting of 0.1 M phosphate buffer (pH 6.5), 1.2 mM GSH, and 1.23 mM solution of 1-chloro-2,4-dinitrobenzene (CDNB) in ethanol, was prepared just before the assay.

GST activity was then measured spectrophotochemically at 340 nm by the formation of glutathione-CDNB-conjugate.

For all the enzymatic activities, one unit of activity is defined as the amount of enzyme required to transform 1 μmol of substrate/min under the described assay conditions. To estimate the specific enzyme activity, the haemoglobin levels in the extracts were determined using the Drabkin colorimetric method (Spinreact, Spain), with animal-origin haemoglobin (15 g/dL; Spinreact, Spain) as the standard. For all the biochemical variables, two measurements were taken from each aliquot, and the average of these was used in the subsequent statistical analyses. All the biochemical reagents, including substrates, coenzymes, and enzymes, were obtained from Roche (Mannheim, Germany), Sigma Aldrich Chemical Co. (USA), or Merck (Darmstadt, Germany).

Statistical analyses

We used Cleveland plots to check whether the oxidative status biomarkers (MDA, TEAC, CAT, GST and GPX) and haemoglobin concentrations had outliers, and we graphically inspected these for normality (following Zuur et al. 2010). Because none of the biomarkers followed a normal distribution, all of the variables were log-transformed. An outlier was detected for the GST activity of a larger nestling from the holm oak forest. The outlier was probably an artefact as it far exceeded the confidence interval at 95% for GST activity (CI-95% = 14.31–16.80 mU/mg Hb, n = 68; outlier = 74.10 mU/mg Hb). For this reason, we performed the analyses using both the original full dataset and the dataset without this outlier. Herein we report the results without the outlier, in which we have greater confidence.

To determine differences in laying date and brood size between the forests, we used a one way ANOVA test. To check for variability in the prevalence of blowflies and fleas with forest type, given that these variables are frequencies, we used the chi-squared test. We also used a Student's *t*-test to check for differences in laying date and brood size in nests that were infested and non-infested with blowflies and fleas. The correlation between the brood size and laying date was established using the Pearson product-moment correlation. We used Linear Models (LM) to check whether haemoglobin concentrations varied with forest type, nestling rank, laying date, brood size and the prevalence of blowflies and fleas. Because the haemoglobin concentration

did not differ between any of these variables (forest: $F_{3, 65} = 1.69$, $p = 0.177$; nestling rank: $F_{1, 67} = 0.21$, $p = 0.644$; laying date: $F_{1, 67} < 0.01$, $p = 0.994$; brood size: $F_{1, 67} = 1.03$, $p = 0.314$; blowflies: $F_{1, 67} = 0.40$, $p = 0.529$; fleas: $F_{1, 67} = 1.57$, $p = 0.215$), we used the specific enzymatic activity standardised to the total haemoglobin concentration for the subsequent statistical analyses.

To examine whether body mass and tarsus length of the larger and the smaller nestlings varied with forest type, we used Linear Mixed Effects Models of Restricted Maximum Likelihood (REML-LMM) (Zuur et al. 2009) with nest identity as a random factor, and nestling rank (larger versus smaller), forest type (Holm oak, Pyrenean oak, Scots pine, and mixed), and the interaction forest*nestling as predictors. We also used REML-LMMs to test for variation in all oxidative status biomarkers; for this, we first ran full models with the following structure: each biomarker was a dependent variable, the nest identity as a random factor, and forest, nestling rank, interaction forest*nestling, laying date, brood size, the prevalence of fleas, the prevalence of blowflies, tarsus length (log-transformed), and weight (log-transformed) as predictors. Results of full models are given in the supplementary material. Given that tarsus length and body mass were correlated ($r = 0.68$, $p < 0.001$), the simultaneous inclusion of the two variables in the same models could produce collinearity. For this reason, we calculated the Variance Inflation Factor (VIF) for each full model. VIF values were below 10, except for the interaction forest*nestling in a single full model.

To be sure that the results from full models remain robust and were not affected by problems of collinearity or overfitting, we also applied a model selection approach. To select the best models among all possible models, we used the Akaike information criterion (AIC), and we chose those models with a ΔAIC value under 2 (Quinn & Keough 2002). The normality and homoscedasticity of the model residuals were checked following Zuur et al. (2010). The basic statistics are given as mean ± s.e. (standard error). All the analyses were performed using the packages 'nlme' (Pinheiro et al. 2019) and 'MuMIn' (Bartoń 2020) in the R software environment, version 4.0.0. (R Development Core Team 2020).

Results

Descriptive statistics of the study system

Laying occurred between 6 and 24 May (average: 11 May ± 0.58 days). Brood size ranged from 2 to 8 nestlings (mean: 5.49 ± 0.19). Laying date and brood size did not significantly differ between forests (respectively, $F_{3, 31} = 2.59$, $p = 0.071$; $F_{3, 31} = 2.50$, $p = 0.078$). Brood size tended, although not significantly, to decrease with laying date ($r = -0.33$, $p = 0.056$). Eight out of 35 nests were infested with fleas, but this frequency did not differ with forest type ($\chi^2_3 = 1.22$, $p = 0.749$). Flea infestation did not differ with laying date or brood size (in the two cases, $|t_{33}| < 0.10$, $p > 0.90$). However, blowfly nest infestation differed significantly between forests ($\chi^2_3 = 12.89$, $p = 0.005$). All the nests sampled in the Scots pine (n = 8) and mixed (n = 5) forests were infested with blowflies, while 7 out of 15 nests in the Pyrenean oak forest and only 2 out of 7 nests in the holm oak forests were infested. Overall, 22 out of 35 nests were infested with blowflies. Blowfly infestation did not vary with laying date or brood size (in the two cases, $|t_{33}| \leq 1.20$, $p > 0.20$). The tarsus length of blue tit nestlings did not differ between the forests (REML-LMM; forest: $\chi^2_3 = 1.81$, $p = 0.61$; nestling rank (larger versus smaller): $\chi^2 = 7.79$, $p = 0.005$; interaction forest*nestling: $\chi^2_3 = 1.10$, $p = 0.78$). The body mass of blue tit nestlings neither differed among the forests (forest: $\chi^2_3 = 6.34$, $p = 0.09$; nestling rank: $\chi^2 = 22.01$, $p < 0.0001$; interaction forest*nestling: $\chi^2_3 = 3.74$, $p = 0.29$).

Biomarkers of oxidative stress

Table 1 summarises the MDA level, TEAC, the activity of the antioxidant enzymes and the haemoglobin concentrations in the larger and smaller nestlings in each of the four forests. The results of the model selection are summarised in Table 2. The best model for MDA included only tarsus length as a predictor variable, which positively correlated with MDA level ($\chi^2 = 7.80$, $p = 0.005$; Fig. 2). The second-best model included tarsus length and weight, but this was significantly worse ($\Delta AIC > 2$). For GPX activity, the best model included only forest type as a predictor ($\chi^2_3 = 29.60$, $p < 0.0001$). The other two models were not significantly worse ($\Delta AIC < 2$), but these included forest type together with tarsus length, and tarsus length plus weight as predictors.

Table 1. Lipid peroxidation level (MDA), total antioxidant capacity (TEAC), antioxidant enzymatic activity and haemoglobin concentration (Hb) for larger and smaller blue tit nestlings from the four forests. The table shows the mean values, standard error (s.e.) and sample size (n) in brackets.

	Holm oak forest		Pyrenean oak forest		Scots pine forest		Mixed forest	
	Larger	Smaller	Larger	Smaller	Larger	Smaller	Larger	Smaller
MDA (μM)	9.38 ± 1.59 (6)	8.06 ± 0.86 (7)	8.20 ± 0.41 (15)	7.14 ± 0.44 (15)	7.52 ± 0.88 (7)	7.04 ± 0.60 (8)	8.62 ± 1.55 (5)	5.86 ± 0.79 (5)
TEAC (μM eq. Trolox)	99.63 ± 17.41 (6)	105.51 ± 27.02 (7)	82.26 ± 14.31 (13)	76.65 ± 21.52 (13)	105.43 ± 16.72 (8)	102.05 ± 19.92 (8)	142.00 ± 30.20 (5)	107.55 ± 67.46 (4)
CAT (U/mg Hb)	14.48 ± 1.29 (6)	17.14 ± 2.45 (7)	21.45 ± 2.71 (15)	19.75 ± 1.03 (15)	16.05 ± 2.41 (8)	18.46 ± 1.53 (8)	17.36 ± 3.53 (5)	14.49 ± 1.39 (5)
GPX (mU/mg Hb)	24.13 ± 5.85 (2)	21.44 ± 6.49 (5)	23.74 ± 2.51 (13)	23.25 ± 2.72 (14)	39.37 ± 8.62 (7)	39.96 ± 8.06 (6)	8.53 ± 2.11 (4)	6.98 ± 0.78 (4)
GST (mU/mg Hb)	13.78 ± 1.18 (6)	16.94 ± 3.38 (6)	16.01 ± 1.57 (15)	16.90 ± 1.45 (15)	13.22 ± 1.00 (8)	14.76 ± 1.39 (8)	17.20 ± 2.37 (5)	13.93 ± 0.58 (5)
Hb (mg/mL)	21.78 ± 1.04 (6)	20.81 ± 2.29 (7)	21.72 ± 1.25 (15)	21.85 ± 1.13 (15)	21.30 ± 3.22 (8)	16.71 ± 1.42 (8)	18.66 ± 2.34 (5)	21.85 ± 1.48 (5)

MDA = malondialdehyde, TEAC = trolox-equivalent antioxidant capacity, CAT = catalase, GPX = glutathione peroxidase, GST = glutathione-S-transferase, Hb = haemoglobin.

Table 2. AIC values and AIC increment (ΔAIC) of the linear mixed models for oxidative status biomarkers, indicating the model predictors (nest identity was the random factor in all models). The significant predictors ($p < 0.05$) are marked in bold.

Variable	AIC	ΔAIC
MDA		
Log(tarsus)	25.95	0.00
TEAC		
Log(tarsus)	144.05	0.00
Log(tarsus), log(weight)	144.52	0.47
Log(tarsus), blowflies	145.80	1.75
Null model [a]	146.01	1.96
CAT		
Log(tarsus)	57.53	0.00
Null model [a]	57.96	0.43
GST		
Null model [a]	40.93	0.00
GPX		
Forest	87.56	0.00
Forest, log(tarsus)	87.64	0.07
Forest, log(tarsus), log(weight)	88.87	1.31

[a] Null model included only the intercept.

In these models, however, only forest type significantly affected GPX activity. Specifically, the GPX activity was less in the mixed forest, higher in the Scots pine forest, and intermediate in holm oak and Pyrenean oak forests (Fig. 3). Post-hoc analyses indicated that the GPX activity in the mixed forest differed significantly from the other forests (in all cases: $p < 0.05$). For the other dependent variables (TEAC, CAT and GST), no model differed significantly from the null model (in all cases: ΔAIC < 2).

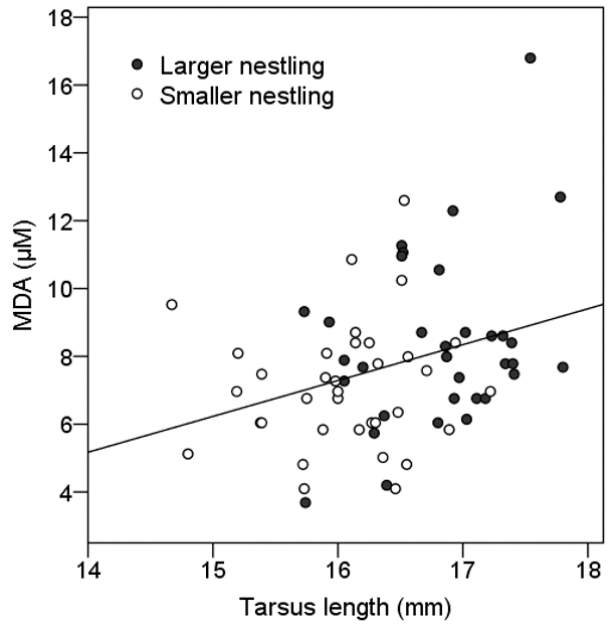

Figure 2. Relationship between tarsus length and lipid peroxidation level (MDA) in blue tit nestlings. Larger nestlings: filled circles; smaller nestlings: open circles.

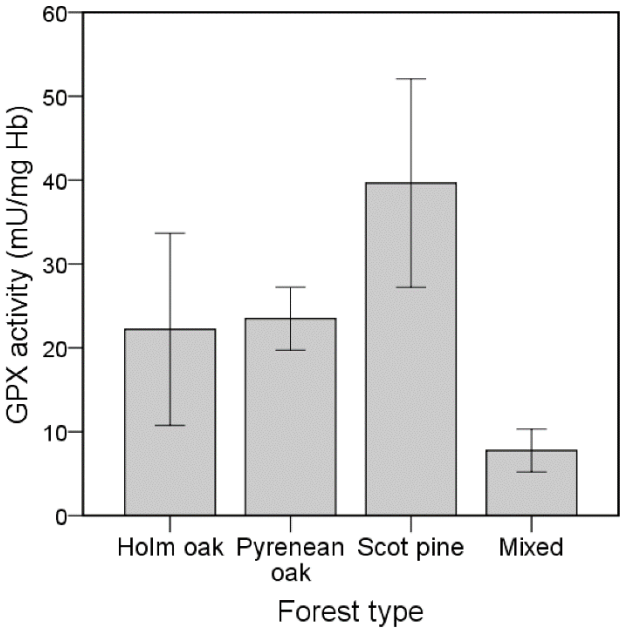

Figure 3. Mean values (showing the confidence interval 95%) of the glutathione peroxidase (GPX) activity in the blue tit nestlings from the four forests.

Discussion

Our results show that the glutathione peroxidase (GPX) activity differed among the forests, being the highest in the Scots pine forest, the lowest in the mixed forest, and intermediate in the dry zone forests, suggesting that habitat heterogeneity can shape the expression of some antioxidant enzymes, such as GPX (Norte et al. 2009a). Furthermore, the lipid peroxidation, a biomarker of oxidative damage, increased with nestling tarsus length. Larger nestlings suffered more oxidative damage, suggesting that this damage is a consequence of growth costs (see below). In contrast, although brood size, laying date, and ectoparasites have been shown to alter nestling oxidative status in other bird species (Costantini et al. 2006; Bourgeon et al. 2011; Hanssen et al. 2013; López-Arrabé et al. 2015), we did not find evidence that these factors can alter the oxidative status in blue tit nestlings.

Forest type modulates the GPX activity

The GPX activity varied between the forest types, being the lowest in the mixed forest and highest in the Scots pine forest, both located in the humid area, while intermediate values were found in the dry zone forests. Glutathione peroxidase is one of the main components of the cell enzymatic antioxidant system, which converts hydrogen peroxide to water using reduced glutathione (Costantini 2014). It has previously been shown that the activity of this enzyme varies relative to nest rearing conditions in great tit nestlings (Norte et al. 2009b). In the same way, adult great tits show greater concentration of glutathione (an endogenously synthetized metabolite that intervene in the antioxidant function of the GPX) in pine and larch forests than in oak forests (Isaksson 2013). In poor-quality habitats, with low food availability, the intake of dietary antioxidants is limited and birds are expected to respond by upregulating the production of endogenously derived antioxidants, such as GPX (Monaghan et al. 2009). This upregulation of GPX could explain the among-forest variation in its activity, as pine forests are typically poorer in antioxidants than oak forests (Tálos-Nebehaj et al. 2017).

We found that ectoparasites are not an important cause of the among-forest variation in the GPX activity of the blue tit nestlings in our population, since their antioxidant systems did not change in the presence of blowflies and fleas. Nevertheless, a greater infection by other parasites not examined in this study, such

as haemosporidians, may to have increased the GPX activity in nestlings from the Scots pine forest (de Angeli Dutra et al. 2017). Also, during an immune challenge, an organism can downregulate certain antioxidant enzymes, such as GPX, in order to keep the production of some ROS (e.g. hypochlorous acid) up, thereby increasing the effectiveness of the immune response (Costantini 2014). In other unpublished studies, we found that the blue tit nestlings from the humid zone were more probable to be parasitised by the haemosporidian *Leucocytozoon* and had more leucocytes than those from the dry area; therefore, it is plausible that GPX activity was downregulated in the mixed forest as a consequence of a greater immune challenge.

Being larger results in higher oxidative damage

We found that the larger the tarsus length, the higher the lipid peroxidation suffered by blue tit nestlings. The greater oxidative damage suffered by larger nestlings could be attributed to accelerated growth. A faster growth rate results in greater oxidative stress in terms of increased oxidative damage, but not in terms of reduced antioxidant levels (Smith et al. 2016). Concretely, in nestlings, an accelerated growth rate may provoke an increment in oxidative damage (Alonso-Álvarez et al. 2007; Costantini 2010; Moreno-Rueda et al. 2012; Stier et al. 2014). In asynchronously hatched blue tits, parents typically feed larger nestlings to the detriment of smaller nestlings (Dickens & Hartley 2007; García-Navas et al. 2014), meaning early-hatched nestlings grow faster than last-hatched nestlings (Björklund 1997). Furthermore, the extra resources provided by parents to core nestlings are invested in growth, rather than in reducing oxidative damage (Hall et al. 2010). Therefore, our findings suggest that an accelerated growth comes at the expense of an oxidative cost. Still, final tarsus length may determine survival prospects in blue tits (Charmantier et al. 2004), and so nestlings are probably selected for faster growth to attain a structurally large body size. Moreover, a reduction in the duration of the nestling period by an accelerated growth in birds is selected to escape both from parasites and predators (Møller 2005; Cheng and Martin 2012).

Nestling oxidative status unaffected by ectoparasites, brood size and laying date

We also found that nestling oxidative status did not vary with the prevalence of blowflies and hen fleas in the nest. These ectoparasites alter the expression of stress proteins and negatively impact haematocrit, body condition, immune response and growth rate of blue tit nestling (Hurtrez-Boussès et al. 1997; Tripet & Richner 1997; Arriero et al. 2008; Pitala et al. 2009; Brommer et al. 2011), hence oxidative stress was predicted to be higher in parasitised nestlings. However, studies examining the relationships between nest-dwelling ectoparasites and the oxidative status of nestlings suggest complex relationships, with ectoparasites altering the oxidative status of nestlings in various ways (Hanssen et al. 2013; López-Arrabé et al. 2015), although these do not always cause observable effects (de Coster et al. 2012; Maronde et al. 2018). Removing ectoparasites from raptor nests reduced plasma oxidants and led to a higher total antioxidant capacity in the chicks (Hanssen et al. 2013). In contrast, removing nest-dwelling ectoparasites provoked no change in MDA levels or total antioxidant capacity in pied flycatcher (*Ficedula hypoleuca*) nestlings, although the concentration of glutathione, an important non-enzymatic intracellular antioxidant, was higher when ectoparasites were absent (López-Arrabé et al. 2015). However, and in line with our results, experimental manipulation of flea infestations had no influence on oxidative status in great tit nestlings (Maronde et al. 2018). Highlighting the complexity of the interrelationships between oxidative status and ectoparasites, de Coster et al. (2012) found that maternal effects may reduce oxidative stress in great tit nestlings parasitised with fleas, but the effect was only found in female nestlings. The results of our study, together with those of the aforementioned studies, suggest that exposure to ectoparasites does not necessarily affect the oxidative status of nestlings, probably due to the complex interactions between parasite pressure, immune function, and the oxidative challenge.

Some studies examining the oxidative stress of nestlings in broods of different sizes, or which were experimentally enlarged or reduced, have found that nestling oxidative stress (i.e. higher oxidative damage or lower antioxidant defence) is greater in larger broods (Alonso-Álvarez et al. 2006; Costantini et al. 2006; Bourgeon et al. 2011). However, other studies have not found this pattern (Hall et al. 2010; Losdat et al. 2010; López-Arrabé et al. 2016; this study). The discrepancies between these

studies may be because it is difficult to determine to what degree the different behaviours and factors within the nests, including begging, scramble competition between nestlings, parental care, nestling growth rates, and quantity of food resources, contribute to modulate the oxidative status of nestlings. Moreover, most of these studies examined only one component of the redox system, which may not be sufficient to detect a significant increase in oxidative stress. Furthermore, inter-year variations in environmental quality can confound brood-size effects on nestling oxidative stress (Bourgeon et al. 2011). The oxidative costs of larger broods can be also masked by, for instance, the quality of the food provided by the parents, given that, with a high-quality diet, nestlings can allocate resources to both growth and antioxidant defences (Hall et al. 2010).

In our study population, we found no relationship between the laying date and nestling oxidative status. As the environmental temperature increases and food abundance normally decreases throughout the breeding season, it was predicted that the condition and physiology of the nestlings would be impaired. In Eurasian kestrel (*Falco tinnunculus*) nestlings, no relationship was found between oxidative stress and hatching date (Costantini et al. 2006). However, great tit nestlings can show a negative correlation between GPX activity and the hatching date (Norte et al. 2009b). In contrast, in other great tit populations, nestling resistance to oxidative stress (measured as enzymatic and non-enzymatic antioxidants combined) was found to increase with laying date in some years, although no relationship was evident in others (Losdat et al. 2010, Losdat et al. 2011). Similarly, a positive association between hatching date and non-enzymatic antioxidants was found in pied flycatcher nestlings (López-Arrabé et al. 2016). These results suggest that local environmental factors that fluctuate during the breeding season, including food abundance and temperature, influence the relationship between laying date and oxidative status in nestlings, in different ways, making it difficult to draw a general conclusion.

Conclusions

Although we are aware that determining the natural causes of variation in oxidative stress is complex, our findings suggest that the local environmental conditions where the nestlings develop, such as habitat type, shape some components of their oxidative status. Specifically, environmental conditions are responsible for the variation in the

expression of certain components of the antioxidant system of nestlings, such as GPX, which varies among forests. GPX activity was lowest in the mixed forest and highest in the Scots pine forest. Moreover, larger nestlings showed more oxidative damage than smaller nestlings, since reaching a larger tarsus length implied suffer more from lipid peroxidation, suggesting that faster growth involves an oxidative cost. This study, therefore, helps to improve our knowledge of the environmental causes of variation concerning some components of the oxidative status of birds, highlighting the importance of habitat heterogeneity in the physiology of organisms.

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Supplementary material 1

Table S1. Temperature, relative humidity, solar radiation, insolation, NDVI, caterpillar abundance and ectoparasite prevalence (fleas and blowflies) for the four forests of the study area. The table shows the mean values, standard deviation (except for prevalences), statistic and *p*-value.

Factor	Forest type				Statistic	*p*-value
	Holm oak forest	Pyrenean oak forest	Scots pine forest	Mixed forest		
Temperature (ºC) [1]	-	17.81 ± 6.47	-	16.80 ± 6.19	$t_{1338} = 14.11$	< 0.001
Relative humidity (%) [1]	-	41.37 ± 18.02	-	42.99 ± 17.90	$t_{1338} = 9.21$	< 0.001
Solar radiation (kWh/m²/day) [2]	6.46 ± 0.08	6.53 ± 0.09	6.34 ± 0.08	6.35 ± 0.08	$\chi^2_3 = 7.48$	0.058
Insolation (h) [3]	12.83 ± 0.32	13.46 ± 0.34	12.19 ± 0.26	11.98 ± 0.21	$\chi^2_3 = 62.48$	< 0.001
NDVI [4]	0.40 ± 0.02	0.56 ± 0.08	0.64 ± 0.04	0.60 ± 0.04	$\chi^2_3 = 26.06$	< 0.001
Caterpillar abundance (caterpillars/10 shots/4 m²) [5]	-	7.13 ± 8.18	-	12.87 ± 14.11	$\chi^2 = 47.90$	< 0.001
Flea prevalence [6]	0.14	0.20	0.40	0.25	$\chi^2_3 = 1.22$	0.749
Blowfly prevalence [6]	0.29	0.47	1.00	1.00	$\chi^2_3 = 12.89$	0.005

[1] Data obtained from dataloggers iButton® installed in outer walls of nest-boxes. Year: 2019. Statistic: *t*-test.

[2] Data obtained from software ArGis Desktop 10.3.1, using the "Area solar radiation" algorithm. Solar radiation obtained every five days for breeding seasons of 2016, 2017, 2018 and 2019. Statistic: Kruskal–Wallis test.

[3] Data obtained from software GRASS SIG 7.8.2, integrated in QGIS 3.10.5, using the "r.sun.insoltime" algorithm. Insolation obtained every five days and covering the breeding season of 2017. Statistic: Kruskal–Wallis test.

[4] Data obtained from software QGIS 3.10.5 using Landsat-8 satellite images covering the breeding seasons of 2016, 2017, 2018 and 2019. NDVI calculated based on Landsat-8 bands B4 (red) and B5 (near infrared) as: (B5 – B4) / (B5 + B4). Statistic: Kruskal–Wallis test.

[5] Data obtained from 2018 and 2019. The caterpillar abundance was calculated as the number of caterpillars per 10 shots to a branch, and collected in a 2 × 2 blanket. Statistic: GLM with a Poisson distribution and a logit-link function, with forest type as the predictor.

[6] Data from 2017. Statistic: chi-squared test.

Supplementary material 2

Table S2.1. Results of the full model for MDA, with log-transformed MDA level as the dependent variable and nest identity as the random factor.

	Wald χ^2	Degrees of freedom	*p*-value
Intercept	0.002	1	0.96
Forest	3.24	3	0.36
Nestling rank	0.71	1	0.40
Laying date	0.002	1	0.96
Fleas	0.83	1	0.36
Blowflies	0.08	1	0.77
Brood size	0.37	1	0.54
Log(tarsus)	2.81	1	0.09
Log(weight)	1.48	1	0.22
Forest*nestling	2.41	3	0.49

Table S2.2. Results of the full model for TEAC, with log-transformed TEAC as the dependent variable and nest identity as the random factor.

	Wald χ^2	Degrees of freedom	*p*-value
Intercept	0.02	1	0.90
Forest	2.14	3	0.54
Nestling rank	0.03	1	0.86
Laying date	0.02	1	0.89
Fleas	1.03	1	0.31
Blowflies	0.04	1	0.84
Brood size	0.02	1	0.88
Log(tarsus)	0.53	1	0.47
Log(weight)	0.03	1	0.86
Forest*nestling	1.22	3	0.75

Table S2.3. Results of the full model for CAT, with log-transformed CAT as the dependent variable and nest identity as the random factor. In bold those predictors that were significant ($p < 0.05$).

	Wald χ^2	Degrees of freedom	*p*-value
Intercept	0.51	1	0.48
Forest	5.57	3	0.13
Nestling rank	0.85	1	0.36
Laying date	0.50	1	0.48
Fleas	2.69	1	0.10
Blowflies	5.44	1	**0.02**
Brood size	1.59	1	0.21
Log(tarsus)	0.03	1	0.87
Log(weight)	0.19	1	0.66
Forest*nestling	2.07	3	0.56

Table S2.4. Results of the full model for GST, with log-transformed GST as the dependent variable and nest identity as the random factor.

	Wald χ^2	Degrees of freedom	*p*-value
Intercept	1.64	1	0.20
Forest	0.42	3	0.94
Nestling rank	0.66	1	0.42
Laying date	1.63	1	0.20
Fleas	0.74	1	0.39
Blowflies	0.82	1	0.37
Brood size	0.40	1	0.53
Log(tarsus)	0.03	1	0.86
Log(weight)	0.33	1	0.57
Forest*nestling	2.54	3	0.47

Table S2.5. Results of the full model for GPX, with GPX as the dependent variable and nest identity as the random factor. In bold those predictors that were significant ($p < 0.05$).

	Wald χ^2	Degrees of freedom	*p*-value
Intercept	0.02	1	0.89
Forest	16.55	3	**< 0.001**
Nestling rank	0.19	1	0.66
Laying date	0.02	1	0.90
Fleas	0.47	1	0.49
Blowflies	0.05	1	0.82
Brood size	0.06	1	0.81
Log(tarsus)	0.76	1	0.38
Log(weight)	< 0.001	1	0.97
Forest*nestling	0.06	3	0.99

Chapter 5

Within-brood body size and immunological differences in blue tit (*Cyanistes caeruleus*) nestlings relative to ectoparasitism

ABSTRACT Several ectoparasites parasitise nestlings decreasing their body condition, growth and survival. To minimise any loss of fitness due to ectoparasites, birds have developed a wide variety of defence mechanisms, potentially including hatching asynchrony. According to the Tasty Chick Hypothesis (TCH), the cost of parasitism would be reduced if ectoparasites tend to eat on less immunocompetent nestlings, typically the last-hatched chick in asynchronously hatched broods, as they are in poor body condition. Two predictions of the TCH are that immune capacity is lower in smaller nestlings than in larger ones and that parasites should provoke a more negative effect on smaller nestlings. Here, we test these predictions in a population of blue tits (*Cyanistes caeruleus*) whose broods are parasitised by hen fleas (*Ceratophyllus gallinae*) and blowflies (*Protocalliphora azurea*). We recorded the presence of both ectoparasites and analysed the immunocompetence (number of leucocytes per 10,000 erythrocytes and cutaneous immune response to PHA) and body condition of smaller and larger nestlings within individual broods. The leucocyte count was higher in smaller nestlings than in larger ones, whereas the cutaneous immune response did not differ between smaller and larger nestlings. Smaller nestlings, but not larger nestlings, had lower body mass when fleas were present. Blowflies, by contrast, had no detectable negative effect on nestlings. Overall, our findings provide partial support to the TCH. Lower immune capacity in smaller nestlings than in larger ones was not supported, but hen fleas seemed to negatively impact on smaller nestlings more than on larger ones.

Keywords: Blowflies, blue tit, *Cyanistes caeruleus*, ectoparasites, hen fleas, immunocompetence, Tasty Chick Hypothesis

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Introduction

Parasites take resources from their hosts and so typically reduce host fitness (Schmid-Hempel 2011). In birds, a range of arthropod ectoparasites, such as fleas, flies, bugs or mites, inhabiting the nest or nestling body are known to reduce chick health, body size and body mass (e.g. Richner et al. 1993; Merino & Potti 1995; Christe et al. 1996; Hurtrez-Boussès et al. 1997; Puchala 2004; Simon et al. 2004), sometimes provoking nestling death (reviewed in Møller et al. 2009). In addition, nest-dwelling ectoparasites may reduce future nestling survival (Brown & Brown 1986; Szép & Møller 2000; Streby et al. 2009), reproductive success (Fitze et al. 2004) or increase the costs of reproduction in parents (Richner & Tripet 1999).

To minimise the detrimental effects caused by nest ectoparasites, birds have developed a wide variety of defence mechanisms, including behavioural strategies (reviewed in Bush & Clayton 2018) and immunological responses (Davison et al. 2008; Owen et al. 2010; Schmid-Hempel 2011). Inflammation is a typical immune response to ectoparasite injuries, which damages parasite tissues and physically prevents parasites from reaching the host bloodstream (Owen et al. 2009). Therefore, inflammation reduces the blood meal consumed by the ectoparasite and, consequently, its fecundity and survival (Tschirren et al. 2007; Bize et al. 2008). However, the maintenance and deployment of immune defence mechanisms are energetically expensive and require limited nutrients such as amino acids (Lochmiller & Deerenberg 2000). As such, nestlings in better body condition are normally more immunocompetent (e.g. Saino et al. 1997; Brinkhof et al. 1999; Westneat et al. 2004; Dubiec et al. 2006; Martínez-de la Puente et al. 2013).

In altricial birds, nestlings often establish a body size hierarchy, mainly due to hatching asynchrony (Clark & Wilson 1981). Size hierarchy implies the nestlings have different reproductive values for parents, so individual broods contain both core high-value and marginal low-value nestlings (Forbes et al. 1997). This means food is distributed unequally among the nestlings, with small ones usually being underfed (Dickens & Hartley 2007; Moreno-Rueda et al. 2009; García-Navas et al. 2014). Several hypotheses have been proposed to explain the adaptive value of hatching asynchrony, mainly in relation to resource availability (Magrath 1990; Stenning 1996). An example is the Tasty Chick Hypothesis (TCH), which suggests that hatching asynchrony may have evolved as an anti-ectoparasite defence (Christe et al. 1998).

The TCH proposes that ectoparasites enhance their fitness by aggregating on the less immunocompetent nestling, which is presumed to be the last chick hatched in asynchronous broods and also the one with the poorest body condition due to a low nutritional status. Hence, the smallest nestling in a brood would be the most attractive (tasty) to parasites. This would have the concomitant effect of reducing the parasitic load for the remaining nestlings, which are of higher reproductive value for parents. According to the TCH, parental fitness would eventually increase with more healthy core nestlings surviving at the expense of sacrificing the highly parasitised last-hatched chick, the "tasty" chick (Christe et al. 1998). In sum, the TCH predicts that: (1) smaller nestlings are less immunocompetent against ectoparasites than larger nestlings; (2) parasites feed mainly on smaller nestlings; and (3) smaller nestlings are more negatively affected by ectoparasites than larger nestlings.

Although the results of some studies support the TCH (Simon et al. 2003; O'Connor et al. 2014), most do not (Descamps et al. 2002; Valera et al. 2004; Roulin et al. 2008; Václav et al. 2008; O'Brien & Dawson 2009) or provide only partial support (Roulin et al. 2003; Bize et al. 2008; Václav & Valera 2018). In some cases, contrasting results have even been reported for the same species of birds and parasites. For example, Descamps et al. (2002) did not find evidence supporting the TCH in blue tit (*Cyanistes caeruleus*) nestlings parasitised by *Protocalliphora* larvae. However, in the same population, Simon et al. (2003) reported that *Protocalliphora* larvae consumed more blood from smaller rather than larger nestlings. The blood-sucking fly *Carnus hemapterus* prefers to feed on larger European roller (*Coracias garrulous*) nestlings (Václav et al. 2008), but also selects less immunocompetent nestlings when hosts have poor body condition (Václav & Valera 2018).

The aim of the present study was to test the aforementioned predictions 1 and 3 of the TCH in a wild population of blue tits parasitised by blowfly larvae (*Protocalliphora azurea*) and hen fleas (*Ceratophyllus gallinae*). We did not test prediction 2 (parasites aggregate on the last-hatched nestling) given that these parasites inhabit inside the nest material, feeding on nestlings usually at night. According to the TCH, we predict that smaller nestlings in the same brood will present lower immunocompetence than larger nestlings (prediction 1). Moreover, if the TCH is applicable in this species, given that ectoparasites would feed mainly on the smaller nestlings, these nestlings will suffer a higher impact from ectoparasitism than larger

nestlings. Consequently, within-brood variation in body mass, tarsus length and body condition are expected to be greater in infested compared to uninfested nests (prediction 3). Specifically, we compared body mass, tarsus length, body condition and immunocompetence (measured as relative leucocyte concentration and immune response to phytohaemagglutinin (PHA)) between the smallest and largest nestlings within a brood according to the prevalence of nest ectoparasites.

Materials and methods

Study area and bird species

Blue tits are Palearctic forest insectivorous birds that nest in secondary cavities in trees, where they typically lay an average of six to nine eggs per clutch (seven in our population) in the Iberian Peninsula (Salvador 2016). Eggs generally hatch asynchronously (hatching spread of two days; Slagsvold et al. 1995; Stenning 2008), leading to within-brood size hierarchy (Stenning 2018). During the breeding season, nests are commonly infested by different ectoparasites such as blowflies, fleas and mites, which usually decreases nestling health, condition and survival (e.g. Hurtrez-Boussès et al. 1997; Tomás et al. 2007; Castaño-Vázquez et al. 2018).

In 2017, we studied a wild population of blue tits inhabiting two contiguous forests, located at 1700–1800 m a.s.l. in the Sierra Nevada National Park (southeastern Spain; 36°57'N, 3°24'W). The two study areas comprised of (1) a dry forest composed of Holm oaks (*Quercus ilex*) and Pyrenean oaks (*Quercus pyrenaica*), and (2) a humid forest characterised by Scots pines (*Pinus sylvestris*), Holm oaks and Pyrenean oaks. These two forests differed in several environmental and biotic factors, such as mean temperature, humidity, solar radiation, insolation time, canopy cover and parasite load (supplementary material; also see Garrido-Bautista et al. 2021). The higher humidity of the humid forest was due to the presence of a river and a stream, and, to simplify, we referred to these two areas as humid and dry forests throughout the text. Blue tits bred in nest boxes (ICONA C model; base area: 196 cm^2; height: 20 cm; hole diameter: 3 cm; material: wood with the outer layer of plastic paint; Moreno-Rueda 2003) hung from branches using metal hooks. They were inspected regularly to determine laying date, hatching date and brood size. Nest boxes were cleaned and

their contents removed at the end of the breeding season to avoid the accumulation of ectoparasites between breeding seasons.

Morphometric measurements and leucocyte count

We considered the hatching day as the day 0. When the nestlings were 13 days old, the age at which body mass and tarsus length reach asymptotic growth (Björklund 1996), nestlings from 37 nests (20 from the dry forest and 17 from the humid forest) were weighed with a portable, digital scale (accuracy, 0.1 g) and their tarsus length measured to the nearest 0.01 mm with a digital calliper. We calculated the body condition index (BCI) of nestlings as the residuals of regressing log body mass on log tarsus length. In 19 nests (dry forest, n = 11; humid forest, n = 8), we also took a 100 µL blood sample from the largest and smallest nestlings (~1% of nestling body mass, which was about 10 g) from the jugular vein using heparinised insulin syringes in sterile conditions (following Owen 2011). Blood sampling has been shown to have negligible effects on tit nestling survival (Schmoll et al. 2004). Handling time was kept to a minimum to reduce nestling stress (de Jong 2019). A drop of blood was smeared on a slide and air-dried following Owen (2011). Blood samples were fixed in absolute methanol for 2 min and stained with a Wright-Giemsa combination stain as follows: (1) Wright stain for 2 min; (2) distilled water for 2 min followed by tap water for a few seconds; (3) Giemsa stain 1:9 diluted in phosphate-buffered saline, pH 7.2, for 10 min followed by tap water for a few seconds; and (4) 0.5% acetic acid for 1 s. Smears were prepared with Eukitt® mounting medium. Samples were viewed with a Zeiss Axiophot microscope at 400 × magnification and 35–40 fields per blood smear were observed (always by the same researcher, A.S.). Fields were photographed with a Zeiss Axiocam camera connected to the microscope. Leucocytes were counted and identified following Campbell & Ellis (2007) and erythrocytes were counted with Mizutama software (Ochoa et al. 2019). The relative leucocyte concentration for each nestling was estimated as the number of leucocytes per 10,000 erythrocytes.

Cutaneous immune response to PHA

We also measured the immune response to PHA in larger and smaller nestlings from 28 broods (dry forest, n = 15; humid forest, n = 13). When the nestlings were 12 days old, we inoculated 0.1 mg of phytohaemagglutinin (PHA-P; Sigma Aldrich, L-8754)

diluted in 0.02 mL of isotonic phosphate-buffered saline in their left wing web (following Smits et al. 1999). PHA is a mitogen that provokes an inflammatory immune response involving different types of cell and can be considered a multifaceted index of cutaneous immune activity (Martin et al. 2006). The inflammatory response to PHA correlates positively with nestling survival to adulthood in blue tits (Cichoń & Dubiec 2005). Prior to inoculation of PHA, we measured the wing web thickness three times with a pressure-sensitive micrometre (Mitutoyo Inc.; accuracy, 0.01 mm) and took the average. The following day, when nestlings were 13 days old, we measured the wing web thickness again following with the same procedure, calculating the inflammatory immune response as the difference between both measurements. All measurements were made by the same researcher (G.M.R.). Some of the nestlings (n = 20) that were inoculated with PHA were measured at the next day for leucocyte count. Although the inoculation of PHA in the wing web of nestlings causes local and cutaneous inflammation, an elevation of T-cells in the bloodstream can occur (Tella et al. 2008). To control for this possible effect, we compared the leucocyte count between those nestlings that were inoculated and not inoculated with PHA.

Ectoparasite abundance and prevalence

Once nestlings fledged and left the nest, we carefully sorted through nest material and recorded the presence and number of blowflies (puparia and larvae) per nest, as well as the presence or absence of hen fleas (larvae or adults). Females of the blowfly lay their eggs in the nests of cavity-nesting birds, so their larvae inhabit inside nest material and feed intermittently on nestlings (Bennett & Whitworth 1991). The haematophagous activity of blowfly larvae provokes several negative impacts on nestlings (Merino & Potti 1995; Hurtrez-Boussès et al. 1997; Puchala 2004). Tits (Paridae) are one of the hosts where the hen flea achieves its higher productivity (Tripet & Richner 1997a). Fleas inhabit inside nest material; flea larvae are saprophyte, but adults take blood from nestlings (Tripet & Richner 1999). The blood-sucking of adult hen fleas has been reported to cause detrimental effects on the growth, body condition, blood count and health of blue tit nestlings (Tripet & Richner 1997b; Pitala et al. 2009; Brommer et al. 2011).

Statistical analyses

Graphical inspection (following Zuur et al. 2010) revealed a normal distribution for all recorded variables, except blowfly abundance. The variation in blowfly and flea prevalence in relation to each forest was tested using the chi-squared test, and the abundance of blowflies between forests was tested with the Mann–Whitney U test. We calculated the within-brood differences in relative leucocyte count, cutaneous immune response, body mass, tarsus length and body condition between larger and smaller nestlings (values for larger nestling minus smaller ones).

The differences in the number of leucocytes between larger and smaller nestlings were initially tested using a Student's *t*-test (Quinn & Keough 2002). We assessed how the relative leucocyte count and the cutaneous immune response varied with forest type and nestling size using two separate linear mixed-effects models of restricted maximum likelihood (REML-LMM) (Zuur et al. 2009), where nest identity was the random factor and nestling rank (larger versus smaller), forest type (humid versus dry) and its interaction were the predictors. Brood size and laying date were included as covariates, but their effects were not significant and so they were removed from the final model. A REML-LMM was also used to test the relationship between body mass and leucocyte count. In this case, nest identity was the random factor and body mass and tarsus length were the covariates. We tested whether the differences in relative leucocyte count were related to blowfly abundance using Spearman's rank correlation. We used a *t*-test to analyse the effect of PHA (nestlings inoculated and non-inoculated with PHA) on relative leucocyte count. We ran two separate linear models to assess how the within-brood differences in relative leucocyte count and cutaneous immune response varied with nest parasitisation by hen fleas and blowflies. These models included forest type, prevalence of fleas and prevalence of blowflies as predictors and nest identity as random factor.

We used *t*-tests to analyse the effect of nest parasitisation by blowflies and fleas (parasitised or nonparasitised) on body mass, tarsus length and BCI and its within-brood differences. REML-LMM were used to test whether the observed ectoparasite-dependent within-brood differences in body mass, tarsus length and BCI were robust when controlled for forest, laying date and brood size, variables which could potentially affect any within-brood variation. In these cases, within-brood differences in the three morphological characters were the dependent variables, and flea or

blowfly prevalence, forest type, brood size and laying date were the predictors. The correlations between body mass of larger and smaller nestlings in nests parasitised and nonparasitised by fleas were established using the Pearson product-moment correlation. The basic statistics are given as mean ± s.e. (standard error). We used the 'nlme' package (Pinheiro et al. 2019) in the software R (R Development Core Team 2020).

Results

Immunological parameters

Blowfly prevalence and abundance were higher in the humid forest (14 out 17 nests infested, abundance: 10.82 ± 2.20 blowflies) than in the dry forest (7 out of 20 nests infested, abundance: 2.45 ± 1.70 blowflies; respectively, $\chi^2 = 8.40$, $p = 0.0038$, Mann–Whitney U test, $z = 2.85$, $p = 0.003$), but flea prevalence did not differ between forests (humid: 7 out of 17 nests, dry: 5 out of 20 nests; $\chi^2 = 1.10$, $p = 0.29$). Larger nestlings had half as many leucocytes (54.85 ± 5.54 leucocytes per 10,000 erythrocytes) as smaller nestlings (119.98 ± 15.05; $t_{18} = -4.75$, $p < 0.001$; Table 1). In fact, the number of leucocytes per 10,000 erythrocytes in a nestling was negatively affected by body mass ($\chi^2 = 12.87$, $p < 0.001$; Fig. 1). This within-brood difference was higher in the humid forest than in the dry forest (Table 1; Fig. 2). However, the within-brood difference in relative leucocyte count between larger and smaller nestlings did not vary with blowfly abundance or prevalence (abundance: $r_s = 0.22$, $p = 0.36$; Table 1) or flea prevalence (Table 1). Leucocyte count was not affected by PHA inoculation, as inoculated nestlings (n = 20) did not statistically differ in leucocyte count to non-inoculated nestlings (n = 18 nests; for larger nestlings: $t_{17} = 0.49$, $p = 0.62$; smaller: $t_{17} = 0.27$, $p = 0.79$). The cutaneous immune response did not differ significantly between larger (0.47 ± 0.04 mm) and smaller nestlings (0.46 ± 0.04 mm; Table 1). The presence of blowflies or fleas did not affect the within-brood difference in immune response (Table 1).

Table 1. Results of the linear mixed-effects models and linear models for the number of leucocytes, cutaneous immune response, and its within-brood differences (values for larger nestling minus smaller ones) of blue tit nestlings. Significant predictors ($p < 0.05$) are marked in bold.

Variable	Chi-square (df = 1)	*p*-value
Number of leucocytes		
Forest type	5.39	**0.02**
Nestling rank	6.27	**0.012**
Forest type*nestling rank	5.81	**0.02**
Cutaneous immune response		
Forest type	0.57	0.45
Nestling rank	0.19	0.66
Forest type*nestling rank	0.24	0.62
Variable	*F*-value (df = 1)	*p*-value
Within-brood difference in leucocytes		
Forest type	6.36	**0.02**
Flea prevalence	0.04	0.84
Blowfly prevalence	1.20	0.29
Within-brood difference in immune response		
Forest type	0.59	0.45
Flea prevalence	1.17	0.29
Blowfly prevalence	0.51	0.48

Body mass, tarsus length and body condition

In flea-infested broods, smaller nestlings tended to have a lower body mass and shorter tarsus than uninfested broods, while larger nestling body mass and tarsus length did not differ with flea prevalence (Table 2). The presence of fleas also affected the within-brood difference in body mass, being higher in parasitised nests (Table 2). The effect of flea prevalence on within-brood differences in body mass remained statistically significant in a linear model when controlling for forest type, laying date, and brood size ($F_{1, 31} = 13.09$; $p = 0.001$; the remaining effects were not significant).

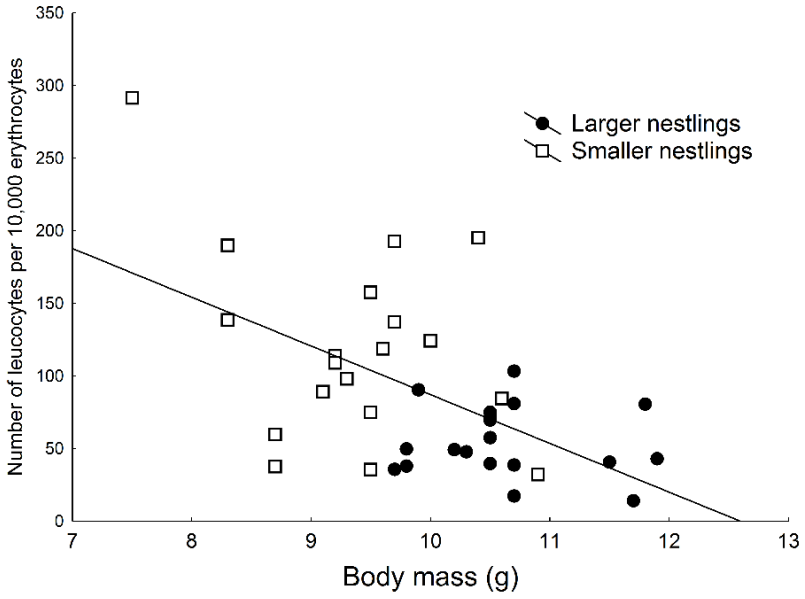

Figure 1. Relationship between body mass and relative leucocyte count in blue tit nestlings. Larger nestlings: filled circles; smaller nestlings: open squares.

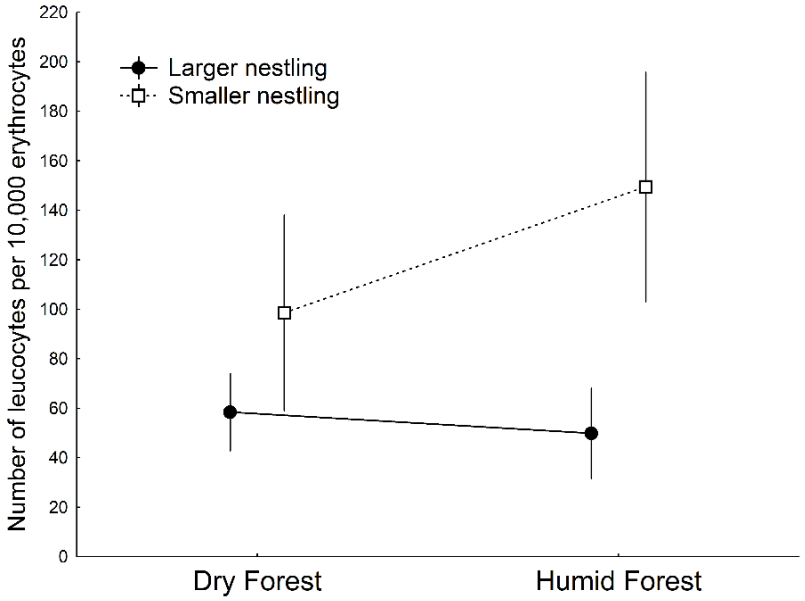

Figure 2. Relative number of leucocytes in larger (filled circles) and smaller (open squares) blue tit nestlings according to forest type. Bars represent the 95% confidence interval.

In non-parasitised broods, there was a strong correlation between the body mass of larger and smaller nestlings (r = 0.93, p < 0.001); however, in broods parasitised by fleas, the body mass of larger and smaller nestlings did not present a significant correlation (r = 0.45, p = 0.15). Flea infestation was unrelated to nestling BCI (Table 2; always p > 0.15). However, within-brood differences in BCI were higher in the humid forest (0.09 ± 0.013) than in the dry forest (0.04 ± 0.013; t_{35} = 2.60, p = 0.014). This difference remained significant when controlling for laying date and brood size ($F_{1, 33}$ = 7.02, p = 0.012; the remaining effects were not significant). Blowfly prevalence held no relation to within-brood differences in tarsus length, body mass or BCI (data not shown for simplicity).

Table 2. Body mass, tarsus length and body condition index (BCI) of smaller and larger blue tit nestlings and the within-brood differences between hen flea-infested and uninfested broods.

	Infested broods (n = 12)	Uninfested broods (n = 25)	*t*-value	*p*-value
Larger nestlings				
Tarsus length (mm)	16.63 ± 0.50	16.79 ± 0.52	-0.92	0.36
Body mass (g)	10.65 ± 0.58	10.62 ± 0.74	0.11	0.92
BCI (residuals)	0.045 ± 0.041	0.027 ± 0.087	0.67	0.51
Smaller nestlings				
Tarsus length (mm)	15.76 ± 0.47	16.19 ± 0.45	-2.70	0.01
Body mass (g)	9.06 ± 0.71	9.54 ± 0.80	-1.78	0.08
BCI (residuals)	-0.042 ± 0.074	-0.029 ± 0.087	-0.44	0.67
Within-brood difference				
Tarsus length (mm)	0.87 ± 0.62	0.60 ± 0.65	1.17	0.25
Body mass (g)	1.59 ± 0.68	1.08 ± 0.30	3.18	0.003
BCI (residuals)	0.087 ± 0.062	0.056 ± 0.058	1.46	0.15

Differences were tested using *t*-tests. Mean values are given with the s.d. (standard deviation).

Discussion

In the present study conducted in a wild population of blue tits parasitised by blowflies and fleas, we tested two predictions of the TCH: whether smaller nestlings within a brood have lower immunocompetence than larger nestlings (Christe et al. 1998) and

whether ectoparasites had a greater negative effect on body mass and body size in smaller nestlings compared to larger nestlings (Szép & Møller 2000). Our results lend some support to the TCH, as the presence of hen fleas in broods was detrimental for smaller blue tit nestlings (negatively affecting their body mass) but did not affect larger nestlings. However, there was no evidence that smaller nestlings are less immunocompetent than larger nestlings: the relative leucocyte count was higher in smaller than in larger nestlings and the immune response to a novel substance, phytohaemagglutinin, did not differ between larger and smaller nestlings. Moreover, the presence of fleas did not influence either of the immune response markers we measured. We did not find any evidence that blowflies affected the body characteristics or immune status of smaller nestlings; so, this ectoparasite species does not appear to apply to the TCH (unlike the findings reported by Simon et al. 2003).

Evidence for the Tasty Chick Hypothesis

We found some evidence that the TCH might apply to the blue tit in our study population. Body mass and tarsus length of smaller nestlings were lower in the presence of fleas (the tarsus length, nonsignificantly), without affecting the body size and mass of larger nestlings. Consequently, within-brood differences in body mass were more marked in broods parasitised by fleas. Similarly, other studies have reported increased within-brood variance in infested nests (Merino & Potti 1995; Christe et al. 1996; Szép & Møller 2000; but no such effect was observed by Descamps et al. 2002; Hannam 2006). Several studies have shown that nest-dwelling ectoparasites, including fleas, have negative effects on nestling body mass (Richner et al. 1993; Merino & Potti 1995; Tomás et al. 2007). Therefore, if these ectoparasites tend to feed on last-hatched nestlings, smaller nestlings would be more negatively affected by parasitism (Szép & Møller 2000). Nevertheless, this lends very indirect support to the TCH and alternative explanations should be explored. Specifically, parasitised broods may suffer a food shortage which causes parents to feed the larger nestlings preferentially (Smiseth et al. 2003), resulting in poorer growth of smaller nestlings. Parents may also feed healthy nestlings preferentially (Saino et al. 2000), hence smaller nestlings might suffer a disadvantage if parents disfavour them for being unhealthy.

Smaller nestlings had significantly more leucocytes than larger ones. This finding, a priori, contradicts a pivotal prediction of the TCH. There is compelling evidence that an increase in the leucocyte count in birds is usually associated with the infestation or infection by different ecto- and endoparasites (Norris & Evans 2000), so the smaller nestlings' greater investment in immune function could be explained by increased exposure to parasites. Similarly, Saino et al. (2001) and Parejo et al. (2007) found that last-hatched nestlings showed a higher immunological profile (level of antibodies) than their core siblings. However, in our study, neither the presence of fleas nor Blowflies affected immune function. A possible explanation is that smaller nestlings mounted an immune response against other parasites that were not analysed in the present study, such as viruses, bacteria, coccidia, haemosporidians or their dipteran vectors. In fact, the relative leucocyte count of smaller nestlings was higher in the humid forest than in the dry forest and, in a previous study carried out in 2016, we found a higher prevalence of the haemosporidian *Leucocytozoon* in the humid forest (Moreno-Rueda et al. submitted). However, we cannot discard that higher leucocyte count in smaller nestlings is due to other causes not related to TCH. For example, parents could improve the immune system of smaller nestlings via resources in ovo (Roulin et al. 2008). Therefore, the overall evidence for the TCH in our study is weak and indirect.

Evidence against the Tasty Chick Hypothesis

However, some of our results do not support the TCH: the cutaneous immune response did not differ between larger and smaller nestlings and was unaffected by ectoparasites. The fact that exposure to parasites activates the immune system means that comparing immune system parameters, such as leucocyte count, is of no use when examining differences in immunocompetence, as these parameters probably mirror a mix of immunocompetence and parasite exposure. Immunocompetence can be measured by challenging the immune system with a novel stimulus to which it has not previously been exposed. PHA is a novel stimulus widely used to quantify immunocompetence (Kennedy & Nager 2006). It has been shown to provoke inflammation with infiltration of different immune cells (Martin et al. 2006). Various studies have found that PHA testing normally reflects nestling body condition and nutritional status (e.g. Saino et al. 1997; Westneat et al. 2004; Martínez-de la Puente et al. 2013) and is related to nestling ectoparasite resistance (Martin et al. 2001;

Tschirren et al. 2007; Bize et al. 2008). Hence, the TCH predicts a weaker inflammatory response to PHA in smaller compared to larger nestlings. This has been confirmed in house martin (*Delichon urbicum*; Christe et al. 1998), black-headed gull (*Chroicocephalus ridibundus*; Müller et al. 2003), barn owl (*Tyto alba*; Roulin et al. 2003), and collared dove (*Streptopelia decaocto*; Eraud et al. 2008) nestlings. However, no within-brood differences in PHA immune response have been observed in great tit (*Parus major*; Roulin et al. 2003; Kilgas et al. 2010), alpine swift (*Tachymarptis melba*; Bize et al. 2005), red-billed chough (*Pyrrohocorax pyrrhocorax*; Banda & Blanco 2008), or blue tit (this study) nestlings. In fact, Saino et al. (2001) even reported a stronger immune response in smaller than in larger barn swallow (*Hirundo rustica*) nestlings. Therefore, there is a lack of evidence to support one of the main predictions of the TCH, namely that smaller nestlings have poorer immune systems than larger nestlings.

For their part, blowflies, unlike fleas, did not affect the body mass or tarsus length of smaller nestlings. Blowfly larvae are known to have negative effects on the physiology, growth and survival of blue tit nestlings (Hurtrez-Boussès et al. 1997; Bouslama et al. 2001; Tomás et al. 2007; Arriero et al. 2008; Castaño-Vázquez et al. 2018). However, these negative impacts of blowflies may vary between years and according to weather conditions, so in some years they cause relatively little harm to nestlings (Merino & Potti 1996; Simon et al. 2004). Furthermore, there is still no consensus on whether blowfly larvae eat preferentially on last-hatched nestlings (Descamps et al. 2002; Simon et al. 2003). Hence, the apparent absence of any impact of blowfly larvae on nestlings in our study may be due to a lack of any significant detrimental effects on blue tit nestlings in our population specifically during the year the study was carried out.

Tasty Chick Hypothesis: state of the art

The TCH predicts that last-hatched (marginal) nestlings must be less immunocompetent than core nestlings. As explained above, several studies performed in various species do not support this prediction (Saino et al. 2001; Roulin et al. 2003; Bize et al. 2005; the present study). In addition, the TCH predicts that nest-dwelling ectoparasites tend to feed more on the least immunocompetent nestling. Although some studies have shown that higher host immunocompetence reduces ectoparasite

feeding, breeding and survival success (Tschirren et al. 2007; Bize et al. 2008), only a few studies support this prediction. Simon et al. (2003) reported that blowfly larvae eat more blood from smaller than larger blue tit nestlings. O'Connor et al. (2014) evidenced aggregation of parasitic larvae of the dipteran *Philornis downsi* on just one nestling per brood. However, nestlings frequently move around more when parasitised, in an attempt to escape from parasites in the nest (Simon et al. 2005; O'Connor et al. 2010), so it is unclear whether the patterns reported by Simon et al. (2003) and O'Connor et al. (2014) are due to the feeding behaviour of parasites or nestling competition for the positions least exposed to ectoparasites. Furthermore, and in clear contrast with the TCH, several studies have shown that parasites prefer to feed on large, well-nourished nestlings before last-hatched nestlings (Roulin et al. 2003; Valera et al. 2004; Bize et al. 2008; Václav et al. 2008; Václav & Valera 2018). Therefore, the overall evidence in favour of the TCH is, at best, only weak.

The evolution of hatching asynchrony in the blue tit

While the TCH seems unable to explain the evolution of hatching asynchrony in the blue tit, other hypotheses may explain this phenomenon in said species. Indeed, hatching asynchrony has been shown to be adaptive in blue tits, as asynchronous broods produce heavier offspring than synchronous broods, even when there was no brood reduction (Slagsvold et al. 1995). The most classical explanation for hatching asynchrony is to facilitate adaptive brood reduction (Stenning 1996). According to this concept, in the scenario of a temporally and spatially unpredictable environment, parents might secondarily adjust their brood size to food availability, with small chicks sacrificed to ensure the survival of the remaining offspring if food resources become scarce (Magrath 1989). Hatching asynchrony may also have evolved to reduce sibling competition, to decrease the peak in parental workload or to minimise predation risk and egg failure in some altricial birds (review in Magrath 1990). Accordingly, Stenning (2008) showed that blue tit nestlings from asynchronous broods spent less time in their nests. Moreover, marginal nestlings from asynchronous broods typically die earlier compared to those in synchronous broods, with the parents saving the energy that they would have invested in chicks that would have likely died anyway.

Conclusions

In conclusion, our study provides some, albeit indirect, evidence that the TCH might be applied to the blue tit and a specific ectoparasite as we found that the presence of hen fleas was more detrimental to smaller than larger nestlings (affecting their body mass). However, most of the results in our study did not support the TCH. Blowflies did not affect nestlings' morphometry and immune system. Moreover, we did not find evidence that smaller nestlings were less immunocompetent than larger nestlings as the immune response to PHA did not differ between both type of nestlings and smaller nestlings had more leucocytes than larger ones.

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Supplementary material

Forest characterization The study area comprises two neighbouring forests typically representative of the Mediterranean habitat (Fig. S1). To characterize forest heterogeneity, we performed several image data analyses and field samplings. In 2019, we installed data loggers iButton® in an outer wall of nest-boxes in the humid and dry forests (five in each location) to measure the environmental temperature (ºC) and humidity (%). Data loggers were placed on 05 May and removed 29 June, and measurements were taken every hour. The temperature of the dry forest was on average 1 ºC higher than the humid forest (mean ± s.e.; dry: 17.81 ± 0.18 ºC, humid: 16.80 ± 0.17 ºC; *t*-test: $t_{1338} = 14.11$, $p < 0.001$) and the humidity of the dry forest was lower than in the humid forest (dry: 41.37 ± 0.49 %, humid: 42.99 ± 0.49 %; *t*-test: $t_{1338} = 9.21$, $p < 0.001$).

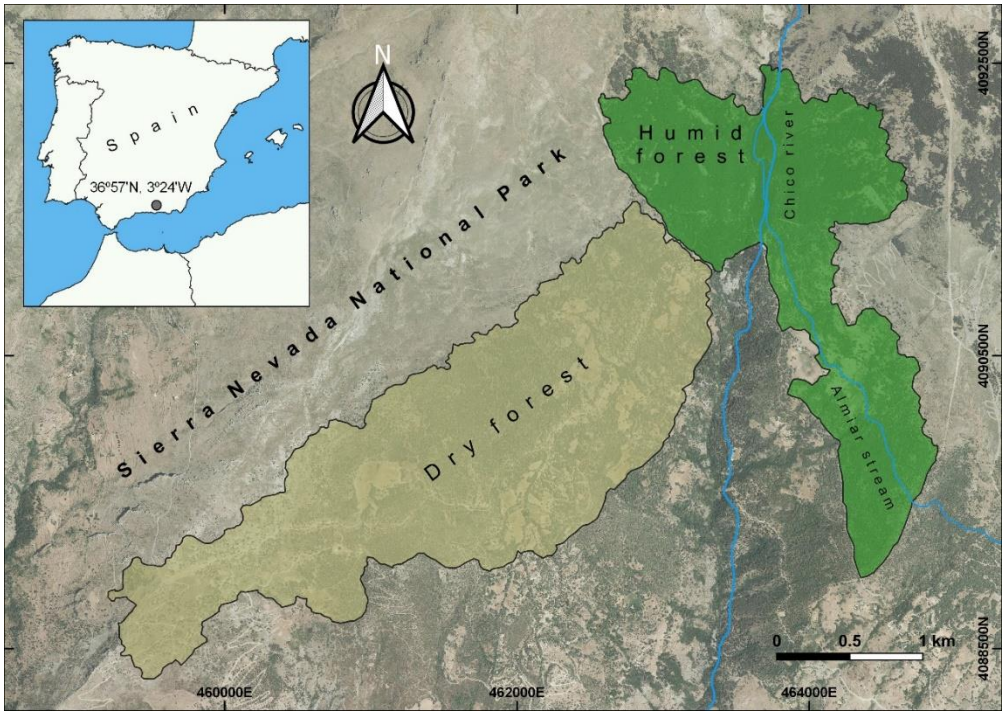

Figure S1. Location of the dry (ocher colour) and humid (green colour) forests in the Sierra Nevada National Park, southern Spain. Notice the presence of the Chico river and the Almiar stream crossing the humid forest.

To calculate solar radiation and insolation time in each forest we used a digital elevation model, with a 5 × 5 m resolution (source: MTN50 project; Spanish *Instituto Geográfico Nacional*, IGN). Solar radiation for each forest was calculated using the "Area solar radiation" algorithm within the Spatial Analyst Tools extension for ArcGIS Desktop 10.3.1 software (ESRI, Redlands, CA, USA). We selected the breeding amplitude (i.e. day from the first egg laid until the day the last fledgling left the nest) of our blue tit population in 2017 (28 April to 30 June). The output raster values, which give information of radiation for cloudless days, were then divided by the number of days of the breeding amplitude (64 days) to obtain the radiation raster units in kWh/m²/day. In addition, we calculated the insolation time (h) for each forest using the "r.sun.insoltime" algorithm within the GRASS SIG 7.8.2 software (GRASS Development Team 2020), integrated into Quantum GIS 3.10.5 software (QGIS Development Team 2020). We fixed the time step for processing the sums of all-day radiation in 1 min and the insolation time was obtained every five days within the breeding amplitude, with the rest of parameters by default. The dry forest had on average 1 h more of insolation than the humid forest (dry: 13.15 ± 0.07 h, humid: 12.09 ± 0.05 h; *t*-test: $t_{19} = 50.79$, $p < 0.001$), so the solar radiation was slighty greater in this dry forest than in the humid forest (mean ± s.d.; dry: 6.38 ± 0.21 kWh/m²/day, humid: 6.22 ± 0.32 kWh/m²/day; Fig. S2).

Caterpillars are the main food of blue tit nestlings, followed by a less but considerable proportion of spiders (unpublished data). Our preliminary results based on video recordings inside the nest boxes showed that 81.58% and 82.36% of all identified preys consumed by nestlings were caterpillars in the dry and humid forest, respectively (data from 2019). The proportions of spiders are 15.07% and 16.15% in the dry and humid forest, respectively. To test whether prey availability differs between both locations, we estimated the arthropod population intensity in the humid and dry forest following Zandt (1994). In 2018, we sampled five randomly branches in three days and in each forest to estimate the arthropod population as the number of arthropods per 10 shoots (sample units per forest; dry forest: 15 branches; humid forest: 15 branches). When shooting a branch, a 2 × 2 m blanket was placed in the ground under the branch, and arthropods were collected in 70% ethanol labelled vials. In the laboratory, arthropods were identified to the order level according to Barrientos (1988). Thus we obtained, for each sample unit, the caterpillar intensity. The humid forest harboured twice caterpillars than the dry forest (mean ± s.e.; humid: 11.30 ±

2.49 caterpillars, dry: 4.77 ± 0.98; GLM linked to a loglineal-Poisson distribution, forest as the predictor: $\chi^2 = 14.43$, $p < 0.001$).

Forest coverage and land use were estimated through the use of vector layers from the SIOSE project (Spanish *Sistema de Información sobre Ocupación del Suelo de España*), downloaded from the REDIAM Spanish program. We calculated the extension (ha) and percentage of land uses (e.g. forest type, shrubland type, grassland, etc.) for each forest by using the "intersection" function in Quantum GIS 3.10.5 software (QGIS Development Team 2020). The two forests differed in the tree and shrub extension, as well as land uses (Table S1).

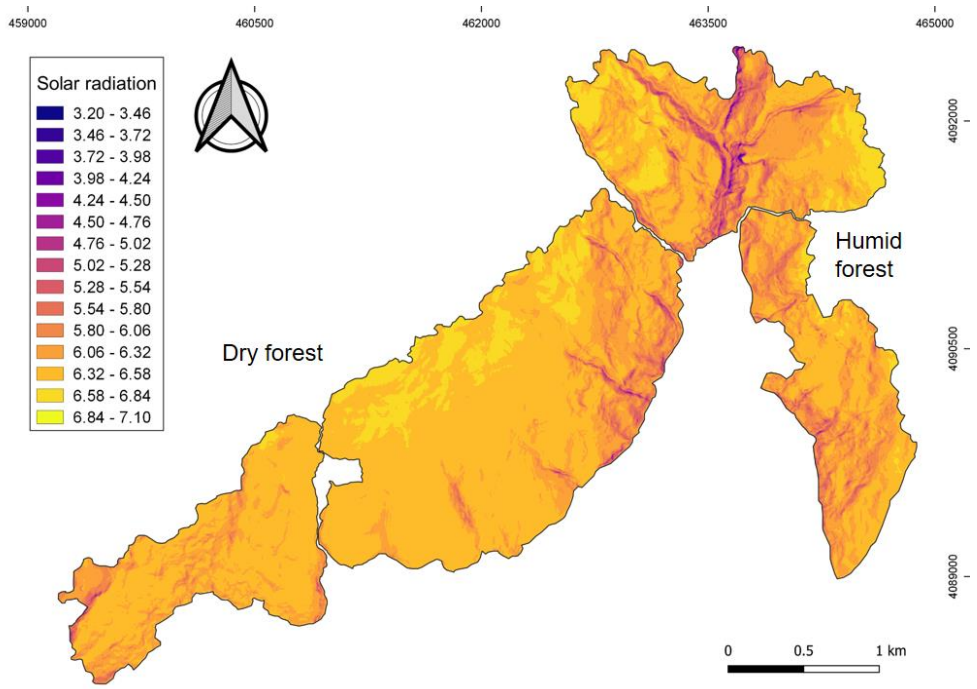

Figure S2. Solar radiation of the two forest types in the Sierra Nevada National Park, southern Spain. Units are expressed in kWh/m²/day.

Nest-dwelling ectoparasites (hen fleas and blowflies) were collected and examined as described in the main manuscript. The variation in the prevalence of both ectoparasites and the abundance of blowflies were determined also as described in the

main manuscript. The blowfly prevalence and abundance were higher in the humid forest than in the dry forest in the year in which the study was done (see Results in the main manuscript), but also when considering several years (2016, 2017 and 2018; dry forest: 42 out of 109 nests infested, humid forest: 90 out of 100 nests infested; chi-square test: $\chi^2_3 = 60.00$, $p < 0.001$; see also Garrido-Bautista et al. 2020). The abundance of blowflies also varied between forests across years (2016, 2017 and 2018; Kruskal–Wallis test: $\chi^2_3 = 62.51$, $p < 0.001$). Although differences in flea prevalence were not significant in the sample size used in this study, when analysing all of our nest-boxes, flea prevalence was higher in the humid forest (2017; dry forest: 11 out of 43 nests infested; humid forest: 20 out of 41; $\chi^2 = 3.91$, $p = 0.048$). This between-forest difference in the prevalence of fleas is maintained when several years are considered (2017 and 2018; dry forest: 19 out of 78 nests infested; humid forest: 46 out of 76 nests infested; $\chi^2 = 19.19$; $p < 0.001$). Moreover, the prevalence of the haemosporidian *Leucocytozoon* spp. differed between forests. The 50% of nestlings were infected by this endoparasite in the humid forest, while the infection rate was lower in the dry forest (26.9%) (data from 2016; methodology and results in Moreno-Rueda et al. submitted).

All statistical analyses were performed according to Quinn & Keough (2002) and Zuur et al. (2009) in the software R (R Development Core Team 2012). We used the 'nlme' package (Pinheiro et al. 2019) when conducting the GLM.

Table S1. Area (ha) and percentage (%; between brackets) of land uses of the two forest types of the study area. Land use categories have been extracted from the SIOSE Spanish program (information about the methodology and categorization of land uses can be found in the webpage of SIOSE).

	Dry forest	Humid forest
Dense forest: conifers	2.48 (0.51)	162.61 (52.70)
Dense forest: oaks	247.93 (50.68)	91.35 (29.60)
Dense forest: mixed	2.92 (0.59)	0 (0)
Dense shrubland: conifers	1.47 (0.30)	15.15 (4.91)
Dense shrubland: oaks	85.94 (17.56)	2.82 (0.91)
Dense shrubland: mixed	10.39 (2.12)	0.03 (0.01)
Scattered shrubland: conifers	0 (0)	10.21 (3.31)
Scattered shrubland: oaks	51.90 (10.61)	4.25 (1.37)
Scattered shrubland: mixed	4.09 (0.84)	4.30 (1.39)
Grassland	41.32 (8.45)	3.23 (1.05)
Mosaic	20.56 (4.20)	0.58 (0.18)
Bare ground	0.69 (0.14)	0 (0)
Rivers and streambeds	0 (0)	3.55 (1.15)
Farmland [a]	7.77 (1.59)	5.57 (1.80)
Others [b]	11.73 (2.40)	4.92 (1.59)

[a] Includes farmlands, irrigation ponds and livestock lands.

[b] Includes forest tracks, firebreaks and forestry facilitations.

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PART II

Genotypic and phenotypic divergence: evolution of fine scale optimal clutch size

Chapter 6

Habitat-dependent breeding biology of the blue tit (*Cyanistes caeruleus*) across a continuous and heterogeneous Mediterranean woodland

ABSTRACT Mediterranean woodland environments are characterised by high spatial and temporal heterogeneity, which means the inhabiting species face a wide variety of selective pressures. Species may respond differently to habitat heterogeneity and so distinct eco-evolutionary scenarios may be responsible for the inter-habitat variability in reproductive strategies observed in certain species. The inter-forest variability of some reproductive traits in passerines has been examined by comparing forest patches or separated fragments. However, there is still little information regarding how such highly mobile animals adjust their breeding performance across continuous and heterogeneous woodlands. Here we studied the reproductive performance of a population of blue tits (*Cyanistes caeruleus*) in an area of continuous Mediterranean woodland that included two mountain slopes and four different types of forest, ranging from deciduous oak forests to perennial non-oak forests. We studied the habitat heterogeneity and inter-forest phenotypic variation in terms of reproductive performance and adult and nestling biometry, besides also exploring the effects of ectoparasites on blue tit reproduction. Eggs were laid earliest in deciduous Pyrenean oak (*Quercus pyrenaica*) forests, while clutch size and the number of fledglings were highest in the humid Pyrenean oak forest, which had the greatest tree coverage and most humid climate, and lowest in the coniferous Scots pine (*Pinus sylvestris*) forest. There were no inter-forest differences in hatching (percentage of nests with at least one egg hatched) and fledging (percentage of nests in which at least one nestling fledged) success. Similarly, there were no inter-forest differences in adult and nestling biometry, but adults that raised more fledglings had a lower body mass, while males whose females laid larger clutches had smaller tarsi. Most ectoparasites did not affect blue tit reproduction, although *Culicoides* had a negative impact on nestling body mass. These results suggest that blue tits can adjust their reproductive effort to the forest where they breed even across a very small spatial scale. Different eco-

evolutionary scenarios, such as phenotypic plasticity or genetic structuring and local adaptation, might explain the phenotypic differentiation in the reproductive strategies observed over small areas in woodlands.

Keywords: Birds, Breeding success, cavity-nesting birds, paridae, passerines, reproduction

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Introduction

Environmental factors vary in space and time, so organisms inhabiting heterogeneous environments face a challenge to match their phenotype to different habitats in order to maximise fitness. The degree of heterogeneity creates a range of habitats available in which to reproduce (Sparrow 1999), forming a mosaic of optimal and suboptimal habitats (Hansson et al. 1995), with important implications for ecological and evolutionary processes. In homogeneous environments, natural selection should favour a common, well-matched phenotype (Edelaar et al. 2017). In heterogeneous environments, by contrast, natural selection should promote a range of phenotypes, as individuals can access different habitats that are optimal for each phenotype (Edelaar & Bolnick 2019; Trevail et al. 2021).

Accordingly, birds should lay clutches of a size that maximises their reproductive success in a given habitat (Stearns 1992), but different eco-evolutionary scenarios may also explain the habitat-related variation observed in avian reproductive performance. For example, some phenotypic variation observed between ecologically divergent conditions may be a consequence of adaptive phenotypic plasticity (Chevin & Hoffmann 2017) and, indeed, phenotypic plasticity provides the potential for birds to rapidly respond to environmental changes (Przybylo et al. 2000; Charmantier et al. 2008; Biquet et al. 2022). Also, the selection of some breeding traits may affect the variation of phenotypic plasticity in populations found in spatio-temporally heterogeneous habitats (Porlier et al. 2012). On the other hand, populations may adapt to environmental heterogeneity through microevolution. Limited gene flow and selection against immigrant genes could be responsible for local adaptation of several avian traits (Blondel et al. 1999; Garant et al. 2005; Postma & van Noordwijk 2005), whilst high levels of gene flow between areas of different habitat quality can maintain non-adaptive and less-than-optimal clutches because of differential costs associated with each habitat type (Blondel et al. 1998; Dhondt et al. 1990; Liou et al. 1993). Overall, both plastic responses to habitat variation and genetically local specialisation can occur simultaneously within the same meta-population (Blondel 2007), with each process depending mainly on individual dispersal ranges (Blondel et al. 2006).

Habitat quality has a strong influence on bird reproduction and can be expressed as a combination of several abiotic and biotic factors, such as vegetation structure, composition and maturity (Arriero et al. 2006; Pimentel & Nilsson 2007; Riddington

& Gosler 2008), food availability and quality (Seki & Takano 1998; Tilgar et al. 1999; Mägi et al. 2009), presence of parasites (Merilä et al. 1995; Arriero et al. 2008; Eeva & Klemola 2013), nest predation risk (Møller 1988; Heske et al. 1999; Morris & Gilroy 2008), or breeding population density (Both 1998; Sillett et al. 2004; Brouwer et al. 2009). In the case of insectivorous passerines, breeding performance is particularly affected by vegetation structure and maturity, which correlates with insect abundance (Tye 1992) — a parameter that ultimately controls all stages of reproduction (Martin 1987). There is compelling evidence that deciduous forests offer a higher quality habitat for most insectivorous birds during the breeding season than evergreen, coniferous forests. Deciduous woodlands typically contain more caterpillars and other invertebrates than evergreen forests (van Balen 1973; Huhta et al. 1998; Tremblay et al. 2003), and the peak of caterpillars usually appears earlier in the season in deciduous patches (Blondel et al. 1991; Tremblay et al. 2003, 2005). Indeed, several forest passerines, such as the great tit (*Parus major*), blue tit (*Cyanistes caeruleus*) or European pied flycatcher (*Ficedula hypoleuca*), lay their eggs earlier (Gezelius et al. 1984; Lemel 1989; Lambrechts et al. 2004; Riddington & Gosler 2008; Mägi et al. 2009), produce larger clutches (van Balen 1973; Gezelius et al. 1984; Lemel 1989; Sanz 1998; Lambrechts et al. 2004; Riddington & Gosler 2008) and eggs (Mägi & Mänd 2004), and rear more (van Balen 1973; Sanz 1998; Lambrechts et al. 2004; Riddington & Gosler 2008) and heavier fledglings (Lambrechts et al. 2004; Riddington & Gosler 2008) in deciduous woodlands than in coniferous or marginal habitats. Nevertheless, a higher reproductive performance was observed in suboptimal coniferous habitats compared to deciduous woods when the latter provided young, secondary stands of non-oak species (Mägi & Mänd 2004; Mägi et al. 2009).

Blue tits are small, Palearctic forest-dwelling, insectivorous passerines that nest in secondary cavities in trees (Stenning 2018), and whose breeding biology and habitat-dependent variation within Europe has been studied extensively in recent decades. Studies examining the inter-habitat variation in the breeding performance of blue tits across the species' entire European distribution have generally found that deciduous forests are the preferred habitat as they correspond to the highest reproductive and rearing parameters (Blondel et al. 1987, 1991, 1999; Dias et al. 1994; Fargallo & Johnston 1997; Tremblay et al. 2003, 2005; Lambrechts et al. 2004; Blondel 2007). Blue tit reproductive parameters are also shaped by other factors besides caterpillar abundance. In heterogeneous forests, for example, females in good body condition

may select high-quality territories (Arriero et al. 2006), with individuals in worse condition occupying poor quality patches and ultimately producing reduced clutch sizes (Dhondt et al. 1992) or impaired offspring condition and physiology (Arriero et al. 2008; Arriero 2009). Lastly, the genes of immigrants from nearby marginal and poor-quality habitats may lead to maladaptation in some reproductive parameters, such as clutch size (Dhondt et al. 1990; Blondel et al. 2006). Nonetheless, most of the aforementioned studies looked at the habitat-dependent variation in blue tit breeding biology or specific life-history traits at the macro-scale (Blondel et al. 1987, 1991; Gil-Delgado et al. 1992; Møller et al. 2014; Vaugoyeau et al. 2016) or at more local spatial scale (Nour et al. 1998; Blondel et al. 1999, 2006; Tremblay et al. 2003, 2005; Lambrechts et al. 2004), usually comparing reproductive parameters between distant geographical regions or forest patches and fragments. In this sense, Lambrechts et al. (2004) reported contrasted differences in the blue tit breeding performance between deciduous and evergreen patches separated on average 5 km, with a minimum between-patch distance of 600 m. So, there is scant information on how the blue tit adjusts its breeding performance at smaller spatial scales throughout continuous and heterogeneous woodlands.

This study tried to answer the question as how a highly mobile animal adjusts its breeding performance at extremely small spatial scale and across a gradient of habitats. To this end, we examined the breeding biology variation in wild blue tits inhabiting a continuous woodland comprising different Mediterranean forestry formations in southeastern Spain. We also explored forest-dependent variations in adult and nestling biometry and the effects of parental biometry on offspring condition. The study area was a continuous woodland located across two opposing mountain slopes, including four habitat types ranging from deciduous to coniferous, evergreen forest formations. We measured and analysed various abiotic and biotic factors, namely solar radiation, vegetation composition and parasite pressure to identify each forest formation's habitat features and hence the environmental pressures the blue tits faced during their breeding seasons. Based on the literature, we predict that breeding performance (in terms of a higher production of fledglings per nest and/or more high-quality fledglings) should be better in deciduous oak forests than in coniferous and evergreen formations within the same woodland, as sclerophyllous Mediterranean habitats are generally known to produce delayed

caterpillar emergence and lower caterpillar populations (van Balen 1973; Blondel et al. 1991, 2006; Tremblay et al. 2003, 2005).

Materials and methods

Study area

The study area was almost 800 ha in total and located 1700–1800 m a.s.l. in the Sierra Nevada National Park (southern Spain; 36°57'N, 3°24'W). It contained four different forestry formations: (1) a Holm oak (*Quercus ilex*) forest, (2) a dry Pyrenean oak (*Quercus pyrenaica*) forest, (3) a Scots pine (*Pinus sylvestris*) forest, and (4) a humid Pyrenean oak forest (Fig. 1), together constituting a continuous woodland representative of the Mediterranean habitat. The two Pyrenean oak forests are referred to as dry and humid forests throughout the text as the higher humidity of the humid Pyrenean oak forest, traversed by the Almiar stream, was one of the main differences between them (Fig. 1). These four forests represented different environmental pressures for blue tits during breeding season, as they differed in a wide variety of factors, such as solar radiation, insolation time, canopy cover, vegetation quality and ectoparasite and vector presence (supplementary material).

Blue tit sampling

This study was performed across 2017 and 2018. We installed and monitored nest boxes throughout the four forests, all of the same type (ICONA C model; basal area: 196 cm^2; height: 20 cm; hole diameter: 3 cm; material: painted wood; more details in Moreno-Rueda 2003). The nest boxes were hung from a tree branch using a metal hook at a height of 3–4 m and their geographical position recorded with a GPS device. The average separation between nest boxes in each forest was 96.56 ± 53.20 m (mean ± standard deviation). We monitored the nest boxes throughout each year's breeding season to determine the standardised laying date (difference between the actual laying date for each nest and the laying date of the first egg laid in each year; day 0 = day the first egg was laid each year), standardised hatching date (day 0 = day the first egg hatched each year), clutch size, brood size (nestlings counted at 3 days after hatching) and number of fledglings (fledglings counted at 13 days after hatching). Brood size was counted at 3 days given that blue tits practice asynchronous hatching and so it

may take 2–3 days for all the eggs to hatch (Stenning 2008). The number of fledglings was recorded when nestlings were 13 days old because they have reached their asymptotic mass and body size by this age (Björklund 1996) and nestlings older than 13 days may jump out of the nest when visiting nest boxes. Hatching success was calculated as the percentage of nests with at least one egg hatched out of all the nests. Fledging success was calculated as the number of nests in which at least one nestling fledged. The number of unhatched eggs per nest was taken as the difference between clutch size and brood size, and the number of eggs per nest that did not produce a fledgling was determined from the difference between clutch size and number of fledglings. We also calculated the percentage of eggs that produced fledglings for each nest (completely failed nests excluded). In total, we monitored breeding in 175 nest boxes (Holm oak: 26 boxes, dry Pyrenean oak: 63, Scots pine: 25, humid Pyrenean oak: 61), but the sample size varied slightly depending on each analysis (see below).

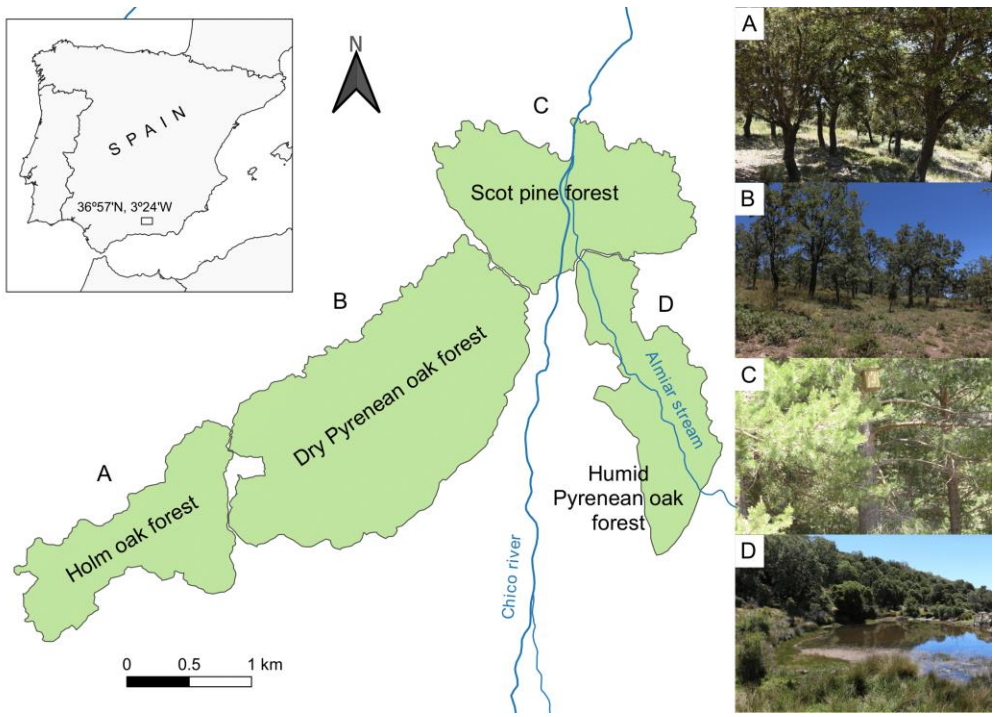

Figure 1. Locations of the four forest types in the Sierra Nevada National Park (southern Spain). (A) Holm oak forest, (B) dry Pyrenean oak forest, (C) Scots pine forest, (D) humid Pyrenean oak forest.

Adult birds were captured when the nestlings were between 8 and 11 days old (day 0 = hatching day) using scuttles that closed the nest box opening when they entered to feed the nestlings. We chose this age range to ensure the nestlings would not be harmed, given that the parents do not return to their nest box immediately (Schlicht & Kempenaers 2015), as tit nestlings develop thermoregulation from day 8 (Perrins 1979). Once captured, we sexed adults by examining for brood patches in females, measured their tarsus length with a digital calliper (accuracy: 0.01 mm) and weighed them to the nearest 0.1 g with a digital portable scale. The adults were banded with aluminium rings for further identification and liberated within 10 m of their nest boxes. In total, we measured 116 adults (Holm oak: 12 adults; dry Pyrenean oak: 41; Scots pine: 15; humid Pyrenean oak: 48). In 2017, when the nestlings were 13 days old, we measured their tarsus length and body mass (as explained above for adults), and they were banded with aluminium rings. All adult and nestling birds were measured by the same researcher (G.M.R.). For each nest, we calculated the mean brood mass and standard deviation of nestling body mass within broods. For logistic reasons, fledgling biometry could not be recorded in 2018. In total, we measured 445 fledglings corresponding to 80 nests (Holm oak: 76 fledglings, dry Pyrenean oak: 146, Scots pine: 47, humid Pyrenean oak: 176).

Ectoparasite estimation

We carefully revised the nest material in the nest boxes once all fledglings left their nests to obtain an estimate of ectoparasite pressure. We recorded the prevalence and intensity (i.e. number of parasites in infested nests) of *Protocalliphora azurea* blowfly larvae and puparia and the prevalence of *Ceratophyllus gallinae* hen flea larvae and adults. In total, we obtained nest-dwelling ectoparasite data from 154 nest boxes. Blowfly larvae feed on nestling blood, which has a negative effect on their physiology and growth (Hurtrez-Boussès et al. 1997; Arriero et al. 2008). Although hen flea larvae are saprophytic, adults suck nestling blood to the detriment of their physiology, health and feather growth (Pitala et al. 2009; Brommer et al. 2011).

We also estimated the intensity and prevalence of biting midges (genus *Culicoides*) and black flies (Simuliidae) in nests following protocols described elsewhere (detailed in Garrido-Bautista et al. 2022a). Briefly, the procedure involved placing a 60 mm Petri dish layered with a drop of body oil gel (Johnson's© Baby Camomile, Johnson

& Johnson, Dusseldorf, Germany; gel composition in Tomás et al. 2008a) in each next box. The petri dishes were placed in the nest boxes when the nestlings were 12 days old and collected the next day. They were then taken to the laboratory and any flying insects removed from the dishes by applying xylene for a few seconds and immediately transferred to absolute ethanol for a few minutes at 25 °C. Finally, they were stored in 70% ethanol until their identification and quantification. Other arthropods that accidently adhered to the dishes were excluded from subsequent analyses. We obtained data for biting midges and black flies from 77 nest boxes. Both biting midges and black flies are vectors of different avian blood parasites (Votýpka et al. 2002; Martínez-de la Puente et al. 2011) and can diminish nestling condition and body mass (Tomás et al. 2008b; Martínez-de la Puente et al. 2010).

Forest characterization: GIS and remote sensing analyses

Forest heterogeneity was characterised using various image analysis techniques. All geographical data and images were processed with Quantum GIS 3.10.5 software (QGIS Development Team 2020), unless indicated otherwise. First, we downloaded a digital elevation model (5 m × 5 m resolution) covering the study area (source: MTN50 project; Spanish *Instituto Geográfico Nacional*) to obtain forest orientation, forest slope, solar radiation and insolation time. Before making any calculations, the spatial extent of each forest was delimited and clipped based on its natural extension (Fig. 1). The mean forest orientations were obtained by reclassifying the raster pixel values into four categories: 315–45° (north), 45–135° (east), 135–225° (south) and 225–315° (west).

Solar radiation was determined using the 'Area solar radiation' algorithm within the Spatial Analyst extension of the ArcGIS Desktop 10.3.1 software (ESRI, Redlands, CA, USA). We calculated solar radiation every five days in the 2017 and 2018 breeding seasons starting from the day the first egg was laid until the day the last fledgling left the nest. The 2017 and 2018 breeding seasons covered from April 28 to June 30 and May 1 to July 31, respectively. Radiation was expressed in units of kWh/m^2/day. The insolation time (the hours per day a forest receives solar radiation) was estimated with the 'r.sun.insoltime' algorithm in the GRASS GIS 7.8.2 software (GRASS Development Team 2020), which is integrated within Quantum GIS 3.10.5 (QGIS Development Team 2020). We set the time step for processing the sum of all-day radiation to 1 min.

The mean insolation time was obtained every five days from April 28 to August 1 to cover the two breeding seasons.

Forest coverage and land use were estimated using vector layers obtained from the SIOSE project (Spanish *Sistema de Información sobre Ocupación del Suelo de España*), which was downloaded from the REDIAM website (*Red de Información Ambiental de Andalucía*). We calculated the extension (ha) and percentage of land uses with the intersection function. Further details on the method used to categorise land uses can be found in the public repository of the SIOSE project (see supplementary material). Vegetation quality was characterised based on Landsat-8 satellite images (30 m × 30 m resolution) of the entire study area that were acquired throughout the 2017 and 2018 breeding seasons. We obtained two satellite images for 2017 and three for 2018, while five other images were excluded because of cloud cover. The normalised difference vegetation index (NDVI) was calculated based on bands B4 (red: 0.64–0.67 μm) and B5 (near infrared: 0.85–0.88 μm) as: (B5–B4)/(B5+B4). NVDI values range from −1 to 1, where negative values correspond to an absence of vegetation and values close to 1 equate to dense vegetation coverage (Pettorelli et al. 2005).

Statistical analyses

Although there was a total of 175 occupied nest boxes, sample sizes varied depending on the analysis being carried out. Not all nest boxes could be followed to the end of the study to obtain all the reproductive parameters due to predation, desertion or unidentified causes of nestling death. Consequently, the sample sizes were 171 next boxes for laying date, 174 for clutch size, 172 for brood size, and 171 for the number of fledglings. The variables (environment: insolation time, solar radiation, NDVI; ectoparasites: intensities of blowflies, biting midges and black flies; reproductive parameters: laying date, clutch size, brood size, number of fledglings, number of unhatched eggs and number of eggs that failed to produce a fledgling; biometry: tarsus length and body mass) were plotted and tested for normality following Zuur et al. (2010). We checked for outliers using Cleveland dot plots and did not find any abnormalities in any of the variables. As insolation time, solar radiation and NDVI were not normally distributed, we used the Kruskal–Wallis test to examine the differences between forests for these variables. In the case of NDVI, one value was

excluded from the statistical analysis because a forest patch was covered by clouds (supplementary material).

The residuals of all models in the following sections were checked for normality following Zuur et al. (2010). The basic statistics are given as mean ± standard error (s.e.). All the analyses were performed in the R software environment, version 4.0.0 (R Development Core Team 2020), using the 'nlme' (Pinheiro et al. 2019) and 'lme4' packages (Bates et al. 2020), and graphs were constructed using the 'ggplot2' package (Wickman 2016).

Ectoparasites We used the chi-squared test to check for inter-forest variability in the prevalence of ectoparasites and the Kruskal–Wallis test to examine differences between forests in the intensity (i.e. number of parasites in infested nests; Rózsa et al. 2000) of blowflies, biting midges and black flies. Linear models were employed to determine the variation in the intensity of blowflies, biting midges and black flies depending on the laying date. Linear mixed-effects models of restricted maximum likelihood (REML-LMM) (Zuur et al. 2009) were applied to examine whether the presence of ectoparasites affected blue tit breeding biology. The models had the following structures: (1) for blowflies and fleas: clutch size, brood size and the number of fledglings were the dependent variables in separate models; forest, year and the prevalence of fleas and blowflies were the predictors; standardised laying date was the covariate; and nest identity was the random factor; (2) for biting midges and black flies: clutch size, brood size and the number of fledglings were the dependent variables in separate models; forest, year and the prevalence of biting midges and black flies were the predictors; standardised laying date was the covariate; and nest identity was the random factor. We also used REML-LMMs to determine if the presence of ectoparasites had an impact on nestling biometry. We ran two separate REML-LMMs for the nestlings' body mass and tarsus length applying the following structure: (1) forest, the presence of fleas and the presence of blowflies were the predictors, standardised laying date the covariate and nest identity the random factor; (2) forest, the presence of biting midges and the presence of black flies were the predictors, standardised laying date the covariate and nest identity the random factor. The nestling tarsus length (log transformed) was also included as a covariate to control for structural size in the model for nestling body mass.

Breeding biology and nestling biometry An REML-LMM was used to assess whether laying date varied with forest type. In the final model, the standardised laying date was the dependent variable, nest identity was the random factor, and forest and year were the predictors. The interaction between forest and year was removed from the final model because it was not significant. We also used separate REML-LMMs to check whether clutch size, brood size, number of fledglings and percentage of eggs that produced fledglings (arcsine transformed) varied with forest type. In these cases, the final models had the following structure for each dependent variable: the nest identity was the random factor, forest and year were the predictors and standardised laying date was the covariate. Interactions between forest and year and between forest and laying date were removed from all models because none of them were significant. Lastly, separate REML-LMMs were also applied to examine the variation in nestling body mass (log transformed) and nestling tarsus length (log transformed) across the forests. In both cases, nest identity was the random factor, forest type the predictor and standardised laying date the covariate. However, in the model for nestling body mass, the nestling tarsus length (log transformed) was again included as a covariate to control for structural size.

Correlations between laying date and clutch size, brood size, number of fledglings and nestling body mass were examined using the Pearson product-moment correlation coefficient. We used the chi-squared test to check for inter-forest variability in hatching success and fledging success, given that these variables are frequencies. As the number of unhatched eggs and eggs that did not produce fledglings had left-skewed distributions, we used generalised linear mixed models (GLMM) with a Poisson distribution and a log link-function to study if the two parameters varied with forest type. We ran two separate models for each variable with the following structure: nest identity was the random factor, forest and year were the predictors and laying date was the covariate.

Adults biometry We used REML-LMMs to examine the variation in body mass and tarsus length of males and females depending on forest type. The log transformed body mass and log transformed tarsus length of males and females were the dependent variables in separate models, while forest and year were the predictors and nest identity was the random factor (the model for body mass also included log transformed tarsus length as a covariate). The effects of adult biometry on

reproduction were tested using REML-LMMs; all subsequent models were run separately for males and females. The models for adult body mass had the following structure: clutch size, brood size and number of fledglings were the dependent variables in separate models; nest identity was the random factor; forest and year were the predictors and standardised laying date, adult log body mass and adult log tarsus length were the covariates. The interactions between forest and year and between forest and laying date were removed from these models because they were not significant. As nestlings were only measured in one year, the variation in mean brood mass and standard deviation of nestling mass within broods against adult biometry was examined with linear models. Separate models took brood mass and its standard deviation as the dependent variables, forest type as the predictor and log adult body mass and log adult tarsus length as the covariates.

Results

Environmental differences between forest types

The mean orientations of the Holm oak and dry Pyrenean oak forests were 171.44° and 148.32°, respectively, while the Scots pine and humid Pyrenean oak forests were oriented at 190.42° and 237.66°, respectively (supplementary material, Fig. S1). Accordingly, the two forests on the southeastern slope (Holm oak and dry Pyrenean oak forests) received more solar radiation (approx. 0.15 kWh/m²/day) and had 1 h more of insolation per day than the forests on the southwestern slope (Scots pine and humid Pyrenean oak forests) (radiation: $\chi^2_3 = 23.74$, $p < 0.001$; insolation: $\chi^2_3 = 62.49$, $p < 0.001$; supplementary material, Figs. S2, S3 and S4; Table S1).

The four forest types differed in tree and shrub structure as well as land uses (supplementary material, Fig. S5; Table S2). The Holm oak forest was the most open, with the lowest percentage of dense tree cover (15.63%). The dry Pyrenean oak forest had a higher percentage of dense tree cover (approx. 65%), but less than the forests on the southwestern slope, which had the highest level of dense tree cover (approx. 82% in both the Scots pine and humid Pyrenean oak forests; supplementary material, Table S2). The NDVI differed between forest types ($\chi^2_3 = 21.49$, $p < 0.001$; supplementary material, Table S3), with the Holm oak forest presenting the lowest values (0.40 ± 0.004) and the Scots pine forest with the highest (0.66 ± 0.004). The

dry and humid Pyrenean oak forests were found to have intermediate NDVI values (0.59 ± 0.01 and 0.62 ± 0.003, respectively).

Differences in ectoparasite presence between forest types

Overall, 65 out of 154 nests (42.21%) were infested with fleas, with nest infestation rates differing between forest types (chi-squared test, $\chi^2_3 = 24.51$, $p < 0.001$; Fig. 2A). Only 9.09% of nests sampled in the Holm oak forest and 30.36% in the dry Pyrenean oak forest were infested with fleas, while they were found in 52.17% and 64.15% of the nests in the Scots pine and humid Pyrenean oak forests, respectively. Blowflies infested 100 out of 154 nests (64.94%). As observed for fleas, blowflies were less prevalent in the Holm oak (40.90% of nests infested) and dry Pyrenean oak (41.07%) forests than in the Scots pine (95.65%) and humid Pyrenean oak (86.79%) forests ($\chi^2_3 = 40.24$, $p < 0.001$; Fig. 2A). Considering only infested nests, there were no significant differences in blowfly intensity within each nest across the four forest types (Holm oak: 12.11 ± 4.30; dry Pyrenean oak: 12.78 ± 2.03; Scots pine: 15.09 ± 2.57; humid Pyrenean oak: 17.20 ± 1.42; Kruskal–Wallis test, $\chi^2_3 = 5.87$, $p = 0.118$).

Across all forest types, biting midges infested 48.05% and black flies 23.38% of nests (n = 77 for both parasites). In contrast to nest-dwelling ectoparasites, there were no differences between forests in the frequency of nest infestation by biting midges and black flies (in both cases, $\chi^2_3 > 5.09$, $p > 0.12$; Fig. 2B). Similarly, the intensity of biting midges and black flies did not vary between forest types (biting midges: $\chi^2_3 = 5.49$, $p = 0.139$; black flies: $\chi^2_3 = 4.33$, $p = 0.228$). Nor did the intensity of blowflies ($F_{1, 98} = 0.80$, $p = 0.37$), black flies ($F_{1, 39} = 0.21$, $p = 0.65$) and biting midges ($F_{1, 10} = 2.32$, $p = 0.20$) vary with laying date.

Flea-infested nests contained a significantly higher number of fledglings than uninfested nests (infested nests: 6.31 ± 0.15; uninfested nests: 5.17 ± 0.17; Table 1). The presence of blowflies, biting midges and black flies in blue tit nests was not significantly associated with clutch size, brood size or number of fledglings (Table 1). The presence of fleas, blowflies and black flies did not affect nestling tarsus length ($p > 0.05$ in all cases, data not shown for simplicity) or body mass ($p > 0.05$ in all cases, data not shown for simplicity), but the presence of biting midges had a negative impact on body mass (infested nests: 9.20 ± 0.09 g; uninfested nests: 10.15 ± 0.06 g; $\chi^2 = 6.19$, $p = 0.01$).

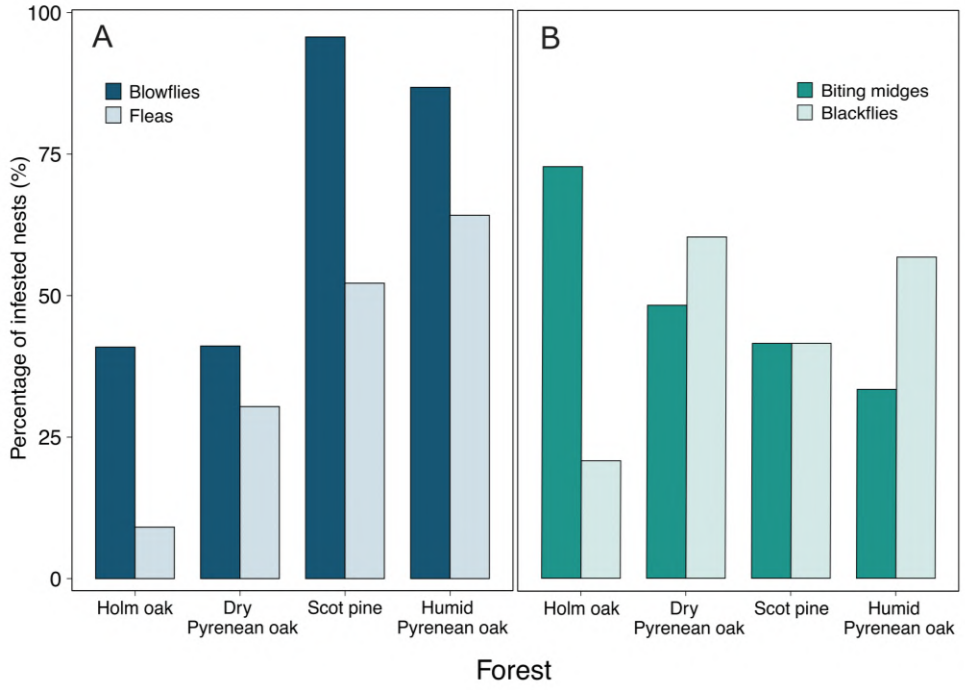

Figure 2. Percentage of blue tit nests infested by (A) nest-dwelling ectoparasites and (B) flying ectoparasites in the four forest types.

Forest-dependent variation in reproductive parameters

The laying date was later in the Holm oak and Scots pine forests than in the dry and humid Pyrenean oak forests ($\chi^2_3 = 9.89$, $p = 0.019$; Fig. 3; Table 2). Clutch size ranged from 3 to 15 eggs and the mode was 7 eggs (18.97%). After adjusting for the year and laying date, the largest clutches were in the humid Pyrenean oak forest and the smallest in the Scots pine forest, whilst intermediate values were found in the Holm oak and dry Pyrenean oak forests ($\chi^2_3 = 19.54$, $p < 0.001$; Fig. 4A; Table 2). Across all forests, clutch size decreased with laying date ($\chi^2 = 62.41$, $p < 0.001$), as they showed a negative correlation ($r = -0.34$, $p < 0.001$). In total, 10 out of 172 clutches failed to hatch (i.e. none of the eggs hatched). There was no inter-forest variability in hatching success ($\chi^2_3 = 0.49$, $p = 0.92$; Table 2) or the number of unhatched eggs per nest, which also showed no variation with laying date (forest: $\chi^2_3 = 2.26$, $p = 0.52$; laying date: $\chi^2 = 0.68$, $p = 0.41$; Table 2).

Table 1. Results of the linear mixed-effects models (LMM) for clutch size, brood size and number of fledglings for the blue tit (*Cyanistes caeruleus*) population in the Sierra Nevada National Park, southern Spain, with the prevalence of different ectoparasites: blowflies, fleas, biting midges and black flies. Statistical significance was set at $p < 0.05$ (marked in bold).

Predictor	Dependent variable								
	Clutch size			Brood size			Number of fledglings		
	Wald χ^2	df	*p*-value	Wald χ^2	df	*p*-value	Wald χ^2	df	*p*-value
Models for nest-dwelling ectoparasites									
Forest	17.73	3	**< 0.001**	15.52	3	**0.001**	17.56	3	**< 0.001**
Year	54.50	1	**< 0.001**	24.48	1	**< 0.001**	16.25	1	**< 0.001**
Laying date	41.86	1	**< 0.001**	18.47	1	**< 0.001**	16.22	1	**< 0.001**
Blowflies	0.33	1	0.56	2.92	1	0.09	3.03	1	0.08
Fleas	0.08	1	0.77	2.31	1	0.13	5.42	1	**0.02**
Models for flying ectoparasites									
Forest	13.25	3	**0.004**	14.43	3	**0.002**	7.61	3	**0.050**
Year	19.06	1	**< 0.001**	10.86	1	**< 0.001**	7.89	1	**0.005**
Laying date	1.16	1	0.28	0.69	1	0.41	0.63	1	0.43
Biting midges	0.64	1	0.42	3.25	1	0.07	0.30	1	0.58
Black flies	0.42	1	0.51	0.91	1	0.34	0.95	1	0.33

Brood size ranged from 0 to 10 with a mode of 6 nestlings (19.77%). There were no statistically significant differences in brood size between forest types, but it tended to follow the same pattern as clutch size ($\chi^2_3 = 6.52$, $p = 0.089$; Fig. 4B; Table 2). As observed for clutch size, brood size also decreased with the laying date in all forests ($r = -0.26$; $\chi^2 = 25.62$, $p < 0.001$).

Of all the nests that produced at least one nestling (161 out of 171), only seven (4.35%) did not manage to raise any nestlings. Causes of nestling mortality in these seven cases included predation (n = 1), nest abandonment by parents (n = 1), starvation (n = 3) and unidentified causes (n = 2). Fledging success did not vary between forest types ($\chi^2_3 = 1.99$, $p = 0.57$; Table 2). Over the two years, the percentage of eggs that produced fledglings did not differ between forest types ($\chi^2_3 = 1.13$, $p = 0.77$; Table 2), nor was it affected by laying date ($\chi^2 = 0.02$, $p = 0.89$).

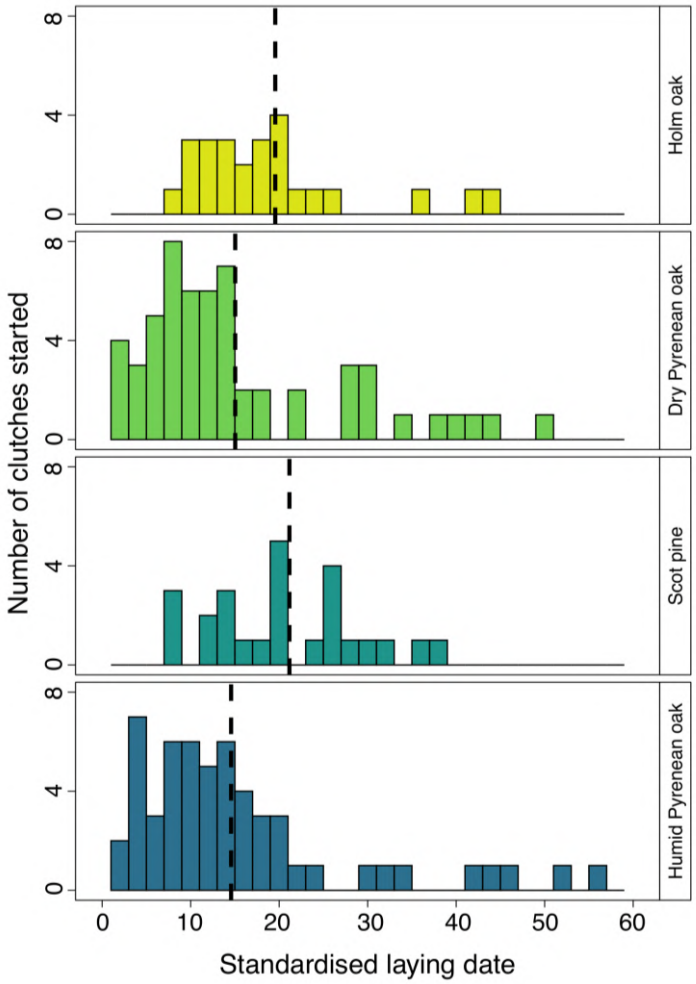

Figure 3. Frequency distribution of blue tit pair laying dates in the four forest types. Standardised laying dates are the differences between the actual laying date for each nest and the laying date of the first egg laid in each year (day 0 = first egg laid each year). Laying dates are shown as 10 day periods. Dashed line indicates the mean.

However, when considering all nests, the number of fledglings per nest showed the same pattern of variation between forests as clutch size (χ^2_3 = 8.58, p = 0.035; Fig. 4C). Blue tits breeding in the humid Pyrenean oak forest had the highest number of fledglings (5.92), while those in the Scots pine forest had the lowest productivity (4.44), with intermediate values recorded for the Holm oak (5.08) and dry Pyrenean oak forests (4.58) (Table 2). As for clutch and brood size, the number of fledglings

decreased with laying date in all forest types ($r = -0.20$; $\chi^2 = 14.81$, $p < 0.001$). There was no variation in the number of eggs that failed to produce a fledgling across the forest types ($\chi^2_3 = 1.90$, $p = 0.59$) or in terms of the laying date ($\chi^2 = 3.08$, $p = 0.08$; Table 2).

Neither the tarsus-corrected body mass ($\chi^2_3 = 0.58$, $p = 0.90$) nor the tarsus length of nestlings ($\chi^2_3 = 2.41$, $p = 0.49$) differed between forest types (Table 2). Nestling body mass, but not tarsus length, diminished with the laying date in all forests (body mass: $\chi^2 = 13.18$, $p < 0.01$; $r = -0.35$, $p < 0.01$; tarsus length: $\chi^2 = 0.35$, $p = 0.56$; $r = -0.06$, $p = 0.22$).

Table 2. Reproductive parameters for blue tits (*Cyanistes caeruleus*) and biometric measurements of blue tit nestlings in four forest types in the Sierra Nevada National Park, southern Spain. Reproductive parameters include data for 2017 and 2018, but nestling biometry is for 2017 only. The table shows the mean values, standard error (s.e.) and sample size (n) in parentheses.

Parameter	Holm oak	Dry Pyrenean oak	Scots pine	Humid Pyrenean oak
Laying date [a]	19.56 ± 0.72 (25)	15.25 ± 0.91 (60)	21.16 ± 0.64 (25)	14.57 ± 0.99 (61)
Clutch size	7.42 ±0.11 (26)	6.89 ± 0.14 (62)	5.88 ± 0.07 (25)	7.74 ± 0.14 (61)
Brood size	5.72 ± 0.18 (25)	5.39 ± 0.18 (62)	5.04 ± 0.13 (25)	6.35 ± 0.19 (60)
Number of fledglings	5.08 ± 0.21 (26)	4.58 ± 0.20 (59)	4.44 ± 0.14 (25)	5.92 ± 0.19 (61)
Hatching success (%)	92.0 ± 0.02 (25)	93.6 ± 0.02 (62)	96.0 ± 0.01 (25)	95.0 ± 0.02 (60)
Number of unhatched eggs	1.72 ± 0.18 (25)	1.50 ± 0.17 (62)	0.84 ± 0.09 (25)	1.26 ± 0.13 (60)
Number of eggs that failed to produce a fledgling	2.34 ± 0.21 (26)	2.22 ± 0.18 (59)	1.44 ± 0.13 (25)	1.82 ± 0.15 (61)
% eggs that produced fledglings [b]	81.74 ± 0.01 (22)	76.20 ± 0.02 (52)	81.10 ± 0.02 (23)	81.08 ± 0.02 (57)
Fledging success (%)	84.62 ± 0.03 (26)	88.14 ± 0.02 (59)	92.00 ± 0.02 (25)	93.44 ± 0.02 (61)
Nestling body mass (g)	9.79 ± 0.05 (76)	9.83 ± 0.05 (145)	9.47 ± 0.05 (47)	10.09 ± 0.04 (176)
Nestling tarsus length (mm)	16.52 ± 0.03 (76)	16.44 ± 0.03 (144)	16.25 ± 0.03 (47)	16.47 ± 0.03 (176)

[a] 0 = day the first egg was laid each year.

[b] Nests without fledglings (predated, deserted, etc.) were excluded.

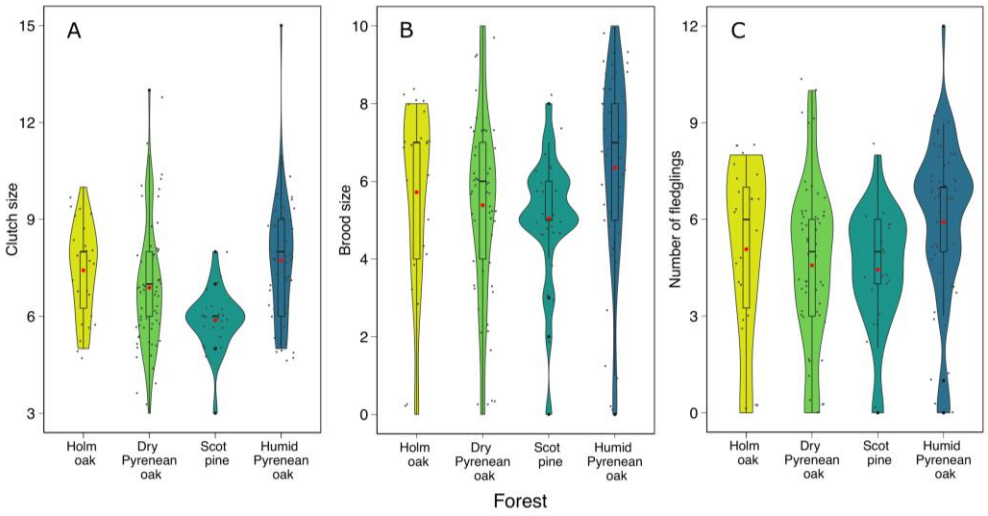

Figure 4. Variation in blue tit clutch size (A), brood size (B) and number of fledglings (C) across the four forest types. The red point shows the mean for each forest, the horizontal line the median and the boxplot represents the interquartile range. The kernel density plot shows the probability density of data at different values. *Note:* the maximum number of fledglings is higher than the maximum brood size in the humid Pyrenean oak forest because brood size could not be recorded in a nest with a clutch of 15 eggs.

Effect of adult biometry on breeding success

As seen with nestlings, the tarsus-corrected body mass (males: $\chi^2_3 = 4.77$, $p = 0.19$; females: $\chi^2_3 = 1.56$, $p = 0.67$) and tarsus length of adults (males: $\chi^2_3 = 1.59$, $p = 0.66$; females: $\chi^2_3 = 3.79$, $p = 0.28$) did not differ between forest types. The body masses of both males and females did not correlate with clutch or brood size ($p > 0.05$ in all cases, data not shown for simplicity), but significant associations were found for the number of fledglings. Adults that produced more fledglings weighed less when they were measured (when nestlings were 8–11 days old; males: $\chi^2 = 4.80$, $p = 0.028$; females: $\chi^2 = 8.00$, $p = 0.005$; Fig. 5). There was no relationship between the tarsus length of females and clutch size, brood size or the number of fledglings ($p > 0.05$ in all cases, data not shown for simplicity). However, males whose females laid larger clutches had smaller tarsi ($\chi^2 = 6.62$, $p = 0.01$). The body mass and tarsus length of adult males and females showed no variation with brood mass or the standard deviation of nestling mass within broods ($p > 0.05$ in all cases, data not shown for simplicity).

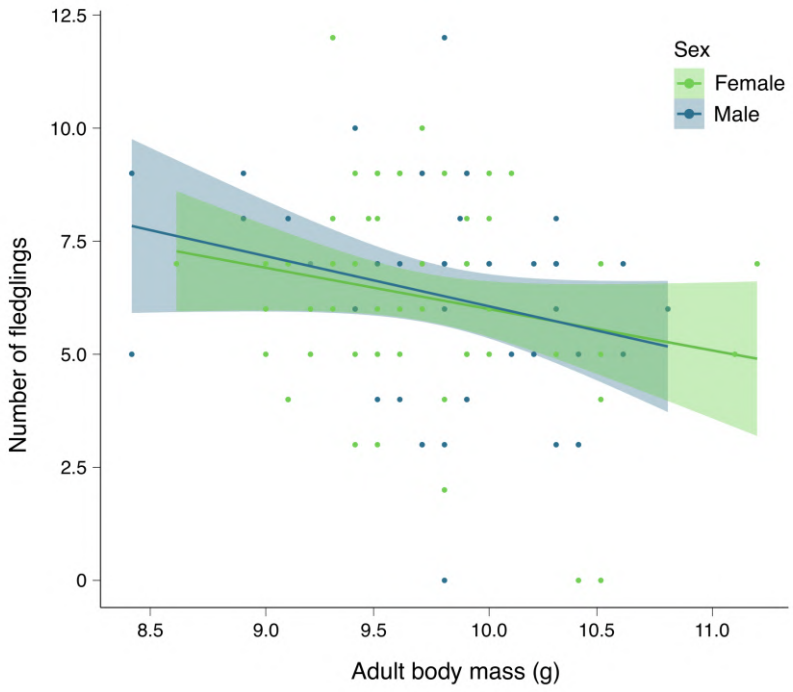

Figure 5. Relationship between the body mass (g) of adult blue tits (*Cyanistes caeruleus*) and the number of fledglings produced depending on sex (green: females, blue: males). The shaded areas correspond to the 95% confidence interval.

Discussion

Mediterranean environments are typically characterised by high spatial heterogeneity, which means species face a wide range of selective pressures in small areas (Blondel et al. 2010). In our study area, for example, the blue tits reproduced in a small, continuous woodland formed by four well-differentiated forestry formations that were characterised by both their solar radiation and their tree cover and composition. The woodland was located across two mountain slopes that were separated by a river (Chico river), hence the west-facing slope — which was also crossed by a stream (Almiar stream) — received less solar radiation than the east-facing slope. The east-facing slope was composed of Holm and Pyrenean oaks, while the west-facing slope contained Scots pines and Pyrenean oaks, thus providing a more humid and productive environment. Given that water availability is a limiting factor on tree productivity in Mediterranean habitats (Príncipe et al. 2022), the higher

productivity of the humid Pyrenean oak forest should be reflected in a greater percentage of dense tree cover (as observed), which could presumably support more caterpillars, the key food source for blue tits during spring (Blondel et al. 1991; Bańbura et al. 1999; Tremblay et al. 2005). Indeed, in a previous study we found that caterpillar abundance was higher in the humid Pyrenean oak forest on the west-facing slope than the dry Pyrenean oak forest on the east-facing slope (Garrido-Bautista et al. 2021). Unfortunately, the same information was not recorded for the years included in this study. The Scots pine forest, however, was expected to produce fewer caterpillars despite its high tree cover because new leaves develop more slowly in sclerophyllous trees (Orshan 1989) and pine needles contain a relatively high concentration of tannins (Achotegui-Castells et al. 2013), so these forests only support low caterpillar populations (Tremblay et al. 2003). Overall, the study area's particular topography and vegetation structure create a microscale geographic variation similar to the 'evolution canyon' models described by Nevo (2006, 2009, 2012), in which opposite, yet still closely neighbouring, slopes display marked microclimatic and biotic contrasts with potentially different eco-evolutionary processes developing on each slope (see below).

The aforementioned inter-forest environmental differences were expected to modulate blue tit breeding performance, specifically, we believed females would lay larger clutches in more productive forests. As predicted, the clutch size and number of fledglings per nest were highest in the humid Pyrenean oak forest and lowest in the coniferous Scots pine forest, a pattern to be expected based on previous evidence (Blondel et al. 1987, 1991; Dias et al. 1994; Fargallo & Johnston 1997; Tremblay et al. 2003, 2005; Lambrechts et al. 2004; Blondel 2007). Besides the low production of caterpillars, coniferous forests also impose foraging costs to blue tits (Díaz et al. 1998), consequently increasing the impacts on the blue tit reproductive output. However, despite statistically different clutch sizes between forest types (ranging from approximately 6 to 8 eggs on average), the fledging success was similar in all forests; with a range of 76–82% of eggs producing fledglings and 1.4–2.3 eggs per clutch failing to produce fledglings. Nestling body mass and tarsus length, predictors of nestling survival and recruitment in the population (Nur 1984a; Blondel et al. 1998; Charmantier et al. 2004), did not differ between forest types, which suggests that the blue tits produced similar quality fledglings in each forest. Although there was a tendency on the east-facing slope to lose one more egg per nest than the west-facing

slope, our results indicate that the blue tits successfully adjusted their reproductive effort to the rearing conditions in the forest where they bred. For example, females started laying eggs earlier in deciduous (dry and humid Pyrenean oak) forests than in evergreen (Holm oak) and coniferous (Scots pine) forests, probably because spring development of caterpillars occurs later in evergreen forests (Blondel et al. 1999; Tremblay et al. 2003). Also, when comparing the two deciduous forests, blue tits in the dry Pyrenean oak forest laid 6.9 eggs producing 4.6 fledglings, whereas birds in the humid Pyrenean oak forests laid 7.7 eggs and produced 5.9 fledglings. Hence, on average blue tit pairs lose two eggs/nestlings per clutch in both forests and in fact, in our study area, two eggs per brood typically hatched asynchronously with a hatching spread of two days (unpublished data). These late-hatched nestlings are therefore apparently marginal and have a low reproductive value for parents (Forbes et al. 1997; Stenning 2008).

In summary, our results showed that, in relative terms, the fledging success of blue tits did not differ between the four forest types, but fitness — measured quantitatively as the number of fledglings per nest — did. The optimal clutch size, which can be indirectly estimated from a population's average clutch size (Liou et al. 1993), for example, appears to be the highest in the humid Pyrenean oak forest, with one more egg on average than the dry Pyrenean oak forest and two more than the Scots pine forest. This pattern may have emerged because of individual optimization of breeding performance, which means that individuals are able to produce the number of eggs, or nestlings, they can successfully rear themselves based on individual-specific condition (Perrins & Moss 1975; Pettifor et al. 1988, 2001; Pettifor 1993), but also on the spatial and temporal variability in food availability in the rearing environment (van Balen 1973; Blondel et al. 1991; Tremblay et al. 2003, 2005). Further cross-fostering studies altering brood sizes should be conducted to identify the optimal clutch size for each forest. Still, optimal clutch sizes from blue tit populations of the Mediterranean basin are expected to be lower than those populations from higher latitudes, mainly as consequence of habitat-specific constraints in food availability (e.g. Blondel et al. 2006; Ziane et al. 2006; Charmantier et al. 2016). An important question still remains as to what extent this variation in clutch size, which ultimately determines the number of fledglings a blue tit pair can raise (see results), is due to phenotypic plasticity or a local adaptation process.

The different production of fledglings from all blue tit pairs breeding in each habitat could determine the overall reproductive output within each forest type, which may promote source-sink population dynamics. Although large geographical distances between populations that limit gene exchange typically lead to population structuring (Slatkin 1987), microscale population differentiation can occur between closely located populations of migrant and resident bird species (Garant et al. 2005; Postma & van Noordwijk 2005; Blondel et al. 2006; Senar et al. 2006; Ortego et al. 2011; Arnoux et al. 2014; García-Navas et al. 2014; Camacho et al. 2016). Individual dispersal range is an important factor in the genetic population structure of passerines (Blondel et al. 2006). The Blue tit is a highly mobile passerine, but it is reported to have a relatively low dispersal capacity that follows a non-random pattern. Females disperse over longer distances than males (Ortego et al. 2011), but they reduce their dispersal distance if hatching success was high in the preceding breeding season (García-Navas & Sanz 2011), which means females with low dispersal distances have higher offspring recruitment rates (García-Navas et al. 2014). Therefore, if low immigration rates and selection against immigrants takes places in a meta-population (e.g. Postma & van Noordwijk 2005), then philopatric females can obtain more local recruits than immigrant counterparts (García-Navas et al. 2014), which may encourage genetic population structuring and local adaptation. The fine-scale genetic population structuring process may be accompanied by phenotypic divergence in reproductive strategies (Blondel et al. 1999; Postma & van Noordwijk 2005) or morphological traits (Blondel et al. 1999; Garant et al. 2005; Senar et al. 2006; Camacho et al. 2013, 2016). The data revealed that blue tits in our study area had low dispersal ranges, with individuals being recruited locally within their own slope (only one individual showed a dispersing behaviour between slopes), and that there was a well-established and significant genetic population structure between the two woodland slopes (microsatellite-based analysis: F_{ST} = 0.016, p < 0.001; Garrido-Bautista et al. submitted). However, in contrast to other passerine meta-populations (Garant et al. 2005; Senar et al. 2006; Camacho et al. 2013, 2016), we did not detect an inter-forest phenotypic differentiation in morphometry. The lack of any inter-forest differences in female body mass (which might have explained the different clutch sizes; Haywood & Perrins 1992) also suggests that the between-forest variation in the reproductive strategies of the blue tits inhabiting our 'evolution canyon'

woodland were due to a fine-scale, local population adaptation process rather than plastic responses.

Furthermore, we found a higher prevalence of nest-dwelling ectoparasites (fleas and blowflies) in the forests on the west-facing slope, i.e. the humid Pyrenean oak forest and the Scots pine forest, than those on the opposite slope. As nest-dwelling ectoparasites feed on nestlings, the number of nestlings in a nest is expected to modulate the presence of such parasites in the different habitats (Hurtrez-Boussès et al. 1999; Arriero et al. 2008); we duly found that nests housing more fledglings were more frequently parasitised by fleas. Environmental factors, such as ambient temperature and humidity, may also play an important role in the presence of nest-dwelling ectoparasites in nests within a given habitat (Heeb et al. 2000; Castaño-Vázquez et al. 2018, 2021; Garrido-Bautista et al. 2020; Mennerat et al. 2021; Moreno-Rueda 2021). A combination of these factors could explain why fleas and blowflies were more common in nests occupying the west-facing slope than those on the east-facing slope, as the latter produced fewer nestlings and a drier environment for ectoparasites. Both fleas and blowflies are known to have an impact on nestling blood physiology, growth and survival (Merino & Potti 1995; Hurtrez-Boussès et al. 1997; Puchala 2004; Pitala et al. 2009; Brommer et al. 2011), but they may also affect several current and future reproductive metrics, such as egg size (Potti 2008), number of fledglings (Lemoine et al. 2012) and lifetime reproductive success (Fitze et al. 2004). Thus, we expected the nests on the west-facing slope to suffer more parasitism, in terms of reproduction costs, than nests on the opposite slope. In previous studies, we found that nestlings did not suffer from any ectoparasite-induced physiological costs, neither to the immune system (Garrido-Bautista et al. 2022b) nor in terms of oxidative status (Garrido-Bautista et al. 2021), and we did not detect any effect of ectoparasites on nestling survival or body size in the present work. The scant effect of ectoparasites on reproductive output and offspring condition across the woodland suggest that the subjects experienced low or negligible parasite-imposed costs. Blue tits can tolerate ectoparasites in their nests by increasing feeding rate (Johnson & Albrecht 1993; Christe et al. 1996; Tripet & Richner 1997), but the parents' body mass may decline if they produce a lot of fledglings, which could impair winter survival or subsequent breeding attempts (Nur 1984b). In effect, in our study, parent body mass decreased with the number of fledglings produced. Nevertheless, further experiments

designed to alter the ectoparasite load in the two slopes would help elucidate the underlying mechanisms (Lemoine et al. 2012).

On the other hand, and in contrast with nest-dwelling ectoparasites, there was no variation in the prevalence of biting midges and black flies between forests. We would expect the presence of biting midges and black flies to be determined mainly by environmental factors, as these vectors are only sporadic visitor in nests for blood meals. The type of forest and environmental conditions do indeed have a significant effect on the biting midges and black flies found inhabiting an area, which ultimately determines their abundance in bird nests (Tomás et al. 2008b, 2020; Martínez-de la Puente et al. 2009, 2010). In fact, in a previous study, we showed that forest type is the main determinant of biting midge abundance and there was no association between nestling characteristics and the number of flies entering nests (Garrido-Bautista et al. 2022a). Both biting midges and black flies are known to reduce nestling condition and body mass (Tomás et al. 2008b; Martínez-de la Puente et al. 2010), and our results reaffirmed this observation as we found a negative correlation between biting midges in nests and nestling body mass. This association may be due to the direct effects of biting midges (e.g. draining blood resources and causing skin inflammation) or the indirect impact of the blood-borne parasites they can transmit, such as avian malaria-like disease caused by *Haemoproteus* (Martínez de-la Puente et al. 2011), which can produce anaemia and decreased body mass (Tomás et al. 2008b; Martínez-de la Puente et al. 2010). On the other hand, and to the best of our knowledge, there is no evidence to suggest that these parasites have an impact on adult reproductive performance, probably because of their ephemeral activity within bird nests and weak evolutionary influence on avian reproduction in contrast to nest-dwelling or blood parasites (e.g. Martin et al. 2001).

Conclusions

Our results suggest that, in highly heterogeneous Mediterranean environments, blue tits adapt to different environmental conditions across a woodland by adjusting clutch size to an optimal level for reproductive success in each habitat. Across four types of forest with different environmental conditions within a single woodland, blue tits differed in clutch size but fledging success and fledgling tarsus length and body mass were very similar. Specifically, blue tits were most productive (i.e. in terms of the

number of fledglings) in a deciduous forest with a humid environment. However, pairs nesting in lower-quality forests reduced their clutch size, and hence the number of fledglings reared, but still maintained a similar level of fledging success and nestling quality.

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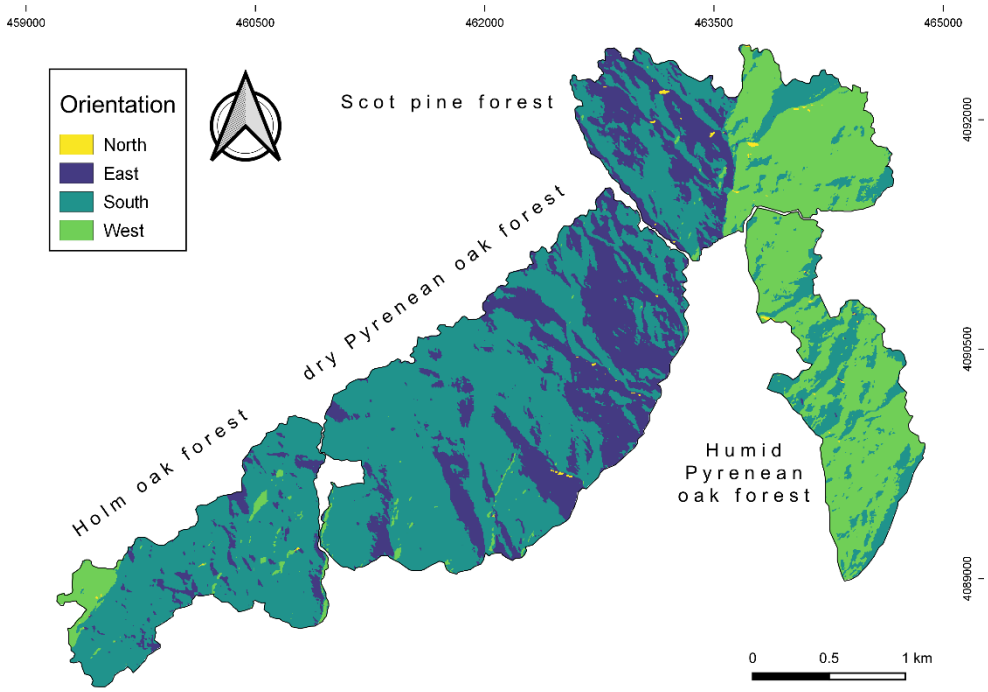

Figure S1. Mean forest orientation in degrees. Pixel values represent: North (315–45°), East (45–135°), South (135–225°) and West (225–315°).

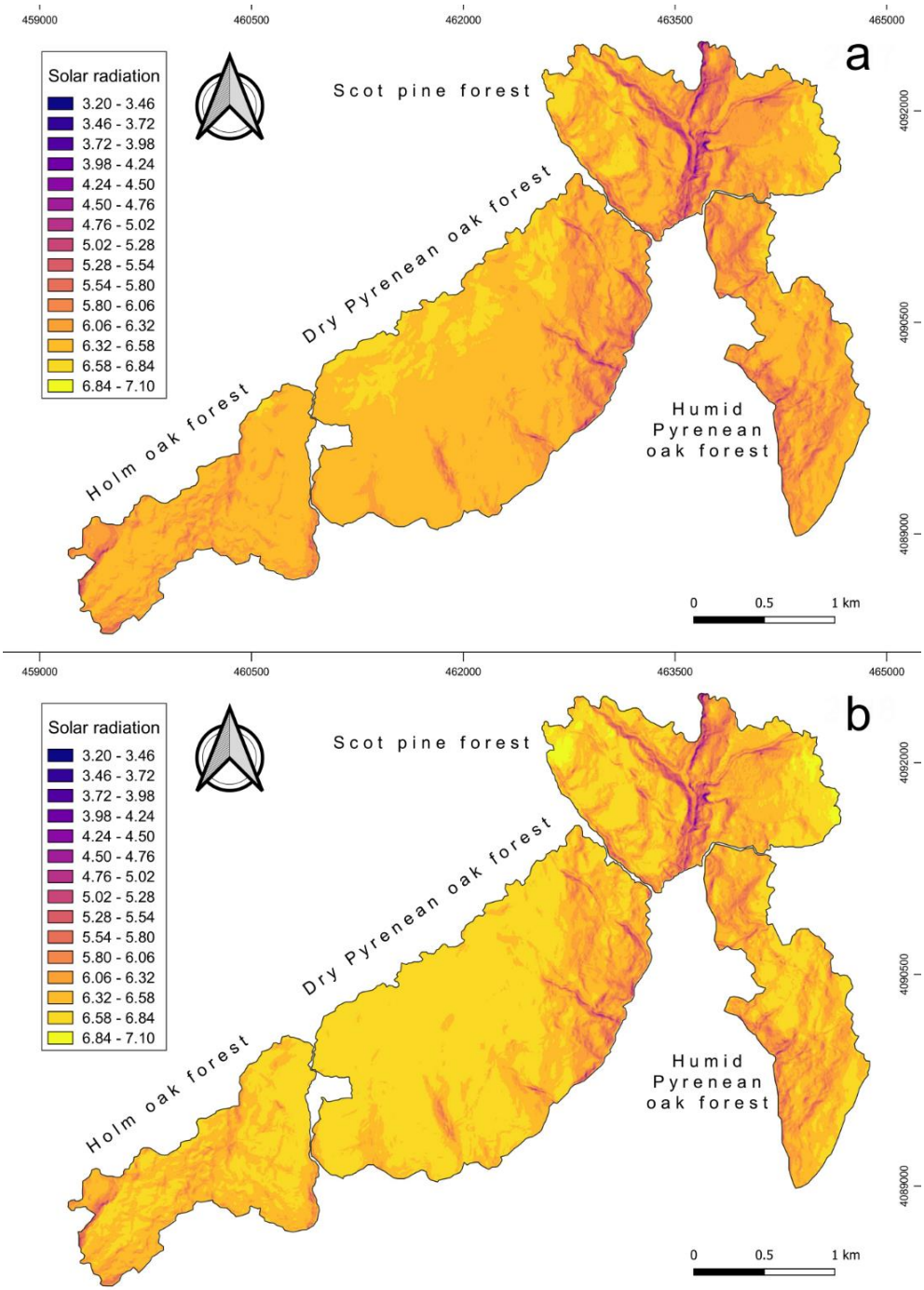

Figure S2. Mean forest solar radiation during the breeding seasons of 2017 (a) and 2018 (b). Units are expressed in kWh/m²/day.

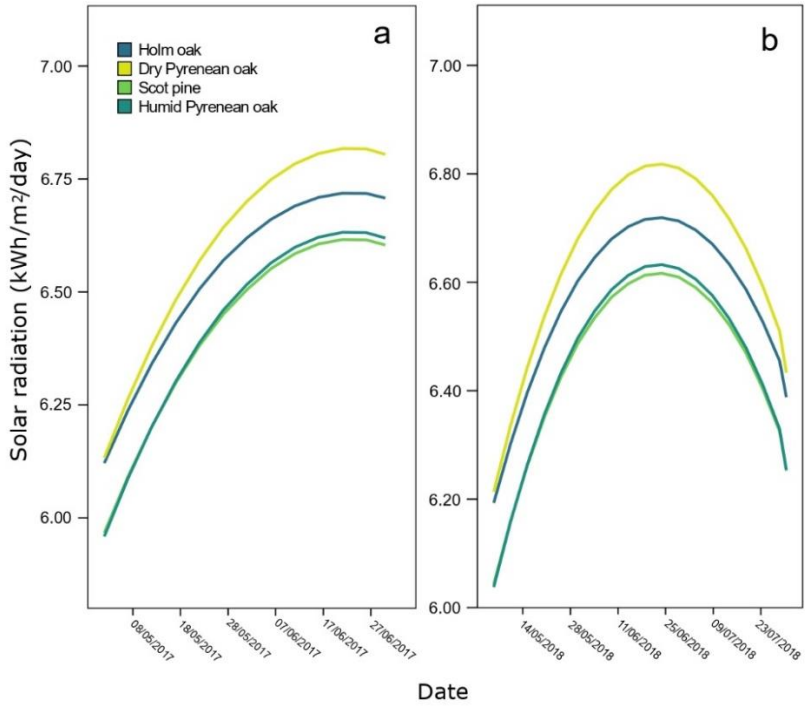

Figure S3. Mean forest solar radiation in five-day periods during the breeding seasons of 2017 (a) and 2018 (b). Units are expressed in kWh/m²/day.

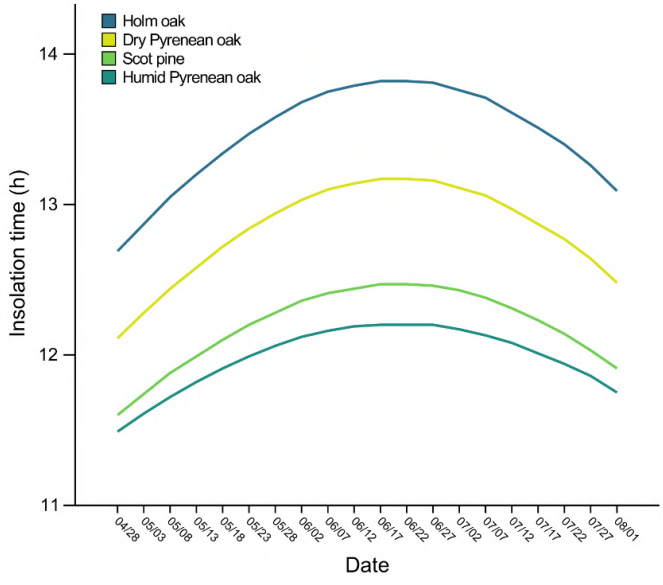

Figure S4. Mean forest insolation time per day in five-day periods during the whole breeding season.

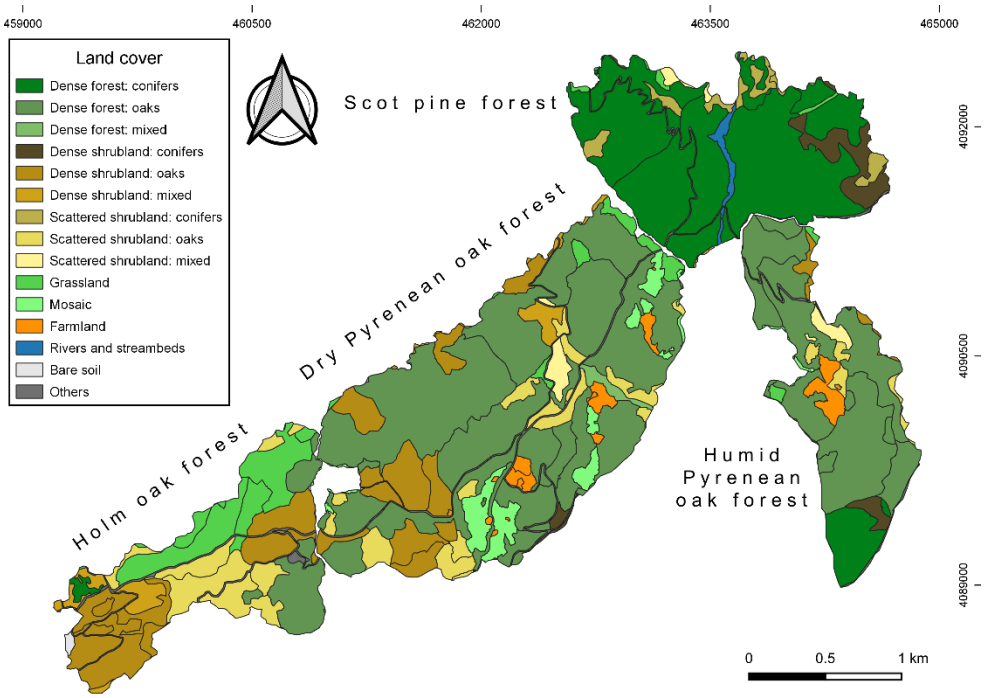

Figure S5. Proportions of land cover in the four forest types. Land use categories have been extracted from the SIOSE Spanish program (information about the methodology and categorization of land uses can be found in the webpage of SIOSE).

Table S1. Mean solar radiation (kWh/m²/day) and insolation time per day (h) for the four forest types during the breeding seasons of 2017 and 2018. Table shows the mean, the standard deviation (s.d.) and the range in parentheses.

	Holm oak	Dry Pyrenean oak	Scots pine	Humid Pyrenean oak
Solar radiation				
2017	6.34 ± 0.19 (4.55–6.64)	6.40 ± 0.23 (4.40–6.68)	6.22 ± 0.39 (3.23–6.83)	6.23 ± 0.25 (4.45–6.67)
2018	6.50 ± 0.19 (4.67–6.81)	6.56 ± 0.23 (4.49–6.85)	6.38 ± 0.40 (3.32–7.00)	6.38 ± 0.26 (4.58–6.84)
Insolation time	12.83 ± 0.32 (12.11–13.17)	13.46 ± 0.34 (12.69–13.82)	12.19 ± 0.26 (11.60–12.47)	11.98 ± 0.21 (11.49–12.20)

Table S2. Area and proportion of land uses of the four forest types. Table shows the area (ha) and percentage (%; in parentheses). See the main manuscript and Fig. S5 for explanations of methodology and categorization of land uses.

Land use	Holm oak	Dry Pyrenean oak	Scots pine	Humid Pyrenean oak
Dense forest: confiers	1.74 (1.26)	0.74 (0.21)	148.96 (81.92)	13.65 (10.77)
Dense forest: oaks	19.81 (14.37)	228.12 (64.93)	0.11 (0.06)	91.24 (71.99)
Dense forest: mixed	0 (0)	2.92 (0.83)	0 (0)	0 (0)
Dense shrubland: confiers	0 (0)	1.47 (0.42)	12.39 (6.81)	2.76 (2.18)
Dense shrubland: oaks	43.97 (31.89)	41.97 (11.95)	0 (0)	2.82 (2.23)
Dense shrubland: mixed	5.01 (3.64)	5.38 (1.53)	0.03 (0.02)	0 (0)
Scattered shrubland: conifers	0 (0)	0 (0)	10.21 (5.62)	0 (0)
Scattered shrubland: oaks	27.29 (19.79)	24.61 (7.00)	0 (0)	4.25 (3.35)
Scattered shrubland: mixed	0 (0)	4.09 (1.16)	1.47 (0.81)	2.83 (2.23)
Grassland	34.13 (24.75)	7.19 (2.05)	1.93 (1.06)	1.30 (1.02)
Mosaic	0.12 (0.09)	20.44 (5.82)	0 (0)	0.58 (0.46)
Bare soil	0.69 (0.50)	0 (0)	0 (0)	0 (0)
Rivers and streambeds	0 (0)	0 (0)	3.55 (1.95)	0 (0)
Farmland [a]	0 (0)	7.77 (2.21)	0 (0)	5.57 (4.39)
Others [b]	5.13 (3.72)	6.60 (1.88)	3.18 (1.75)	1.74 (1.37)
Total area	137.88 (100)	351.31 (100)	181.84 (100)	126.74 (100)

[a] Includes farmlands, irrigation ponds and livestock lands.

[b] Includes forest tracks, firebreaks and forestry facilities.

Table S3. Normalized difference vegetation index (NDVI) of the four forest types during the breeding seasons of 2017 and 2018. Table shows the mean, the standard deviation (s.d.) and the range in parentheses.

Date	Holm oak	Dry Pyrenean oak	Scots pine	Humid Pyrenean oak
2017				
02/05/2017	Cloud cover 4.89%			
18/05/2017	Cloud cover 15.21%			
03/06/2017	0.41 ± 0.09 (0.16–0.75)	0.60 ± 0.12 (0.11–0.81)	0.57 ± 0.19 (0.04–0.81) [a]	0.63 ± 0.10 (0.24–0.80)
19/06/2017	0.35 ± 0.07 (0.16–0.64)	0.46 ± 0.11 (0.24–0.79)	0.65 ± 0.07 (0.26–0.79)	0.62 ± 0.11 (0.22–0.84)
2018				
05/05/2018	Cloud cover 40.03%			
21/05/2018	Cloud cover 56.82%			
06/06/2018	Cloud cover 73.79%			
22/06/2018	0.42 ± 0.08 (0.18–0.70)	0.60 ± 0.08 (0.33–0.76)	0.62 ± 0.06 (0.29–0.75)	0.60 ± 0.08 (0.27–0.74)
08/07/2018	0.42 ± 0.10 (0.14–0.75)	0.61 ± 0.10 (0.25–0.80)	0.68 ± 0.08 (0.27–0.86)	0.62 ± 0.10 (0.22–0.82)
24/07/2018	0.41 ± 0.11 (0.15–0.71)	0.60 ± 0.10 (0.23–0.81)	0.68 ± 0.08 (0.25–0.88)	0.61 ± 0.11 (0.19–0.84)

[a] Approximately 10% of the extension of the Scots pine forest was covered by clouds. This value was excluded from the statistical analysis.

Chapter 7

Fine-scale genetic structure and phenotypic divergence of a passerine bird population inhabiting a continuous Mediterranean woodland

ABSTRACT Genetic differentiation between populations inhabiting ecologically different habitats might appear because of limited dispersal and gene flow, which may lead to patterns of phenotypic divergence and local adaptation. In this study, we use dispersal, genotypic (24 microsatellite loci), and phenotypic (body size and clutch size) data to analyze possible patterns of genetic population structuring and phenotypic divergence in a blue tit (*Cyanistes caeruleus*) population inhabiting a continuous and heterogeneous woodland along a valley. The two slopes of the valley have different forest formations and are markedly different in terms of environmental conditions (especially the humidity degree). Natal dispersal showed that most blue tits reproduced within their natal slope. Accordingly, microsatellite analyses revealed the presence of two genetic clusters along the woodland, with blue tits of each genetic cluster associated to the slope they inhabit. The two genetic populations showed divergence in clutch size, exceeding the level of differentiation expected based on genetic drift, hence suggesting divergent selection on this life-history trait. Our findings reveal that restricted dispersal and spatial heterogeneity may lead to genetic differentiation among bird populations at a surprisingly small scale. It is worth highlighting that such differentiation occurs for an organism with high dispersal capacity (a songbird) and within a continuous woodland. Moreover, we show that small-scale ecological differences, together with limited gene flow, can result in selection favoring different phenotypes even within the same continuum population and without any geographical barrier.

Keywords: Blue tit, *Cyanistes caeruleus*, dispersal, neutral divergence, population differentiation

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Introduction

Dispersal is a key life-history trait playing a crucial role in the evolutionary dynamics of species and determining the levels of gene flow among populations (Clobert et al. 2012). Limited or restricted gene flow due to geographical barriers often has been regarded as the main cause of genetic divergence (Slatkin 1987). Indeed, simulation and empirical data show that genetic differentiation between populations typically increases with geographical distance (the so-called isolation by distance; Slatkin 1993; Hutchison & Templeton 1999; Clegg & Phillimore 2010). However, patterns of genetic structuring also emerge at a small scale because dispersal, and consequently gene flow, can be non-random (reviewed in Edelaar & Bolnick 2012) or because landscape configuration and local ecological conditions may limit gene flow in several ways (e.g. Coulon et al. 2006; Quéméré et al. 2010; Ferrer et al. 2016a). Limited gene flow allows natural selection to adapt a population to local environmental conditions, given that gene flow is assumed to introduce maladaptive foreign alleles into locally adapted populations (Lenormand 2002; Bolnick & Nosil 2007). Nonetheless, adaptive divergence among populations may occur even at intermediate levels of gene flow (Garant et al. 2007, and references therein).

When dispersing from their natal territory, individuals have to cope with new environmental conditions to survive and reproduce in an ecologically different habitat to which they are not adapted (Nosil et al. 2005; Postma & van Noordwijk 2005; Garant et al. 2007). Accordingly, it is expected that selection against immigrant genes would reduce gene flow among populations experiencing contrasting ecological conditions, thus leading to genetic differentiation between populations, phenotypic divergence and local adaptation (Blondel et al. 1999; Garant et al. 2005; Postma & van Noordwijk 2005; Coulon et al. 2008; Porlier et al. 2012; García-Navas et al. 2014a). Population fragmentation because of restricted gene flow can favor the maintenance of local adaptations when genetic diversity is high enough (Smith et al. 2005; Milá et al. 2009). On the other hand, genetic differentiation may reduce the local genetic diversity and impact local adaptation if the effective population size is small (reviewed in Willi et al. 2006). Local adaptation could also be constrained by immigrant genes from marginal and poor-quality habitats (Dhondt et al. 1990; Blondel et al. 2006), and dispersal can benefit populations by reducing close inbreedings as well as the

consequent loss of genetic diversity (Keller et al. 2001; Szulkin & Sheldon 2008; García-Navas et al. 2014a).

Some studies evidence that non-random dispersal and genetic population differentiation can occur between neighboring populations of highly motile animals, such as birds, in some cases with important consequences for phenotypic divergence (Garant et al. 2005; Postma & van Noordwijk 2005; Blondel et al. 2006; Senar et al. 2006; Björklund et al. 2010; Ortego et al. 2011; Porlier et al. 2012; Arnoux et al. 2014; García-Navas et al. 2014a; Camacho et al. 2016; Ferrer et al. 2016a; Menger et al. 2017; Recuerda et al. 2023). For example, a genetic structuring process may be accompanied by a phenotypic differentiation in reproductive strategies (Blondel et al. 1999; Postma & van Noordwijk 2005), behavior (Dubuc-Messier et al. 2018), coloration (Recuerda et al. 2023) or morphological traits, such as body mass (Garant et al. 2005), tarsus length (Senar et al. 2006; Camacho et al. 2013, 2016), wing length (Senar et al. 2006) or culmen length (Blondel et al. 1999). However, the non-experimental basis of these studies does not allow making robust conclusions about patterns of local adaptation (Kawecki & Ebert 2004; Blanquart et al. 2013; but see Dubuc-Messier et al. 2018). There are several logistic concerns when studying the adaptive significance of phenotypic divergence in birds (e.g. difficulty to carry out common garden or reciprocal transplant experiments; Wadgymar et al. 2022). One way to study potential local adaptation is to measure both the neutral genetic divergence and the quantitative phenotypic variation among populations, and compare them using the P_{ST}–F_{ST} method. This approach allows to infer the role of selection and genetic drift in shaping the phenotypic variation among populations (see definition and limitations of the P_{ST}–F_{ST} comparison approach in Sæther et al. 2007; Pujol et al. 2008; Brommer 2011). In short, the P_{ST}–F_{ST} comparison quantifies the phenotypic differentiation (P_{ST}) in relation to the level of divergence expected on the basis of genetic drift alone (F_{ST}). In fact, several studies that used this approach have found signs of local adaptation in avian coloration, morphometry, behavior and reproductive parameters (Lehtonen et al. 2009; Antoniazza et al. 2010; Santure et al. 2010; Holand et al. 2011; Edelaar et al. 2012; Kekkonen et al. 2012; García-Navas et al. 2014a; Dubuc-Messier et al. 2018).

In the present study, we analyzed the natal dispersal, the genetic differentiation, and the phenotypic divergence in a population of blue tits (*Cyanistes caeruleus*) inhabiting a continuous Mediterranean woodland located along a valley. The two

slopes of the valley not only show different forest formations, but also differ in environmental conditions (especially the degree of humidity). Despite their potential high dispersal capacity, blue tits disperse over short distances, typically less than one km from their natal territory, with females dispersing over longer distances than males (Matthysen et al. 2005; Foerster et al. 2006; Parejo et al. 2007; Ortego et al. 2011; García-Navas et al. 2014a). This restricted and sex-biased dispersion reduces the gene flow between nearby blue tit populations and enhance genetic differentiation (Ortego et al. 2011; Porlier et al. 2012; Ferrer et al. 2016a), conducting to local adaptation (Blondel et al. 1999; Porlier et al. 2012; García-Navas et al. 2014a). Nonetheless, studies thus far reported a genetic structure and/or local adaptation processes among blue tit populations from high to medium spatial scales (1–28 km), usually comparing genotypic and phenotypic parameters between distant geographical regions or habitat patches in a mosaic landscape. To our knowledge, the only exceptions examining these processes at a continuous space in birds are from Garant et al. (2005) and Garroway et al. (2013), who documented a local adaptation and spatial genetic structure processes, respectively, in the great tit (*Parus major*). Here, we show that limited dispersal can lead to genetic differentiation in a blue tit population at a surprisingly small spatial scale, concretely within a single continuous woodland formed by different forest formations and without any geographical barrier. Moreover, we found evidence of local adaption for clutch size; since there was a divergence in this trait exceeding the level of divergence expected according to genetic drift alone.

Materials and methods

Study area and sampling

Fieldwork was carried out during the springs of 2017, 2018 and 2019 in a continuous woodland located at 1700–1800 m above sea level (a.s.l.) in the Sierra Nevada National Park (southeast Spain, 36°57'N, 3°24'W). This woodland is located along a valley. The east-facing slope of the valley is composed by two contiguous main forest formations of Holm oaks (*Quercus ilex*) and Pyrenean oaks (*Q. pyrenaica*), while the west-facing slope is composed by Scots pines (*Pinus sylvestris*) and Pyrenean oaks (Fig. 1). The Pyrenean oak forests from the east- and west-facing slopes are referred

as dry and humid, respectively, because they differ in their humidity degree, being the latter crossed by the Almiar stream (Fig. 1). Consequently, the two slopes display marked environmental and biotic contrasts during the spring (supplementary material 1; also see Garrido-Bautista et al. 2023).

Blue tits bred in nest boxes, all of the same type (ICONA C model; more details in Moreno-Rueda 2003), and were checked regularly throughout the breeding season in order to determine general breeding parameters: laying date, clutch size, hatching success and fledgling success (Garrido-Bautista et al. 2023). Parents were captured in their next boxes using scuttles that closed the nest box entry when the bird entered to feed the nestlings at 8–11 days of age (day 0 = hatching day). Adults were individually sexed and banded with aluminium rings for further identification. Females were checked for the presence of brood patches. Birds were weighed to the nearest 0.1 g using a digital portable scale, and their tarsus length was measured to the nearest 0.01 mm using a digital calliper (always by the same researcher, GMR). In 2017, we measured the same parameters in nestlings on day 13 after hatching. Clutch size and morphological traits were used for subsequent analysis of population phenotypic divergence (see the supplementary material 3 for more details). Before releasing adults, 100 μL blood samples (approximately 1% of blue tit body mass) were taken from their jugular vein using disinfected, heparinised insulin syringes (following Owen 2011). Blood sampling was done by the same researcher (GMR). When collecting the blood samples, handling time was minimised, as much as possible (always less than 1 min), to reduce bird stress (de Jong 2019). All samples were preserved in 1.5 ml tubes in absolute ethanol and stored at -20 ºC.

Natal dispersal

In 2016, we monitored the breeding biology of blue tits as described above. Therefore, capture–mark–recapture data from 2016–2019 was used to examine natal dispersal of blue tits (i.e. movements from their natal to their respective first-breeding nest box) between the four forest formations and the two slopes of the valley. Because of small sample size (only 11 nestlings recaptured as adults), no statistical analysis was performed with capture–mark–recapture data.

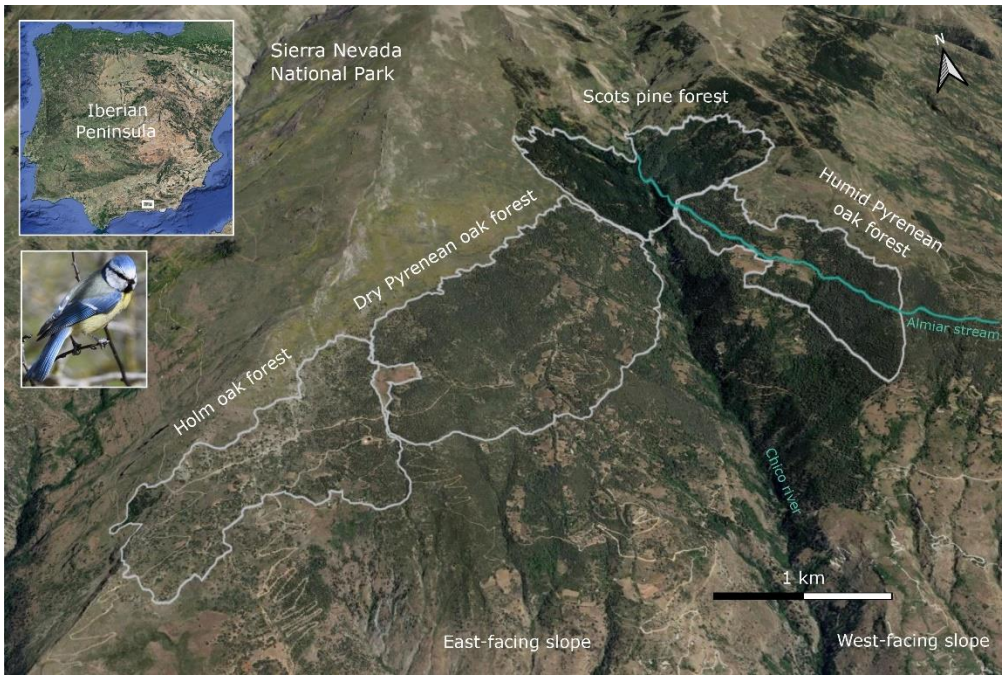

Figure 1. Map of the study area showing the valley where blue tits (*Cyanistes caeruleus*) reproduced. Note the difference in forest density (the east-facing slope being formed by Holm oaks and Pyrenean oaks, and the west-facing slope being formed by Scots pines and Pyrenean oaks) and the presence of the Almiar stream crossing the west-facing slope. Map created using the software QGIS 3.10.5 connected to Google Earth.

Microsatellite genotyping

We genotyped a total of 171 blue tits (east-facing slope: 78, west-facing slope: 93). Approximately 10 µL of blood per blue tit was used for DNA extraction using the commercial kit DNeasy Blood & Tissue (Qiagen, Hilden, Germany) following manufacturer's instructions except for the sample heating time, which was increased to 1 hour, and the final elution volume, which was reduced to 50 µl. DNA quantification was measured using a NanoPhotometer P300 (Implen GmbH, Munich, Germany). An initial set of 27 polymorphic and cross-specific microsatellite markers previously developed for different bird species, including the blue tit, was tested for all blood samples. Microsatellite genotyping is described in the supplementary material 2.

A screening process is necessary to evaluate candidate loci before conducting genetic analyses (Selkoe & Toonen 2006). We tested for the presence of null alleles using Micro-Checker 2.2.3 (van Oosterhout et al. 2004). Linkage disequilibrium (LD) between loci and deviations from Hardy–Weinberg equilibrium (HWE) were tested with GENEPOP web version 4.7 (Raymond & Rousset 1995; Rousset 2008). Probabilities of significance for LD and HWE were computed applying a Markov chain method as implemented in GENEPOP, using a dememorization number of 10,000 with 1,000 batches and 10,000 iterations per batch. Sequential Bonferroni corrections were applied to test for multiple comparisons (Rice 1989). Polymorphism information content (PIC), which measures how polymorphic a locus is, was calculated in CERVUS 3.0.7 (Kalinowski et al. 2007). Selective neutrality is required for microsatellite markers to be used for population studies, but some of the microsatellite markers used in this study are flanking coding genes (Olano-Marin et al. 2011a,b), and hence they could be under selection. So, we used BAYESCAN 2.1 (Foll & Gaggiotti 2008) to detect non-neutral outlier loci. This software implements a Bayesian method to estimate population- and locus-specific F_{ST} coefficients. We conducted 10 pilot runs of 10,000 iterations and an additional burn-in of 100,000 iterations. Outlier loci were identified based on the 99.5% confidence levels. Lastly, we did not perform quality control tests for potential non-Mendelian inheritance and incidence of homoplasious alleles per locus because parental inheritance information and microsatellite sequences are not known given the nature and purpose of this study (Selkoe & Toonen 2006). Microsatellite marker screening analysis allowed us to discard 3 loci. The remaining 24 loci were neutral to selection and were used for subsequent genetic analyses (supplementary material 2).

Genetic diversity

Multilocus genotypes were used to estimate genetic diversity for the two slopes of the valley and for the overall population. Estimates included the number of alleles (K), and the observed and expected heterozygosity (H_O and H_E, respectively), calculated in ARLEQUIN 3.5 (Excoffier & Lischer 2010). Genetic diversity estimates (K, H_O, H_E) were also calculated for all loci, including those that were not finally used in subsequent genetic analyses (supplementary material 2).

Population differentiation and structure

We analysed patterns of spatial genetic structure using the model-based Bayesian Markov chain Monte Carlo clustering method implemented in the software STRUCTURE 2.3.4 (Pritchard et al. 2000; Falush et al. 2007; Hubisz et al. 2009), which assigns individuals to inferred populations based on their multilocus genotypes. We run STRUCTURE assuming correlated allele frequencies, admixture and sampling locations as priors (i.e. the two slopes of the valley). We conducted five independent runs for each value of cluster K (1–10), with 1,000,000 Markov chain-Monte Carlo (MCMC) iterations followed by a burn-in of 100,000 steps. The optimum number of genetic clusters was estimated with the log probabilities [Pr($X|K$)] and Evanno's ΔK method (Evanno et al. 2005), implemented in STRUCTURE HARVESTER (Earl & vonHoldt 2012). Optimal alignments of replicates of the same K and graphical representation of selected clusters were performed in the software CLUMPAK (Kopelman et al. 2015). Since patterns of genetic structure and gene flow in the blue tit may be affected by male-biased philopatry (Ortego et al. 2011; García-Navas et al. 2014a), analyses on spatial genetic structure were performed both for all sampled individuals together and considering male and female genotypes separately.

The inbreeding and outbreeding coefficients for each population were defined previously through results of genetic population structuring conducted in STRUCTURE 2.3.4 (Pritchard et al. 2000; Falush et al. 2007; Hubisz et al. 2009). The extent of geographical structuring of genetic variation between individuals from the two slopes of the valley was evaluated by F_{ST} statistics, using the analysis of molecular variance (AMOVA) (Excoffier et al. 1992), implemented in ARLEQUIN 3.5 (Excoffier & Lischer 2010). The significance of variance components and F-statistics (F_{ST}, F_{IS}, F_{IT}) were assessed by 10,000 permutations. As above, F-statistics were also calculated considering male and female genotypes separately.

Phenotypic population differentiation

In a previous work, we found that adult body size did not vary among the woodland, but females adjusted their clutch size to the slope where they bred, which ultimately determined the number of fledglings produced by a blue tit pair (Garrido-Bautista et al. 2023). However, back them, we could not ascertain whether local adaptation or genetic drift was the underlying process responsible for such phenotypic

differentiation. Here, we used the F_{ST} – Q_{ST} (P_{ST}) comparison approach to examine the relative importance of genetic drift and natural selection in explaining the variation in such life-history (clutch size) and morphological (body mass, tarsus length) traits between the two slopes of the valley. F_{ST} estimates the extent of population genetic differentiation, while Q_{ST} estimates the differentiation of quantitative genetic traits (Spitze 1993). However, Q_{ST} uses purely additive genetic variance, and its estimation requires rearing individuals from different populations in a common environment (Dubuc-Messier et al. 2018), which was unfeasible for this study. For this reason, we used P_{ST}, analogous to Q_{ST}, to quantify the between population variance in quantitative phenotypic traits (Raeymaekers et al. 2006; Brommer 2011). P_{ST} was calculated as follows:

$$P_{ST} = \frac{g\ \sigma^2{}_B}{g\ \sigma^2{}_B + 2\ h^2\ \sigma^2{}_W}$$

where $\sigma^2{}_B$ is the phenotypic variance between populations, $\sigma^2{}_W$ is the phenotypic variance within populations, h^2 is the heritability (i.e. the proportion of additive genetic differences between individuals within populations), and the scalar g expresses the proportion of additive genetic differences between populations (i.e. proportion of the total variance due to additive genetic effects across populations). We calculated the phenotypic differentiation (P_{ST}) for each quantitative trait: clutch size, body mass, and tarsus length. The calculation of each parameter of the equation is detailed in the supplementary material 3.

The F_{ST} – P_{ST} comparisons allows detecting potential local adaptation and testing the relationship between neutral and quantitative genetic variation among populations. There are three possible outcomes from the F_{ST} – P_{ST} comparisons, each of which has a unique interpretation (Merilä & Crnokrak 2001; McKay & Latta 2002). First, if P_{ST} is higher than F_{ST}, then the degree of divergence in phenotypic quantitative traits exceeds that achievable by genetic drift alone (measured through neutral markers), and consequently, natural selection is favoring different phenotypes in every different population (diversifying selection). Second, if P_{ST} equals F_{ST}, then the relative effects of natural selection and genetic drift are indistinguishable, and there is no need to evoke natural selection on population differentiation. Third, if P_{ST} is lower than F_{ST}, then natural selection is favoring the same phenotype in different

populations, so phenotypic differentiation is less the expected on the basis of neutral divergence (Merilä & Crnokrak 2001). See the supplementary material 3 for more details.

Results

Natal dispersal movements

We recaptured 11 blue tits that were ringed as nestlings, 7 individuals at the east-facing slope and 4 at the west-facing slope (Table 1). Only one blue tit (1 out of 11; 9.09%) dispersed between slopes, being born in the humid Pyrenean oak forest (west-facing slope), and reproducing for first time in the dry Pyrenean oak forest (east-facing slope) (Table 1).

Table 1. Number of blue tits (*Cyanistes caeruleus*) recaptured in each forest formation across the two slopes of the valley. The table shows the location of the natal and first-breeding nest box for each capture. The blue tit that showed a dispersing behaviour among slopes is marked in bold.

First-breeding box \ Natal box		Holm oak	Dry Pyrenean oak	Scots pine	Humid Pyrenean oak
		East-facing slope		West-facing slope	
Holm oak	East-facing slope	0	1	0	0
Dry Pyrenean oak		3	2	0	**1**
Scots pine	West-facing slope	0	0	0	0
Humid Pyrenean oak		0	0	1	3

Genetic diversity

Between 2 and 40 alleles were detected per locus (mean = 14), with a mean H_O of 0.58 (range = 0.05–0.87) and a mean H_E of 0.73 (range = 0.45–0.95) (Table S1). The mean H_O of the east-facing and west-facing slope populations were 0.58 and 0.54, respectively, and their mean H_E were 0.73 and 0.70, respectively.

Genetic population differentiation and structure

The extent of geographical structuring inferred from the microsatellite markers between the two slopes of the valley revealed that most of the variation lies within individuals (Table 2). The fixation index F_{ST} among populations indicated a significant genetic divergence between both slopes (F_{ST} = 0.016, p < 0.001, 1.57 % of variance; Table 2). In accordance, F_{IS} estimate significantly differed from zero (average F-statistic over all loci, F_{IS} = 0.220, p < 0.001, 18.94 % of variance; Table 2), suggesting outbreeding within both genetic populations. Also, F_{IS} estimates of each genetic population were statistically significantly different from zero (east-facing slope = 0.176, west-facing slope = 0.207; in both cases, p < 0.001). Lastly, F_{IT} estimate indicated that most of the variation lies within individuals (average F-statistic over all loci, F_{IT} = 0.231, p < 0.001, 79.5% of variance; Table 2). When considering male and female genotypes separately, the same pattern emerged among populations when considering the F_{ST} estimate (F_{ST} for males = 0.018, p < 0.001; F_{ST} for females = 0.015, p < 0.001; Table 2), suggesting that both males and females from one slope are genetically distinct from the males and females of the opposite slope. The F_{IS} and F_{IT} estimates for males and females separately revealed the same pattern as that observed when considering all individuals (F_{IS} for males = 0.203, p < 0.001; F_{IS} for females = 0.188, p < 0.001; F_{IT} for males = 0.218, p < 0.001; F_{IT} for females = 0.200, p < 0.001; Table 2).

Structure analyses considering all individuals revealed a maximum ΔK and $\Pr(X|K)$ for K = 2, indicating the presence of two genetic clusters across the woodland (Fig. 2a). Genetic differentiation occurred mainly between the east-facing and west-facing slopes of the valley, although structure analyses revealed high levels of genetic admixture (Fig. 2a). Analyses considering only male and female genotypes separately also revealed a maximum ΔK and $\Pr(X|K)$ for K = 2 (Figs. 2b and 2c, respectively), with a similar pattern of genetic structure to that observed in the analysis considering all sampled individuals. This means that males and females from one slope were genetically distinct from those from those from the opposite slope. Although analyses also indicated high levels of genetic admixture, these were relatively lower in the case of males (Figs. 2b and 2c). Overall, genetic population differentiation and structure analyses indicated the presence of two genetic clusters within the woodland.

Table 2. Results of comparative hierarchical AMOVAs for the two genetic blue tit (*Cyanistes caeruleus*) populations analysed by microsatellite marker data, considering both all sampled individuals and male and female genotypes separately.

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation
All individuals				
Among populations	1	29.426	0.120	1.57
Among individuals within populations	169	1522.840	1.454	18.94
Within individuals	171	1043.500	6.102	79.49
Total	341	2595.766	7.677	100
Male genotypes				
Among populations	1	20.814	0.148	1.88
Among individuals within populations	76	705.257	1.566	19.92
Within individuals	78	479.500	6.147	78.20
Total	155	1205.571	7.862	100
Female genotypes				
Among populations	1	19.161	0.112	1.45
Among individuals within populations	91	817.812	1.424	18.55
Within individuals	93	571.00	6.139	80.00
Total	185	1407.973	7.675	100

Phenotypic population differentiation

When considering the null assumption where g / $h^2 = 1$ (i.e. when the proportion of variance between populations due to additive genetic effects equals the proportion of variance due to genetic effects within populations), only the P_{ST} estimate for clutch size (0.052) was higher than the global pairwise F_{ST} (body mass: 0.013, tarsus length: 0.005). Comparisons of quantitative trait differentiation (P_{ST}) with its expectation under neutrality (F_{ST}) revealed evidence of divergent selection for clutch size ($P_{ST} > F_{ST}$). The P_{ST} values for clutch size were higher than the global F_{ST} when altering the assumptions about heritability and the values of additive genetic proportion. On the other hand, the P_{ST} values for tarsus length never exceeded the global F_{ST}, while the P_{ST} values for body mass were higher than the global F_{ST} only at medium g values when

heritability was relatively low (see supplementary material 3). The results from F_{ST} – P_{ST} comparisons suggest that clutch size was under diversifying selection, while natural selection favoured a similar tarsus length between slopes. On the contrary, body mass differentiation equalled neutral divergence (however, results derived from the P_{ST} values for body mass should be taken with caution due to variability of results when altering variance assumptions; see supplementary material 3).

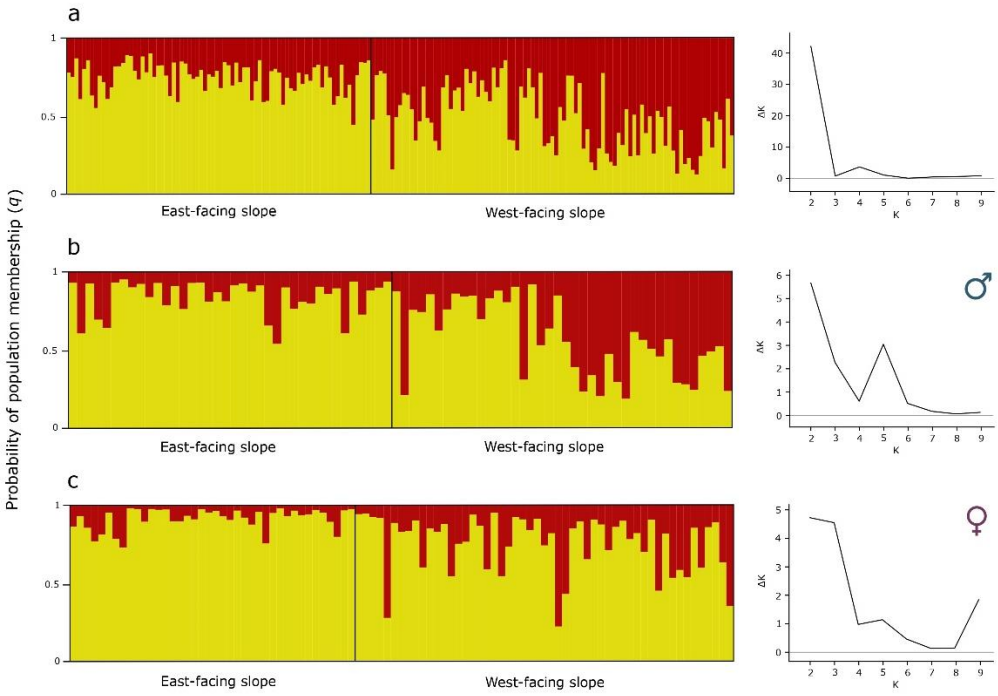

Figure 2. Results of the genetic assignment based on the Bayesian clustering analysis implemented in STRUCTURE software, considering (a) all individuals, (b) male genotypes only, and (c) female genotypes only. Each individual is represented by a vertical line, which is partitioned into two coloured segments representing the individual's probability of pertaining to one of the two populations (q) that the genetic clustering gave. Right: results of the Bayesian clustering into the most probable number of genetic clusters according to ΔK values. The analyses were performed considering (a) all individuals, (b) male genotypes only, and (c) female genotypes only.

Discussion

Our findings reveal that genetic and phenotypic differentiation can occur within a single continuous and heterogeneous woodland in a species with a potential high dispersal capacity, the blue tit. This process was mainly driven by non-random dispersal – as suggested by both the genetic and the capture–mark–recapture data – associated with environmental heterogeneity within the woodland. The concordance between dispersal movements and patterns of genetic population structure agrees with previous studies in which capture–mark–recapture data provide accurate information on recent levels of gene flow among bird populations (Coulon et al. 2008; Ortego et al. 2008, 2011; García-Navas et al. 2014a; Botero-Delgadillo et al. 2017; Li et al. 2019; but see Alcaide et al. 2009). Widespread highly-motile species are likely to experience several environmental conditions and geographical barriers, and thus they are expected to experience genetic population structure at the macro-scale (Adams & Burg 2015; Adams et al. 2016; Lemoine et al. 2016; Perrier et al. 2020), supposedly following an isolation-by-distance pattern (e.g. Clegg & Phillimore 2010; Lemoine et al. 2016; de Greef et al. 2022). However, genetic structures can be observed at the small scale depending on the landscape composition and environmental conditions (Ferrer et al. 2016a), which can restrict dispersal and gene flow among populations when organisms match habitat selection to natal conditions (Edelaar et al. 2008). Hence, our results show that genetic structure (and probably for local adaptation) can occur even between smaller population patches than previously thought (but see Garant et al. 2005), since we showed genetic structuring associated to environmental heterogeneity even within the same woodland and for species with potential high dispersal capacity.

The blue tits inhabiting the continuous woodland displayed a limited dispersal, with all individuals, except for one, reproducing within their natal slope of the valley (Table 1). The strong philopatry of both male and female blue tits seems to have promoted the genetic population differentiation among the two slopes of the valley covered by the woodland. Moreover, blue tits generally inbreed with related partners and females show less promiscuity with genetically distant males (Foerster et al. 2006; see also Szulkin et al. 2009), thus contributing to the pattern of genetic population structure. Nonetheless, we still observed a considerable gene flow between the two genetic clusters of the studied population and the genetic differentiation

between the two slopes of the valley was relatively low but significant, with a pairwise F_{ST} value of 0.016 for all individuals, 0.018 for male genotypes, and 0.015 for female genotypes. Interestingly, our data were similar to those reported in other studies that examined patterns of genetic structure between neighboring and fragmented populations of tits (Paridae) (Table 3) and other passerine birds (Arnoux et al. 2014; Camacho et al. 2016). Surprisingly, we found a level of genetic differentiation higher than expected given the extremely short distance that separates the two slopes of the valley (see Table 3 for geographical and genetic distances in other blue tit populations), and the fact that the two slopes of the valley are connected through a pine forest formation and, thus, conforming a continuum (Fig. 1).

Despite the statistically significant genetic population differentiation of the two clusters, blue tits still showed some degree of outbreeding within both slopes of the valley, suggesting the existence of some gene flow, an expected finding if one considers the short geographical distance that separates the two blue tit clusters (e.g. Ortego et al. 2011). This scenario, *a priori*, could prevent strong genetic and phenotypic divergence between the two clusters, and gene flow might introduce maladaptive alleles into locally adapted clusters (Lenormand 2002; Bolnick & Nosil 2007). Nonetheless, divergent selection can maintain local adaptation and adaptive divergence despite the homogenizing effects of gene flow (Yeaman & Otto 2011; Butlin et al. 2014; Clark et al. 2022), especially when environmental heterogeneity generates spatially contrasting selection pressures (Edelaar & Bolnick 2012; Edelaar et al. 2012). This would occur if local populations retain high genetic diversity (Smith et al. 2005; Milá et al. 2009). The blue tits inhabiting the continuous woodland studied here exhibited moderate to high levels of genetic diversity (heterozygosity) both at population and loci levels (see Results and supplementary material 2), and gene flow seems to maintain such genetic diversity in each slope of the valley. A number of studies have revealed significant associations between individual genetic quality and several fitness-related traits in the blue tit, such as the probability of infection by parasites (Ferrer et al. 2014), carotenoid-based feather coloration (García-Navas et al. 2009; Ferrer et al. 2015), egg quality (García-Navas et al. 2009), clutch size (Olano-Marin et al. 2011a), and the probability of local recruitment (Olano-Marin et al. 2011a; Ferrer et al. 2016b). Therefore, it seems that reproductive success is not constrained by potential genetic inbreeding within each cluster, while phenotypic divergence between both clusters still seems to be maintained by diversifying selection.

Table 3. Summary of studies reporting the geographical and the genetic distance among natural populations of tits (Paridae), namely blue tits (*Cyanistes caeruleus*), great tits (*Parus m*ajor) and black-capped chickadees (*Poecile atricapillus*). The table summarizes the studies examining genetic structure among populations at medium to low geographical distances (continental large-scale studies are excluded)

Species	Region	Country	Geographical distance [a, b]	Genetic distance (F_{ST}) [a, c]	Habitat types (dominant tree species)	Genetic marker	Reference
Blue tit	Granada	Spain	0 km	**0.016**	Continuous woodland (*Quercus pyrenaica*, *Q. ilex*, *Pinus sylvestris*)	Microsatellites	This study
	Toledo	Spain	< 2 km	0.006	Mosaic of forest patches (*Quercus pyrenaica*)	Microsatellites	Ferrer et al. (2015)
	Toledo	Spain	1–28 km	0.000–0.017	Mosaic of forest patches (*Quercus pyrenaica*, *Q. rotundifolia*, *Pinus pinea*, *P. pinaster*)	Microsatellites	Ferrer et al. (2016a)
	Antwerp, Ghent and Kortrijk	BBelgium	2–90 km	0.000–**0.010**	Mosaic of forest patches (*Quercus robur*)	Minisatellites	Verheyen et al. (1997)
	Corsica and southern France	France	5–440 km	**0.008–0.087**	Mosaic of forest patches (*Quercus pubescens*, *Q. ilex*)	SNPs	Perrier et al. (2020)
	Corsica and southern France	France	5–440 km	0.009–**0.054**	Mosaic of forest patches (*Quercus pubescens*, *Q. ilex*)	SNPs	Szulkin et al. (2016)

Table 3. (Continued).

Species	Region	Country	Geographical distance [a, b]	Genetic distance (F_{ST}) [a, c]	Habitat types (dominant tree species)	Genetic marker	Reference
Blue tit	Corsica and southern France	France	5–493 km	0.001–0.049	Mosaic of forest patches (*Quercus pubescens*, *Q. ilex*, *Q. suber*)	Microsatellites	Porlier et al. (2012)
	Toledo	Spain	7 km	**0.005**	Mosaic of forest patches (*Quercus pyrenaica*)	Microsatellites	Ferrer et al. (2016b)
	Toledo	Spain	7 km	**0.033**	Mosaic of forest patches (*Quercus pyrenaica*)	Microsatellites	García-Navas et al. (2014a)
	Łódź	Poland	10–50 km	0.002–0.004	Urban parks and forest patches (*Quercus robur*, *Q. petraea*, *Pinus sylvestris*)	Microsatellites	Markowski et al. (2021)
	Toledo and Ciudad Real	Spain	20 km	**0.003**	Mosaic of forest patches (*Quercus pyrenaica*, Q. rotundifolia)	Microsatellites	Ortego et al. (2011)
	Corsica	France	25 km	-0.003 and **0.012** [d]	Mosaic of forest patches (*Quercus pubescens*, *Q. ilex*)	Microsatellites	Blondel et al. (2001)

Table 3. (Continued).

Species	Region	Country	Geographical distance [a, b]	Genetic distance (F_{ST}) [a, c]	Habitat types (dominant tree species)	Genetic marker	Reference
Blue tit	Corsica	France	25 km	**0.004**	Mosaic of forest patches (*Quercus pubescens*, *Q. ilex*)	SNPs	Dubuc-Messier et al. (2018)
	Corsica	France	Not available	**0.007–0.021**	Mosaic of forest patches (*Quercus pubescens*, *Q. ilex*)	Microsatellites	Blondel et al. (2006)
	Barcelona	Spain	500 m–3 km	0.000–**0.190**	Urban parks and forest patch (parks: several tree species and gardes; forest: *Quercus ilex*, *Q. cerrioides*)	Microsatellites	Björklund et al. (2010)
Great tit	Vlieland and Hoge Veluwe	The Netherlands	5–150 km	0.003–**0.011**	Mosaic of forest patches (*Pinus sylvestris*) [e]	Microsatellites	Postma et al. (2009)
	Toledo	Spain	7 km	0.006	Mosaic of forest patches (*Quercus pyrenaica*)	Microsatellites	García-Navas et al. (2014c)
	Łódź	Poland	10–50 km	0.003–**0.010**	Urban parks and forest patches (*Quercus robur*, *Q. petraea*, *Pinus sylvestris*)	Microsatellites	Markowski et al. (2021)

Table 3. (Continued).

Species	Region	Country	Geographical distance [a, b]	Genetic distance (F_{ST}) [a, c]	Habitat types (dominant tree species)	Genetic marker	Reference
Great tit	Wytham and Hoge Veluwe	United Kingdom and The Netherlands	500 km	**0.010**	Mosaic of forest patches (*Quercus robur*, *Fraxinus excelsior*, *Acer pseudoplatanus*, *Pinus sylvestris*) [e, f]	SNPs	van Bers et al. (2012)
Black-capped chickadee	British Columbia	Canada and USA	26–1500 km	**0.009**–0.316	Not reported	MMicrosatellites	Adams et al. (2016)

[a] When more than two populations were compared, table shows a range of values (minimum to maximum geographical distance, and minimum to maximum F_{ST} values).
[b] When geographical distances among populations were not reported in the original study, EEuclidean distances were estimated based on coordinates and map information.
[c] Statistically significant pairwise F_{ST} values are marked in bold.
[d] The study reported the F_{ST} values in two separate years.
[e] Information about habitat type and dominant tree species recovered from van Balen (1973).
[f] Information about habitat type and dominant tree species recovered from Kirby & Thomas (2000).

We found evidence that blue tits were locally adapted to the conditions of each slope of the valley they inhabit, as clutch size was differentiated between both slopes of the valley to a greater extent than expected if we consider genetic drift alone (i.e. $P_{ST} > F_{ST}$), suggesting that selection favors different clutch sizes in the two slopes of the valley – differentiation in body size, on average, did not exceed the genetic neutral divergence –. These findings are congruent with a previous study in which we found that females adjusted their clutch size to the rearing conditions in the slope where they bred, thus maintaining the breeding success constant throughout the woodland (Garrido-Bautista et al. 2023). Concretely, females from the east-facing slope lay on average one eggs less than females from the humid Pyrenean oak forest in the west-facing slope (Garrido-Bautista et al. 2023). The heritability of clutch size is high in the blue tit (García-Navas et al. 2014a; this study) and moderately high in other passerine species (van der Jeugd & McCleery 2002; Sheldon et al. 2003), indicating a substantial additive genetic variance for this life-history trait that, hence, should provide considerable options and responses for selection to act on. However, studies reporting evidence of phenotypic differentiation in avian traits, including the clutch size, exceeding the level of genetic neutral divergence rely on large scales across the species breeding ranges (Lehtonen et al. 2009; Antoniazza et al. 2010; Santure et al. 2010; Holand et al. 2011; Edelaar et al. 2012; Kekkonen et al. 2012; but see García-Navas et al. 2014a). In fact, few studies have found evidence of local adaptation in bird populations at the small scale (i.e. habitat patches separated by few kilometers). For example, Garant et al. (2005) showed that non-random gene flow and divergent selection generate small scale differentiation in the genetic variance for great tit nestling body mass in a continuous woodland. García-Navas et al. (2014a) found that the divergence in clutch size and morphological traits exceeded the neutral genetic differentiation in two blue tit populations separated by 7 km. Charmantier et al. (2016) and Blondel et al. (2006) showed evidence of local adaptation for several traits occurring in blue tit populations inhabiting forest patches separated by few kilometers. Camacho et al. (2013, 2016) documented a genetic and male-biased phenotypic divergence for tarsus length at a short spatial distance (1.1 km) in two pied flycatcher (*Ficedula hypoleuca*) populations. Postma & van Noordwijk (2005) and Postma et al. (2007, 2009) showed that non-random gene flow is the main cause for the genetic differences in the clutch size between two island great tit populations only

a 1.3 km apart. For their part, Garroway et al. (2013) found evidence of fine-scale genetic structure associated with malaria infection risk and local conspecific density.

Our findings and the results from the aforementioned studies reveal that phenotypic divergence, with potential adaptive values, can occur over surprisingly small spatial scales even for species with potential high dispersal capacity, such as songbirds. However, to our knowledge, only our study and those by Garant et al. (2005) and Garroway et al. (2013) prove evidence for such differentiation in phenotypic traits (clutch size, body mass, and resistance to *Plasmodium* parasites, respectively) over continuous systems. Still, the results derived from the $P_{ST} - F_{ST}$ comparisons should be interpreted cautiously due to a number of caveats (summarized in Pujol et al. 2008; Santure et al. 2010; Kekkonen et al. 2012), and common garden or transplant experiments are needed to avoid some of the potential biases (see Dubuc-Messier et al. (2018) for a $P_{ST} - F_{ST}$ comparison and a common garden experiment using blue tits). Therefore, our conclusions should be reinforced with further experiments altering brood sizes in order to elucidate the role of selection acting on clutch size and to identify the optimal clutch size for each slope of the valley (Pettifor et al. 1988; Blondel et al. 1998).

Environmental heterogeneity added to limited dispersal within the woodland seems to be behind the evolutionary process disentangled in this study. The study area's particular topography creates a microscale geographic variation similar to the evolution canyon models described by Nevo (2006, 2009). In these systems, the opposite and closely neighboring slopes of a valley display marked climatic and biotic contrasts which determine the ecological and evolutionary processes developing on each slope. The east-facing slope of the woodland receives more solar radiation, has a higher temperature and lower humidity, less tree cover, lower nest infestation by nest-dwelling ectoparasites and a lower caterpillar abundance during the spring compared to the west-facing slope (Garrido-Bautista et al. 2021, 2022, 2023; supplementary material 1). In evolution canyon models, the microclimatic inter-slope differences determine the level of gene flow among slopes and produce adaptive divergence (Nevo 2012, 2021). Although the microclimatic and biotic contrasts from our study area are not as divergent as those from evolution canyons described by Nevo (2006, 2009, 2012, 2021) in Israel – in which the two slopes unfold xeric, savannoid and shade, forested ecosystems –, they seem to be enough to allow a genetic and phenotypic

divergence within the blue tit population, suggesting that small variation in evolutionary canyons is sufficient to generate divergent evolution. The inter-slope variation in clutch size (Garrido-Bautista et al. 2023) and the selection favoring such different clutch sizes in the two slopes of the valley suggest that blue tits face distinct selective pressures in each slope during their reproductive period. An important factor constraining all stages of avian reproduction is food availability (Martin 1987). Caterpillars are the main food source for blue tits during the spring (Tremblay et al. 2003, 2005; García-Navas et al. 2014b), and we found that their abundance is lower in the east-facing slope (Garrido-Bautista et al. 2021, 2022). Thus, food availability could be one of the most probable factors of selection underlying the phenotypic divergence observed between the two blue tit clusters, and may explain why the clutch size is lower in the east-facing slope than in the west-facing slope of the valley.

Conclusions

Our findings show that habitat heterogeneity can generate genetic and phenotypic divergence even in an animal with potential high dispersal capacity and at surprisingly small scales (inside a continuous woodland). The topography and microclimatic geographic variation from our study area – a Mediterranean woodland located in a valley with two slopes resembling to an evolution canyon model – create different selective pressures when blue tits reproduce. The east-facing slope of the valley has a drier environment, with less tree cover and food availability, while the west-facing slope of the same valley exhibits a more humid environment with more tree cover and diversity, as well as higher caterpillar abundance. Consequently, blue tits adjust their reproductive effort to the slope where they breed through showing different clutch sizes (Garrido-Bautista et al. 2023). Here, we provide evidence that an adaptive process seems to underlie such between-slope phenotypic variation. Concretely, blue tits from the opposite slopes are two differentiated genetic clusters, and selection seems to favor different clutch sizes between these clusters. This evolutionary scenario may seem surprising given that all the forest formations of the woodland that cover the studied valley are connected, thus forming a continuum of habitats, and given that blue tits have a high dispersal potential.

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Supplementary material 1. Environmental differences between slopes

Forest heterogeneity: GIS and remote sensing analyses Forest heterogeneity was characterized using various image analysis techniques. Geographical data and images were processed with Quantum GIS 3.10.5 software (QGIS Development Team 2020) and ArcGIS Desktop 10.3.1 software (ESRI, Redlands, CA, USA). For each forest type, we obtained the following variables: mean orientation, solar radiation (kWh/m^2/day), insolation time (h), tree coverage and land use (%), and normalized difference vegetation index (NDVI). We calculated the solar radiation and NDVI every five days in the 2017 and 2018 breeding seasons. The specific methodology developed to obtain each environmental variable can be found in the chapter 6 (Garrido-Bautista et al. 2023).

The mean orientations of the Holm oak and dry Pyrenean oak forests (east-facing slope) were 171.44° and 148.32°, respectively, while the Scots pine and humid Pyrenean oak forests (west-facing slope) were oriented at 190.42° and 237.66°, respectively. Accordingly, the two forests on the east-facing slope received more solar radiation (mean ± s.e.; Holm oak: 6.55 ± 0.03 kWh/m^2/day; dry Pyrenean oak: 6.62 ± 0.04) than the forests on the west-facing slope (Scots pine: 6.43 ± 0.03; humid Pyrenean oak: 6.44 ± 0.03) (Kruskal–Wallis test: $\chi^2_3 = 23.74$, $p < 0.001$). The same pattern was found for insolation time, meaning the hours per day a forest receives solar radiation (Holm oak: 12.83 ± 0.07 h; dry Pyrenean oak: 13.46 ± 0.08; Scots pine: 12.19 ± 0.06; humid Pyrenean oak: 11.98 ± 0.05) (Kruskal–Wallis test: $\chi^2_3 = 62.49$, $p < 0.001$). The four forest formations differed in tree and shrub structure, as well as land uses. The Holm oak forest was the most open, with the lowest percentage of dense tree cover (15.63%; Table S1). The dry Pyrenean oak forest had a higher percentage of dense tree cover (approximately 65%; Table S1), but less than the forests on the west-facing slope, which had the highest level of dense tree cover (approximately 82% in both the Scots Pine and humid Pyrenean oak forests; Table S1). The Holm oak forest presented the lowest NDVI values (0.40 ± 0.004) and the Scots pine forest had the highest (0.66 ± 0.004). The dry and humid Pyrenean oak forests were found to have intermediate NDVI values (0.59 ± 0.01 and 0.62 ± 0.003, respectively; Kruskal–Wallis test: $\chi^2_3 = 21.49$, $p < 0.001$).

Table S1. Area and proportion of land uses of the four forest types. Table shows the area (ha) and percentage (%; in parentheses). See the chapter 6 (Garrido-Bautista e tal. 2023) for explanations of methodology and categorization of land uses.

Land use	Holm oak	Dry Pyrenean oak	Scots pine	Humid Pyrenean oak
Dense forest: confiers	1.74 (1.26)	0.74 (0.21)	148.96 (81.92)	13.65 (10.77)
Dense forest: oaks	19.81 (14.37)	228.12 (64.93)	0.11 (0.06)	91.24 (71.99)
Dense forest: mixed	0 (0)	2.92 (0.83)	0 (0)	0 (0)
Dense shrubland: confiers	0 (0)	1.47 (0.42)	12.39 (6.81)	2.76 (2.18)
Dense shrubland: oaks	43.97 (31.89)	41.97 (11.95)	0 (0)	2.82 (2.23)
Dense shrubland: mixed	5.01 (3.64)	5.38 (1.53)	0.03 (0.02)	0 (0)
Scattered shrubland: conifers	0 (0)	0 (0)	10.21 (5.62)	0 (0)
Scattered shrubland: oaks	27.29 (19.79)	24.61 (7.00)	0 (0)	4.25 (3.35)
Scattered shrubland: mixed	0 (0)	4.09 (1.16)	1.47 (0.81)	2.83 (2.23)
Grassland	34.13 (24.75)	7.19 (2.05)	1.93 (1.06)	1.30 (1.02)
Mosaic	0.12 (0.09)	20.44 (5.82)	0 (0)	0.58 (0.46)
Bare soil	0.69 (0.50)	0 (0)	0 (0)	0 (0)
Rivers and streambeds	0 (0)	0 (0)	3.55 (1.95)	0 (0)
Farmland [a]	0 (0)	7.77 (2.21)	0 (0)	5.57 (4.39)
Others [b]	5.13 (3.72)	6.60 (1.88)	3.18 (1.75)	1.74 (1.37)
Total area	137.88 (100)	351.31 (100)	181.84 (100)	126.74 (100)

[a] Includes farmlands, irrigation ponds and livestock lands.

[b] Includes forest tracks, firebreaks and forestry facilities.

Temperature and humidity In 2019, we installed data loggers iButton® in an outer wall of nest boxes in the dry and humid Pyrenean oak forests (five in each forest) to measure the environmental temperature (ºC) and humidity (%). Data loggers were placed on 05 May and removed 29 June, and measurements were taken every hour. The temperature of the dry Pyrenean oak forest, located in the east-facing slope (17.81 ± 0.18 ºC), was on average 1 ºC higher than the humid Pyrenean oak forest from the west-facing slope (16.80 ± 0.17 ºC; *t*-test: $t_{1338} = 14.11$, $p < 0.001$). The humidity of the dry Pyrenean oak forest (41.37 ± 0.49 %) was lower than in the humid Pyrenean oak forest (42.99 ± 0.49 %; *t*-test: $t_{1338} = 9.21$, $p < 0.001$).

Nest-dwelling and flying ectoparasites In 2017 and 2018, we carefully revised the nest material in the nest boxes once all fledglings left their nests to obtain an estimate of nest-dwelling ectoparasite pressure. We recorded the prevalence and intensity (i.e. number of parasites in infested nests) of *Protocalliphora azurea* blowfly larvae and puparia, and the prevalence of *Ceratophyllus gallinae* hen flea larvae and adults. We also estimated the prevalence and intensity of biting midges (genus *Culicoides*) and black flies (genus *Simulium*) in nest boxes following the protocol described by Garrido-Bautista et al. (2022). Briefly, the procedure involved placing a 60 mm petri dish layered with a drop of body oil gel in the roof of each nest box. The petri dishes were placed when the nestlings were 12 days old and collected the next day. Flying ectoparasites were removed from dishes, and identified and quantified in the laboratory. We obtained nest-dwelling and flying ectoparasite data from 154 and 77 nest boxes, respectively.

The nest infestation rates by blowflies and fleas differed between forest formations, with both nest-dwelling ectoparasites being less prevalent in the east-facing slope than in the west-facing slope. Only 9.09% of nests sampled in the Holm oak forest and 30.36% in the dry Pyrenean oak forest were infested with fleas, while they were found in 52.17% and 64.15% of the nests in the Scots pine and humid Pyrenean oak forests, respectively (chi-squared test: $\chi^2_3 = 24.51$, $p < 0.001$). Blowflies were less prevalent in the Holm oak (40.90% of nests infested) and dry Pyrenean oak (41.07%) forests than in the Scots pine (95.65%) and humid Pyrenean oak (86.79%) forests (chi-squared test: $\chi^2_3 = 40.24$, $p < 0.001$). There were no significant differences in blowfly intensity within each nest across the four forest formations (Holm oak: 12.11 ± 4.30; dry Pyrenean oak: 12.78 ± 2.03; Scots pine: 15.09 ± 2.57; humid Pyrenean oak: 17.20 ±

1.42; Kruskal–Wallis test: $\chi^2_3 = 5.87$, $p = 0.118$). In contrast to nest-dwelling ectoparasites, there were no differences between forest formations in the frequency of nest infestation by biting midges and black flies (chi-squared test; in both cases, $\chi^2_3 > 5.09$, $p > 0.12$). The intensity of biting midges and black flies did not vary between forest formaitons (Kruskal–Wallis test: biting midges: $\chi^2_3 = 5.49$, p = 0.139; black flies: $\chi^2_3 = 4.33$, $p = 0.228$).

Caterpillar availability Caterpillars are the main food of blue tit nestlings (Bańbura et al. 1999; García-Navas & Sanz 2011; Tremblay et al. 2005). To test whether prey (caterpillars) availability differs between the two slopes of the valley, we estimated the caterpillar abundance in the dry and humid Pyrenean oak forests following Zandt (1994). In 2018 and 2019, we sampled five randomly branches in three days and in each forest type to estimate the caterpillar population as the number of caterpillars per 10 shoots (sample units per forest type; dry Pyrenean oak: 30 branches; humid Pyrenean oak: 30 branches). When shooting a branch, a 2 × 2 m blanket was placed in the ground under the branch, and all arthropods were collected in 70%-ethanol labelled vials. For each sample unit, we obtained the abundance of caterpillars. The humid Pyrenean oak forest harboured twice caterpillars as the dry Pyrenean oak forest (humid: 12.87 ± 2.57 caterpillars, dry: 7.13 ± 1.49; GLM linked to a loglineal-Poisson distribution, forest type as the predictor: $\chi^2 = 47.90$, $p < 0.001$).

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Supplementary material 2. Microsatellite genotyping and screening process

Materials and methods Details about the 27 microsatellite loci are provided in Table S2. All PCRs were carried out in 96-well plates, with a final reaction volume of 10 µL containing 1 µL of template DNA (approximately 2–5 ng), 1× reaction buffer (NH_4 reaction buffer; Meridian Bioscience, USA), 2.5 mM $MgCl_2$, 0.4 mM of each dNTP, 1 µM of each primer (forward primers were labelled with 5'–fluorescent tags: 6-FAM, PET, HEX or TAMRA; Table S2), and 0.1 U of BioTaq DNA polymerase (Meridian Bioscience). The PCR program used for all loci was 5 min denaturing at 95 °C followed by 35 cycles of 30 s at 95 °C, 30 s at the annealing temperature (Table S2) and 25 s at 72 °C, ending with a 10 min elongation step at 72 °C. In all PCR runs, a negative control of nuclease-free water was included. Amplification products were checked by electrophoresis on 2.5 % agarose gels stained with SYBR® Safe DNA stain (Thermo Fisher Scientific, USA), and were then mixed in tubes according to their fluorescent dyes (Table S2). Mixes of products were genotyped as multiplex on an ABI 300 Genetic Analyzer (Applied Biosystems, USA) using the GeneScan 1200 LIZ size standard (Thermo Fisher Scientific). Allele size scoring was performed using the software Peak Scanner 1.0 (Applied Biosystems) through a semi-automated procedure, i.e. the automated allele calling done by the software was posteriorly checked through visual analysis in order to correct scoring-related errors.

Results We did not find evidence of large allele dropout or scoring error due to stuttering, and we did not detect a high frequency of null alleles across loci and locations. After applying Bonferroni corrections to compensate for multiple statistical tests, we observed evidence of significant Linkage Disequilibrium (LD) between pair of loci in 3 out of 1404 forest type-locus combinations (dry Pyrenean oak forest from the east-facing slope: *CcaTgu11*/*Mcyµ4*, *DkiB119*/*Pca3*; humid Pyrenean oak forest from the west-facing slope: *CcaTgu11*/*Mcyµ4*). We also detected significant departures from the Hardy–Weinberg Equilibrium (HWE) for several loci in some forestry formations, but they were not consistent across locations (Table S3). The mean of polymorphic information content (PIC) across loci was 0.70, and all loci showed moderate to high PIC values (Table S2). Analyses of neutrality based on the F_{ST} outlier tests indicated that 3 loci (*CcaTgu28*, *POCC6* and *Tgu07*) deviated significantly from neutral expectations and were under diversifying selection (Fig. S1).

Table S2. Set of microsatellite markers used to genotype blue tits (*Cyanistes caeruleus*). The following information is given: primer sequence (5´–3´), tail fluorescent dye (forward primer 5´ labelled), multiplex group, annealing temperature (T; ºC), number of alleles (*K*), number of blue tits genotyped (*N*), allele size range (base pairs), expected heterozigosity (H_E), observed heterozigosity (H_O), polymorphic information content (PIC) and reference of each locus.

Locus	Primer sequence (5´– 3´)	Dye	Multiplex group	T	*K*	*N*	Allelic range	H_E	H_O	PIC	Reference
ApCo46	F: GCTGCCAGCACTCTGAATGTC R: GATTCAGCAAAATAGGGGTCAGAAG	HEX	1	54	4	167	24	0.65	0.50	0.57	Stenzler & Fitzpatrick (2002)
Ase18	F: ATCCAGTCTTCGCAAAAGCC R: TGCCCCAGAGGGAAGAAG	6-FAM	5	61	13	167	38	0.86	0.77	0.85	Richardson et al. (2000)
CcaTgu1	F: AGAGCCCTGTTYATRGCTGT R: CCACCATGCAAACAYCAR	PET	2	56	7	167	20	0.59	0.45	0.43	Olano-Marin et al. (2010)
CcaTgu2	F: CAGCMSACAAATGCATCTAC R: GAAGGYGAARTGCTGTCTCTT	HEX	3	55	4	167	7	0.45	0.05	0.40	Olano-Marin et al. (2010)
CcaTgu7	F: TTTTTCAGGAAARGGAAACA R: CAAGCTTTTACAGTGCTAWT	6-FAM	3	55	5	164	8	0.72	0.32	0.68	Olano-Marin et al. (2010)
CcaTgu11	F: TGCTTAGGAAATAGGAAGCACA R: CTGCAACTTAAGCARRGTTATGA	TAMRA	1	54	6	169	12	0.64	0.47	0.60	Olano-Marin et al. (2010)
CcaTgu14	F: GTTGTTCYAATTCCAATGC R: CTAAAAATAGCAGTAAAAATACAYAAA	6-FAM	3	54	15	168	46	0.78	0.65	0.76	Olano-Marin et al. (2010)
CcaTgu15	F: TTAATCCTAGGGTGTYGAGAGAAC R: CCTTTTTCCTTAAATTAKCTCAGCTT	PET	3	57	6	164	15	0.55	0.50	0.43	Olano-Marin et al. (2010)
CcaTgu19	F: CTGGACCATGACTGCAAGATT R: CAGTGGCAAAKCAGCACCT	6-FAM	4	59	34	169	63	0.96	0.87	0.95	Olano-Marin et al. (2010)
CcaTgu28	F: TCTGGACTCTTGGCACCTG R: GCTTAAGGAGAAAAYAATCCTTCAC	TAMRA	5	61	6	169	13	0.67	0.11	0.61	Olano-Marin et al. (2010)

Table S2. (Continued).

Locus	Primer sequence (5´– 3´)	Dye	Multiplex group	T	*K*	*N*	Allelic range	H_E	H_O	PIC	Reference
DkiB102	F: TTGCAACAGGAGGACAAGG R: CAGCAGCACTTCCCAATACA	6-FAM	2	58	33	167	55	0.92	0.86	0.91	King et al. (2005)
DkiB119	F: CATACAACTTCATGACTACCATAGCAC R: TCCATAGTGACATAGAACGAGCTG	HEX	3	58	5	167	4	0.53	0.43	0.52	King et al. (2005)
Mcyμ4	F: ATAAGATGACTAAGGTCTCTGGTG R: TAGCAATTGTCTATCATGGTTTG	PET	1	54	16	169	40	0.81	0.81	0.81	Double et al. (1997)
PAT MP 2-43	F: ACAGGTAGTCAGAAATGGAAAG R: GTATCCAGAGTCTTTGCTGATG	TAMRA	2	55	8	170	23	0.55	0.57	0.46	Otter et al. (1998)
Pca3	F: GGTGTTTGTGAGCCGGGG R: TGTTACAACCAAAGCGGTCATTTG	TAMRA	3	58	12	168	73	0.81	0.82	0.78	Dawson et al. (2000)
Pca7	F: TGAGCATCGTAGCCCAGCAG R: GGTTCAGGACACCTGCACAATG	6-FAM	4	60	12	169	21	0.85	0.63	0.85	Dawson et al. (2000)
Pca8	F: ACTTCTGAAACAAAGATGAAATCA R: TGCCATCAGTGTCAAACCTG	HEX	4	60	40	171	179	0.95	0.68	0.95	Dawson et al. (2000)
Pdoμ5	F: GATGTTGCAGTGACCTCTCTTG R: GCTGTGTTAATGCTATGAAAATGG	TAMRA	2	56	24	159	100	0.88	0.63	0.88	Griffith et al. (1999)
PiJ14	F: ATCTGGCATKGAAAACTTGG R: CTCCCTGCACCCCAAAC	TAMRA	5	61	20	166	59	0.87	0.79	0.84	Saito et al. (2005)
PK12	F: CCTCCTGCAGTTGCCTCCCG R: CGTGGCCATGTTTATAGCCTGGCACTAAGAAC	PET	4	59	24	171	55	0.93	0.82	0.91	GenBank accession number: AF041466.1

Table S2. (Continued).

Locus	Primer sequence (5´– 3´)	Dye	MMultiplex group	T	*K*	*N*	Allelic range	H_E	H_O	PIC	Reference
POCC1	F: TTCTGTGCTGCAATCACACA R: GCTTCCAGCACCACTTCAAT	6-FAM	1	54	14	169	35	0.79	0.57	0.77	Bensch et al. (1997)
POCC6	F: TCACCCTCAAAAACACACACA R: ACTTCTCTCTGAAAAGGGGAGC	HEX	5	61	15	168	37	0.87	0.53	0.85	Bensch et al. (1997)
TG05-046	F: AAAACATGGCTTACAAACTGG R: GCTCAGATAAGGGAGAAAACAG	HEX	2	55	2	167	2	0.45	0.37	0.34	Dawson et al. (2010)
TG05-053	F: GCATCATCTGGTTGAACTCTC R: ACCCTGTTTACAGTGAGGTGTT	TAMRA	4	60	14	170	15	0.88	0.54	0.88	Dawson Et al. (2010)
TG12-015	F: ACAACAGTGGCTTTACTGTGTGA R: TACAGCAGCTGCAGCAAAGT	TAMRA	4	60	9	171	41	0.45	0.54	0.26	Dawson et al. (2010)
TG13-017	F: GCTTTGCATCTTGCCTTAAA R: GGTAACTACAACATTCCAACTCCT	HEX	1	55	11	89	18	0.80	0.51	0.81	Dawson et al. (2010)
Tgu07	F: CTTCCTGCTATAAGGCACAGG R: AAGTGATCACATTTATTTGAATAT	HEX	5	61	7	170	12	0.74	0.32	0.70	Slate et al. (2007)

Therefore, based on these screening analyses and given that LD and deviations from HWE were not consistent across loci and locations, 3 out of the 27 microsatellite markers (*CcaTgu28*, *POCC6* and *Tgu07*) were not used in the genetic analyses conducted in this study.

Table S3. Hardy–Weinberg equilibrium (HWE) *p*–values for microsatellite markers from the four forest formations located across the two slopes of the valley. Significant values after sequential Bonferroni corrections are marked in bold.

Locus	East-facing slope		West-facing slope	
	Holm oak	Dry Pyrenean oak	Scots pine	Humid Pyrenean oak
ApC046	0.0277	0.0137	0.6842	0.2211
Ase18	0.0196	0.2158	0.5103	**0.0001**
CcaTgu1	0.1496	0.0007	0.2178	**< 0.0001**
CcaTgu2	0.0093	**< 0.0001**	0.0012	**< 0.0001**
CcaTgu7	0.1205	**< 0.0001**	0.0841	**0.0001**
CcaTgu11	0.5126	0.0004	0.0672	**< 0.0001**
CcaTgu14	**0.0002**	0.1046	0.0414	0.0771
CcaTgu15	0.1000	0.8468	0.0949	**< 0.0001**
CcaTgu15	0.0744	0.0017	0.0058	0.2852
CcaTgu19		**< 0.0001**	0.0010	**< 0.0001**
CcaTgu28	1.0000	0.0569	1.0000	0.1091
DkiB102	0.4840	0.0399	0.0085	0.0307
DkiB119	0.2617	0.0252	0.1434	0.0107
Mcyμ4	1.0000	0.0109	0.4981	**< 0.0001**
PAT MP 2-43	0.0022	0.1261	0.6554	**0.0002**
Pca3	**0.0002**	**< 0.0001**	0.5833	**< 0.0001**
Pca7	**< 0.0001**	0.6836	0.0119	0.0908
Pca8	0.0234	**< 0.0001**	0.7171	0.0058
Pdoμ5	0.2331	**0.0002**	0.3321	**< 0.0001**
PiJ14	0.0146	0.0269	0.1420	**< 0.0001**
PK12	0.0257	**< 0.0001**	0.0075	**< 0.0001**
POCC1	0.1660	**< 0.0001**	0.7836	**< 0.0001**
POCC6	1.0000	0.0514	1.0000	0.1755
TG05-046	**< 0.0001**	**< 0.0001**	0.0772	**< 0.0001**
TG05-053	1.0000	0.9784	1.0000	1.0000
TG12-015	0.0143	**< 0.0001**	0.1151	**< 0.0001**
TG13-017	0.0244	**< 0.0001**	**0.0001**	**0.0001**
Tgu07	0.0196	0.2158	0.5103	**0.0001**

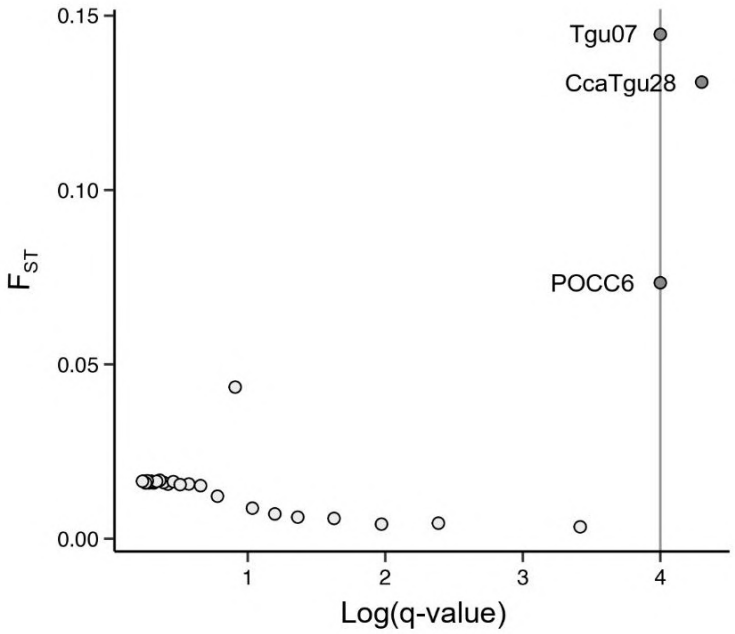

Figure S1. Bayesian test for selection on individual microsatellite markers conducted in BayeScan 2.1. Microsatellite markers to the right of the vertical black line represent outliers with a log(q-value) > 4, classified as markers under diversifying selection.

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Supplementary material 3. Phenotypic differentiation between the two slopes in comparison with the neutral expectations for divergence: F_{ST} – P_{ST} comparison

Materials and methods Between-population differentiation in alleles that are not selected, as quantified by F_{ST}, presents the expectation of the level of differentiation across populations caused by stochastic (neutral) processes, such as genetic drift (Wright 1943). The F_{ST} estimates the level of molecular (i.e. neutral markers) variation among populations and is calculated through a nested analysis of molecular variance (AMOVA). This hierarchical model employs 'within individuals', 'among individuals within populations' and 'among populations' components of diversity, thus reflecting the correlation of molecular diversity at different levels of hierarchical subdivision (see Excoffier et al. 1992; Excoffier et al. 2010). Populations with a low F_{ST} value experience high gene flow between them, homogenizing the gene pool, whereas in populations with a high F_{ST} value, the genetic drift moves the trait averages leading to population divergence. The level of divergence of a quantitative genetic trait (Q_{ST}) can be calculated in a manner analogous to F_{ST} as (Lande 1992; Spitze 1993):

$$Q_{\mathrm{ST}} = \frac{\sigma^2{}_{A_B}}{\sigma^2{}_{A_B} + 2\,\sigma^2{}_{A_W}}$$

where σ^2_{AB} is the additive genetic variance between populations, and σ^2_{AW} is the additive genetic variance within populations. Thus, the Q_{ST} estimates the differentiation of quantitative genetic traits among populations. Under the influence of migration, mutation or genetic drift, the among-population proportion of total genetic variance in phenotypic, quantitative genetic traits is expected to equal that of neutral molecular loci (Lande 1992). However, the calculation of Q_{ST} uses purely additive genetic variance, an information which was absent for this study given that the estimation of additive genetic variance requires rearing individuals from different populations in a common environment (Leinonen et al. 2008). Thereby, Q_{ST} can be approximated by phenotypic differentiation across populations, termed P_{ST}. The quantification of P_{ST} is based on phenotypic measures of a trait in the wild (i.e. no need to rear individuals in a common environment) in several individuals across a number of populations. P_{ST} is calculated as:

$$P_{ST} = \frac{g\ \sigma^2{}_B}{g\ \sigma^2{}_B + 2\ h^2\ \sigma^2{}_W}$$

where σ^2_B is the phenotypic variance between populations, σ^2_W is the phenotypic variance within populations, h^2 is the heritability (additive genetic proportion of differences between individuals within populations), and the scalar g expresses the additive genetic proportion of differences between populations (proportion of the total variance due to additive genetic effects across populations). The approximation of Q_{ST} by P_{ST} thus requires the calculation of parameters g and h^2, which ultimately determines the accuracy of the approximation (Brommer et al. 2011). As described in Brommer et al. (2011), if one would know the values of h^2 and g, the phenotypic divergence of a trait quantified by P_{ST} would equal Q_{ST}. However, estimating both parameters can be challenging, especially the parameter g (Pujol et al. 2008). For this reason, to make acute inferences from an $F_{ST} - P_{ST}$ comparison approach, one should make a sensitive analysis considering a variety of values of h^2 and g (see below).

We followed the procedure described in García-Navas et al. (2014a) and Lehtonen et al. (2010) to estimate P_{ST} for each trait. A trait's heritability (h^2) expresses the proportion of the total variance attributable to the average additive genetic effects and refers to a particular population under particular environmental conditions (Falconer 1989). Because h^2 is specific to a given population and cannot extrapolate trait heritability to other populations, we calculated h^2 for each genetic cluster (east-facing slope of the valley and humid Pyrenean oak forest from the west-facing slope) separately for the three traits considered in this study: clutch size, body mass and tarsus length. The heritability of these traits was estimated based on offspring–parent linear regression β estimates, following Falconer (1989) and Merilä & Wiggins (1995). We calculated the h^2 of clutch size on the basis of a restricted sample of female recruits and their mothers (mother–daughters regressions, n = 11) captured from the 2017–2019 period. The h^2 of body mass and tarsus length was calculated based on offspring–parent regressions (offspring–mother and offspring–father) of trait measurements of offspring on female (n = 165 for body mass, n = 164 for tarsus length) and male (n = 95 for body mass, n = 104 for tarsus length) parents. Heritability estimates for body mass and tarsus length included data from 2017, since we measured nestling body mass and tarsus length of full broods during this year (for logistic reasons, nestling biometry could not be recorded in 2018, and a cross-fostering experiment was

performed in 2019, precluding the use of these data of this year). We then compared and used the estimates of h^2 obtained from the different regressions to calculate P_{ST}. Variance components (σ^2_B and σ^2_W) were estimated using the VAR COMP procedure and applying the ANOVA (type III sum of squares) approach in the software SPSS IBM 22.0. Here, data from all sampled adult blue tits (2017–2019) were used for the estimation of variance components. Lastly, we recalculated P_{ST} for different assumptions about the magnitude of the additive genetic proportion of the between-population variance component (g = 0.1 to 1.0) in a sensitive analysis, following Sæther et al. (2007) and Lehtonen et al. (2009).

Results and discussion The heritability for clutch size was rather high in both populations (east-facing slope: h^2 = 0.58, west-facing slope: h^2 = 0.61). Even with a small sample size, we obtained h^2 values similar to other blue tit populations (García-Navas et al. 2014a), and higher values than those reported from other passerine species (Sheldon et al. 2003), suggesting a substantial additive genetic basis for this life-history trait (see also Postma & van Noordwijk 2005). We calculated the P_{ST} for clutch size using both h^2 values. Heritabilities for body mass were larger in fathers than in mothers in the west-facing slope (fathers = 0.77, mothers = 0.08; Fig. S2A), while in the east-facing slope fathers and mothers showed similar heritability for body mass (fathers = 0.25, mothers = 0.29; Fig. S2B). We obtained a similar sex-dependent pattern of variation among slopes for h^2 for tarsus length (west-facing slope: fathers = 0.66, mothers = -0.02; east-facing slope: fathers = 0.35, mothers = 0.52; Fig. S3). The variability in h^2 for morphological traits among sexes typically arises as a consequence of different rates of extra-pair paternity in blue tit populations (e.g. Kempenaers et al. 1997; Charmantier & Réale 2005; García-Navas et al. 2014b), which indeed may bias the h^2 estimation (Charmantier & Réale 2005). Also, the environmental rearing conditions may influence the expression of genetic variance in morphological traits, and hence, alter the heritability of such traits (Larsson et al. 1997; Merilä 1997). Although some authors argue that heritabilities calculated with mothers are less prone to this potential bias than those made with fathers (see, for example, García-Navas et al. 2014a), to be conservative, we used both paternal and maternal h^2 values to calculate the P_{ST} for body mass and tarsus length (except those negative h^2 values and h^2 values lower than 0.1).

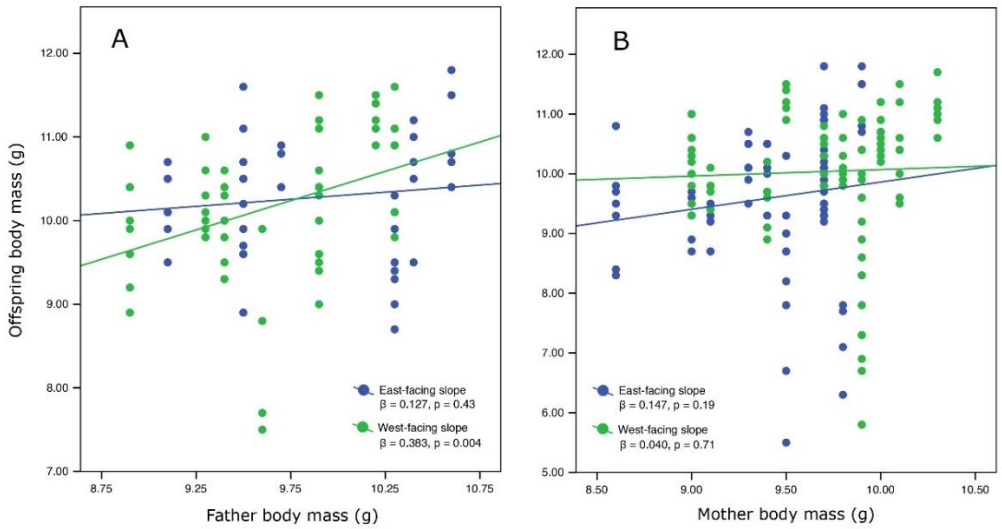

Figure S2. Body mass resemblance estimated by offspring–parent linear regressions (A: father–offspring, B: mother–offspring) in the two blue tit *Cyanistes caeruleus* genetic populations (blue: east-facing slope, green: west-facing slope).

Assuming g / h^2 = 1 (a hypothetical and biologically realistic assumption), P_{ST} values for clutch size, body mass and tarsus length were 0.052, 0.013 and 0.005, respectively, the P_{ST} value for clutch size being the only one exceeding the F_{ST} estimation for genetic population differentiation (F_{ST} = 0.016; see main text). Interestingly, we obtained almost the same results when estimating the P_{ST} values with the actual data varying the h^2 and g values for each trait (Table S4; Fig. S4). The P_{ST} values for tarsus length never exceeded the F_{ST} (Fig. S4), and P_{ST} values for body mass only exceeded the F_{ST} value at medium g scalar values when heritabilities were relatively low (Fig. S4). The P_{ST} values for clutch size, on the other hand, were larger than F_{ST} values across all g scalar assumptions (Fig. S4). Overall, P_{ST} values for the life-history trait (clutch size) were larger than P_{ST} values for morphological traits (body mass and tarsus length).

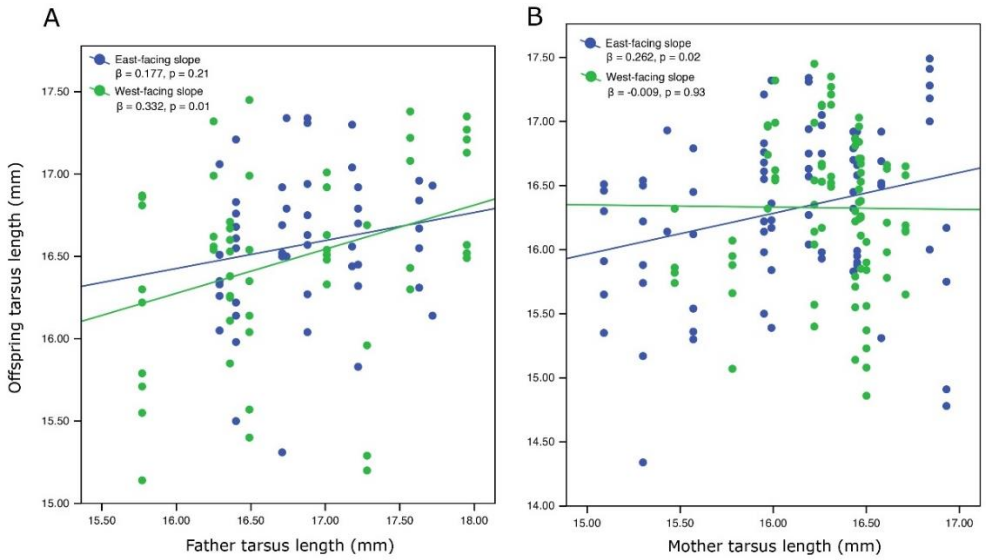

Figure S3. Tarsus length resemblance estimated by offspring–parent linear regressions (A: father–offspring, B: mother–offspring) in the two blue tit *Cyanistes caeruleus* genetic populations (blue: east-facing slope, green: west-facing slope).

P_{ST} values for body mass were higher than P_{ST} values for tarsus length, considering all *g* assumptions, because the phenotypic variance among slopes in relation to phenotypic variance within slopes was higher for body mass. Morphological traits, which have a less polygenic nature than life-history traits, are expected to respond faster to natural selection than life-history traits, and hence have higher P_{ST} values (García-Navas et al. 2014a). However, the results obtained here are in line with our previous work, in which we found that clutch size varied between slopes but neither adult body mass nor tarsus length did (Garrido-Bautista et al. 2023). In that study, we suggested that clutch size could be locally adapted to each forest formation. Here, we found evidence that selection indeed favoured different clutch sizes among slopes ($P_{ST} > F_{ST}$), and that clutch size is probably locally adapted to such habitats.

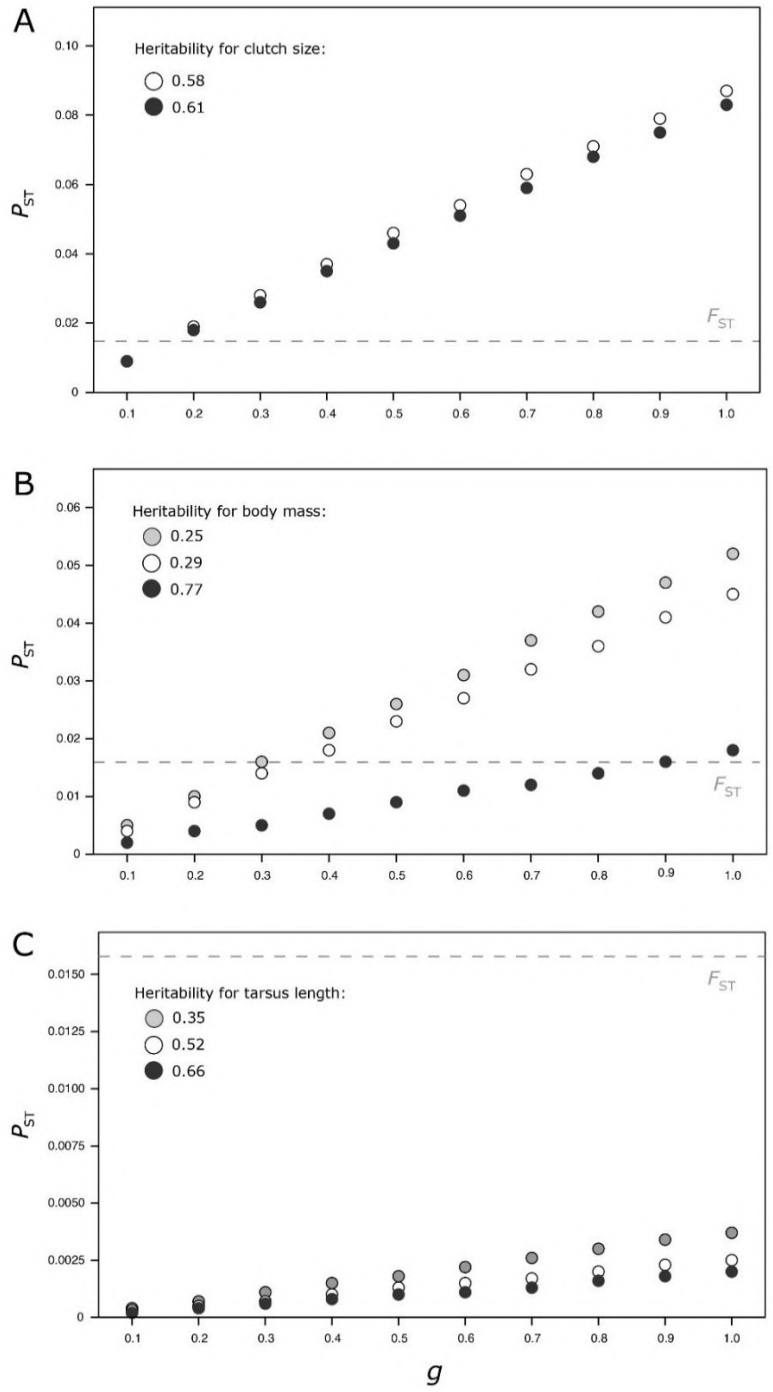

Figure S4. P_{ST} sensitive analysis for variable estimates of the additive genetic proportion among genetic population differences (g) in clutch size (A), adult body mass (B) and adult tarsus length (C), calculated using the different heritability estimates (see text). The global F_{ST} value is represented by the horizontal dotted line.

Table S4. P_{ST} values for clutch size, body mass and tarsus length of blue tits (*Cyanistes caeruleus*) under different assumptions: different heritabilities (h^2) calculated for each trait and different variation due to additive genetic effects (g).

Trait	g									
	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
Clutch size										
$h^2 = 0.58$	0.009	0.019	0.028	0.037	0.046	0.054	0.063	0.071	0.079	0.087
$h^2 = 0.61$	0.009	0.018	0.026	0.035	0.043	0.051	0.059	0.068	0.075	0.083
Body mass										
$h^2 = 0.77$	0.002	0.004	0.005	0.007	0.009	0.011	0.012	0.014	0.016	0.018
$h^2 = 0.29$	0.004	0.009	0.014	0.018	0.023	0.027	0.032	0.036	0.041	0.045
$h^2 = 0.25$	0.005	0.010	0.016	0.021	0.026	0.031	0.037	0.042	0.047	0.052
Tarsus length										
$h^2 = 0.66$	0.0002	0.0004	0.0006	0.0008	0.0010	0.0011	0.0013	0.0016	0.0018	0.0020
$h^2 = 0.52$	0.0003	0.0005	0.0007	0.0010	0.0013	0.0015	0.0017	0.0020	0.0023	0.0025
$h^2 = 0.35$	0.0004	0.0007	0.0011	0.0015	0.0018	0.0022	0.0026	0.0030	0.0034	0.0037

In sum, we found evidence that clutch size has been under diversifying selection ($P_{ST} > F_{ST}$; Fig. S4), with natural selection favouring different clutch sizes between the two slopes of the valley, while similar tarsus lengths were favoured in the two slopes of the valley ($P_{ST} < F_{ST}$; Fig. S4). Phenotypic variation in body mass, on the other hand, cannot attribute to either genetic drift or natural selection ($P_{ST} \simeq F_{ST}$; Fig. S4), given that results derived from body mass P_{ST} values should be taken with caution because conclusions were not robust: variability in h^2 values for this trait, in addition to uncertainties about the additive genetic proportion of inter-population differences (g), gave different results from $P_{ST} - F_{ST}$ comparisons. Nonetheless, the phenotypic variation for body mass was not as high as the phenotypic variation for clutch size, since the former did not show any habitat-dependent significant variation (Garrido-Bautista et al. 2023). Overall, the results suggest that diversifying selection has probably caused the phenotypic divergence in clutch size, although other causes of variance (phenotypic plasticity or local environmental conditions) may have contributed to the observed between-population differentiation.

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Chapter 8

Fine-scale variation in optimal clutch size in a blue tit population

ABSTRACT According to the individual optimization hypothesis, clutch size variation is adaptive in bird populations, since parents lay a clutch size that maximizes offspring recruitment. Given that fledgling condition parameters predict the survival up to the next breeding season, one can test the optimization of clutch size in the short term by studying the variation in nestling body condition – in addition to the physiological mechanisms which traded off with growth – with the manipulated brood size. If clutch size is optimized, then parents rearing an enlarged brood size would produce low-quality nestlings. Here we tested this prediction in a population of blue tits (*Cyanistes caeruleus*) inhabiting two close forests: one dry and one humid. We manipulated the brood size (producing reduced, control, and enlarged broods) within each forest type and evaluated the immune system, oxidative status and morphological parameters of the largest and smallest nestlings within individual broods. We also recorded parental feeding effort and nestling diet composition in each nest. Clutch size was higher in the humid forest, and parents from the two habitats successfully raised the extra nestlings. However, the mean nestling body mass and body condition diminished in enlarged broods compared to control and reduced broods, whilst neither the structural body size, immune system nor oxidative status varied with the brood size manipulation. Parents did not increase their feeding effort in enlarged broods, and nestling diet composition, on average, did not differ between treatments. In contrast, nestlings inhabiting the dry forest showed higher oxidative stress levels. Our findings reveal that, although blue tits were able to raise extra offspring, clutch size may be individually optimized at the fine scale. In each forest, parents similarly failed to adjust their provisioning effort when broods were enlarged, thus producing low-quality nestlings.

Keywords: Body condition, brood size manipulation, *Cyanistes caeruleus*, immune system, individual optimization hypothesis, nestling, optimal clutch size, oxidative status

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Introduction

Individual birds within a population lay clutches of very different sizes. Lack (1954, 1966) proposed that clutch size in altricial birds is adjusted to the maximum number of offspring for which the parents can adequately feed, and that natural selection should favour this 'most productive clutch size'. This idea was refined by Charnov & Krebs (1974), who introduced the concept of 'optimal clutch size', defined as that which maximizes the individual fitness. The optimal clutch size is typically lower than the most productive clutch size – that which produces the most fledglings – because rearing a large number of offspring implies a reduction in the future survival or fecundity of parents (the cost of reproduction hypothesis; Charnov & Krebs 1974), or in the viability of offspring (the individual optimization hypothesis; Perrins & Moss 1975). The individual optimization hypothesis (IOH) states that birds lay a size of clutch (the optimal clutch size) which maximizes the number of fledglings subsequently recruited into the population, and that this optimum varies among individuals (Perrins & Moss 1975). That to say, the clutch size laid reflects the parent's ability to rear offspring surviving to the maturity, based both on its own phenotypic quality and performance (e.g. body condition, coping behaviour or incubation efficiency; Perrins & Moss 1975; Thomson et al. 1998; Nicolaus et al. 2015) and local environmental variation (e.g. food resources or territory quality; Perrins & Moss 1975; Högstedt 1980; Török et al. 2004).

According to the IOH, the experimental addition or removal of nestlings from nests would result in parents recruiting less offspring than if they had reared their own original clutch size. This prediction has been supported by some studies (great tit (*Parus major*): Perrins & Moss 1975; Pettifor et al. 1988, 2001; Tinbergen & Daan 1990; blue tit (*Cyanistes caeruleus*): Pettifor 1993; Knowles et al. 2010; collared flycatcher (*Ficedula albicollis*): Gustafsson & Sutherland 1988; see also Murphy 2000 for short term survival data in the Eastern kingbird (*Tyrannus tyrannus*)), while other studies failed to find support to this prediction (great tit: Dhondt et al. 1990; Sanz & Tinbergen 1999; Tinbergen & Both 1999; Rytkönen & Orell 2001; Tinbergen & Sanz, 2004; blue tit: Nur, 1984a; Dhondt et al., 1990; Blondel et al., 1998; collared flycatcher: Török et al., 2004). Mixed evidence for the hypothesis has even been found in the same population for some species. For example, Nur (1984a) did not find support to the IOH in the population of blue tits from Wytham Woods, in England,

but Pettifor (1993), after reanalysing Nur's data and adding his own, showed that females laid that clutch size which maximized the offspring recruitment. Meanwhile, in the population of great tits from Hoge Veluwe, in the Netherlands, the IOH was initially supported (Tinbergen & Daan 1990) but then rejected (Tinbergen & Both 1999). Other studies found insights of individual optimization, in terms of maximizing the offspring's body mass and fledging success: females optimize their clutch size when they retain high-quality territories (Högstedt 1980) or show reactive behaviours (Nicolaus et al. 2015). These contrasting results might have emerged because the studies did not take into account the dispersal distances, which may be affected by the brood size manipulation (Doligez et al. 2002; Tinbergen 2005).

A potential test of the IOH in the short term is to measure fledgling quality parameters which predict the post-fledging survival, and examine its variation along brood size manipulation (e.g. Nicolaus et al. 2015). Given that in passerines, such as the blue tit, nestling morphological measures at fledging significantly determines recruitment probability (Nur 1984a; Charmantier et al. 2004; Råberg et al. 2005), the IOH would predict that the addition of offspring in the nests should deteriorate the fledgling condition given that parents would be rearing a size of brood different from their original clutch size. Also, parents from control broods (not manipulated) would raise highest quality fledglings. The removal of nestlings probably would not deteriorate fledgling condition regarding control nests, but the number of fledglings would be reduced. In fact, optimal reproductive investment requires trading-off the number versus the quality of offspring (Roff 2002). This means that experimentally enlarged broods would result in more but worse-quality fledglings compared to control and/or reduced broods. This trade-off has been evidenced in a number of bird species, such as the great tit (Lindén 1988; Smith et al. 1989; Sanz & Tinbergen 1999; Losdat et al. 2010; Wegmann et al. 2015), blue tit (Moreno et al. 1996; Fargallo & Merino 1999; Kunz & Ekman 2000), willow tit (*Poecile montanus*) (Orell & Koivula 1988), collared flycatcher (Merilä 1996; Voillemot et al. 2012), pied flycatcher (*Ficedula hypoleuca*) (Silverin 1982), spotless starling (*Sturnus unicolor*) (Gil et al. 2008, 2019), European starling (*Sturnus vulgaris*) (Allen et al. 2023), tree swallow (*Tachycineta bicolor*) (Burness et al. 2000), barn swallow (*Hirundo rustica*) (Saino et al. 1997, 2002, 2003), and alpine swift (*Tachymarptis melba*) (Bize et al. 2010) (recently reviewed by Grames et al. 2023). On the other hand, when brood size is reduced, parents may be able to raise high-quality fledglings without increasing their

provisioning effort (Richner et al. 1995; Sanz & Tinbergen 1999; Musgrove & Wiebe 2014). Even, parents could reduce their feeding effort when brood size is enlarged to ensure their own rather than their offspring's survival (Nur 1984b, 1988; Gustafsson & Sutherland 1988; Pettifor et al. 1988; Rytkönen et al. 1996).

Fledgling recruitment in the population not only depends on body condition, but physiological traits such as nestling immune response and oxidative status also affect fledgling survival (Cichoń & Dubiec 2005; Losdat et al. 2013). In this sense, several studies show that the addition of offspring to the nest impact the nestling immune system by both reducing the capacity to enhance an immune response (Saino et al. 1997, 2002, 2003; Hõrak et al. 1999; Pap & Márkus 2003; Cichoń & Dubiec 2005; Gil et al. 2008) or elevating the immune stress (Ilmonen et al. 2003; Suorsa et al. 2004; Bańbura et al. 2013). Nestlings reared in enlarged broods may also suffer more from oxidative stress than nestlings reared in control and/or reduced broods, in terms of a decrease in the antioxidant defences (Bourgeon et al. 2011) or an increase in the oxidative damage (Reichert et al. 2015), although these changes may be no detectable in some cases (Losdat et al. 2010; Gil et al. 2019). Therefore, the IOH would also predict a clutch size which optimize the immune capacity and oxidative status in fledglings.

Moreover, if individuals adjust their clutch size to local circumstances, then the optimal clutch size would be modulated by habitat quality. In heterogeneous environments, the optimal clutch size is expected to be more variable than in stable environments (Orzack & Tuljapurkar 2001), and unpredictable environmental fluctuations may preclude the individual optimization of clutch size (McNamara 1998). Indeed, individuals might fail to optimize their clutch size if habitats exhibit strong heterogeneity in food resources (caterpillars for most insectivorous birds) (Blondel et al. 1998; Török et al. 2004). A low food availability may select for a low clutch size (Blondel et al. 1998), or may reduce the nestling condition when the experimental brood size is larger than the original clutch size (Tremblay et al. 2003; Harriman et al. 2017). In fact, when food resources are limited in a given habitat, parents do not increase their feeding effort when the brood size is experimentally enlarged (Smith et al. 1988; Rytkönen et al. 1996; Rytkönen & Orell 2001; Musgrove & Wiebe 2014), suggesting that parents adjust their clutch size to the maximum number of nestlings they can successfully rear. Thus, the optimal clutch size is

expected to differ between habitats exhibiting contrasting environmental conditions, even at the small scale. In fact, some studies have reported evidence of optimization for clutch size occurring in bird populations inhabiting habitat patches separated by few kilometres (blue tit: Blondel et al. 2006; García-Navas et al. 2014; Charmantier et al. 2016; great tit: Postma & van Noordwijk 2005; Postma et al. 2007, 2009). However, gene flow between closely populations with different optimal clutch size might preclude habitat-dependent optimization of the clutch size (Dhondt et al. 1990; Blondel et al., 1998).

In this respect, there is even little information on habitat-dependent optimal clutch sizes at small scales. We reported a possible example in a Mediterranean population of blue tits inhabiting a heterogeneous and continuous woodland, extending over a valley, located in the southeast of the Iberian Peninsula. In previous studies, we found that female blue tits adjusted their clutch size to the slope of the valley where they breed (Garrido-Bautista et al. 2023), with an adaptive process underlying such between-slope variation in clutch size (Garrido-Bautista et al. submitted). Concretely, the blue tits from the two slopes, form two differentiated genetic clusters, with selection favouring different clutch sizes between these clusters (females from a dry forest in the east-facing slope of the valley lay on average one egg less than females from a humid forest in the west-facing slope) (Garrido-Bautista et al. 2023; Garrido-Bautista et al. submitted). The environmental heterogeneity within the woodland seems to be behind such adaptive process: the dry forest of the east-facing slope of the valley receives more solar radiation, has a higher temperature and lower humidity, less tree cover, lower nest infestation by ectoparasites and a lower caterpillar abundance during the spring compared to the humid forest of the west-facing slope (Garrido-Bautista et al. 2021, 2022, 2023).

The aim of this study was to test the IOH in the short term in the aforementioned population of blue tits inhabiting the two slopes of the valley. We predicted that, if blue tits optimize their clutch size to their local environmental circumstances (determined by the forest type), the experimental addition of offspring to broods should deteriorate the mean nestling condition in a similar way in each forest type because parents would be rearing a size of brood different to their original (and presumably optimal) clutch size. This should be due to a decrease in the parental feeding effort per chick with the experimental addition of offspring to broods. We also

predicted high nestling condition when the brood size is reduced given than parents would be able to raise high-quality nestlings while ensuring their self-maintenance. By contrast, if clutch size is not optimized in each forest (for example as a consequence of maladaptive gene flow; see Dhondt et al. 1990; Postma & van Noordwijk 2005), the effects of the brood size manipulation should differ between forests. To this end, we experimentally manipulated the brood size (creating reduced, control, and enlarged broods) and measured the condition of the largest and smallest nestlings within individual broods. Concretely, we measured morphological (tarsus length, body mass and body condition) and physiological (immune system and oxidative status) traits when nestlings were 13 days old, as well as the parental feeding rate, nestling diet and food availability. Because blue tit eggs hatch asynchronously, thus leading to a within-brood size hierarchy (Slagsvold et al. 1995; Stenning 2008), by sampling the smallest and largest nestlings we covered the full range of variation in biometry within the broods, as brood rank impacts body mass and condition (see results), as well as immune response (Garrido-Bautista et al. 2022) and oxidative status (Garrido-Bautista et al. 2021).

Materials and methods

Study area and blue tit monitoring

The study area was located at 1700–1800 m above sea level in the Sierra Nevada National Park (southeastern Spain; 36°57'N, 3°24'W). The study was performed in 2021 in the two aforementioned forest types, which are part of a continuum of habitats and extend over the two opposite slopes of a valley (Fig. 1). The two forest types comprised a dry forest composed of Pyrenean oaks (*Quercus pyrenaica*) and a humid forest containing Pyrenean oaks and some Holm oaks (*Quercus ilex*). The dry forest is located in the east-facing slope of the valley, whilst the humid forest extends over the west-facing slope of the valley (Fig. 1). The higher humidity of the humid forest (see results) is due to its southwestern orientation and the presence of a stream (Acequia Almiar; see details in Garrido-Bautista et al. 2023), and, to simplify, we refer to these two areas as humid and dry forests throughout the text. In each forest (dry: 350 ha, humid: 127 ha), we placed 75 nest boxes (ICONA-C model; details in Moreno-Rueda 2003). We hung each nest box in an oak tree's branch at 3–4 m height and

distributed them 100 m apart. We regularly inspected the nest boxes to determine the laying date (date of the first egg laid), clutch size and hatching date (date of the first egg hatched). To measure ambient temperature (ºC) and humidity (%) in the dry and humid forests, we installed data loggers iButtom® in the outer wall of nest boxes (5 in each forest). Data loggers were placed on 01 May and removed on 07 July, and measurements were taken every hour.

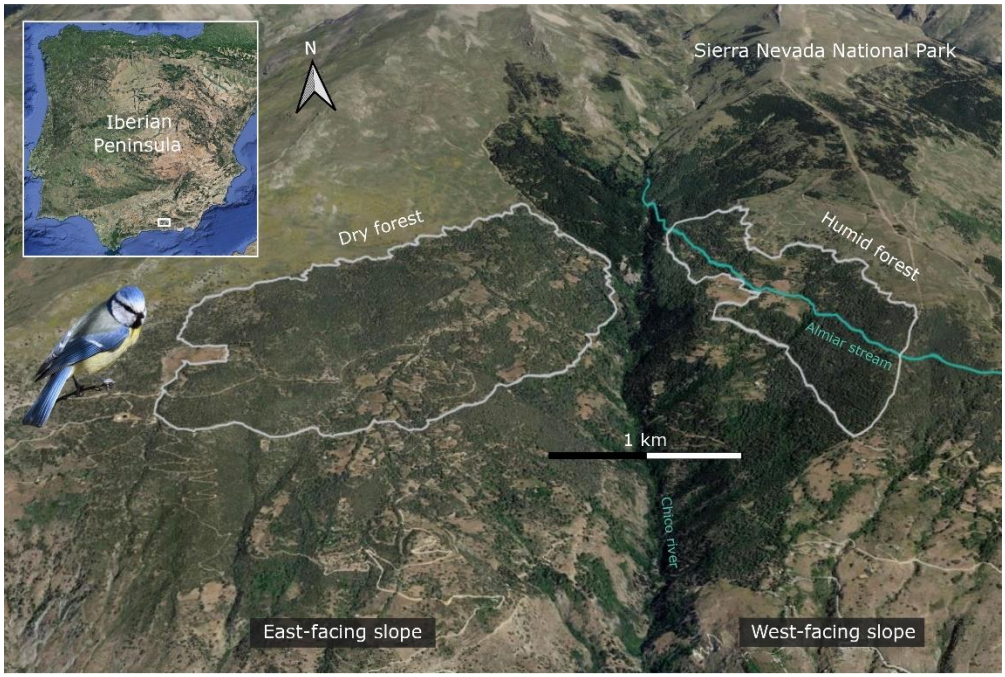

Figure 1. Map of the study area showing the two forests (dry and humid forests) located in the two slopes of the valley (east-facing and west-facing slopes, one facing each other) where blue tits (*Cyanistes caeruleus*) were sampled. Notice that the Chico River separates the two slopes and the Almiar stream crosses the humid forest. Map created with the software QGIS 3.10.5 connected to Google Earth.

Brood size manipulation experiment

In each forest, active nests were randomly assigned to one of three experimental groups (reduced, control, and enlarged), with broods having the same hatching date and being treated in pairs or threesomes when moving the nestlings. In each brood,

3-days-old nestlings (hatching day = 0) were weighed with a portable, digital scale (accuracy, 0.1 g) and they were ranked according to body mass. The largest, smallest and 1-3 randomly medium-size nestlings were marked on the flank and above the humerus with non-toxic paint colours (Edding®, Germany). These body parts are not visible to parents when they feed the nestlings, and this marking has no deleterious effects on nestling body mass or survival (Quesada & Senar 2012). Nestlings were re-marked every 2 days until 13 days old. We performed the manipulation of brood size when nestlings were 3 days old given that blue tits have asynchronous hatching and so it usually takes 2–3 days for all the eggs to hatch (Stenning 2008). To create enlarged and reduced broods, we moved three nestlings from reduced to enlarged broods using warm, breathable bags, and one nestling from the enlarged to the reduced broods. For control broods, two medium-size nestlings were interchanged between two control nests. Thus, we created brood sizes deviating for their original sizes by -2, 0 and +2 nestlings, which received the same perturbation and contained at least one alien nestling. Notice that all interchanged nestlings were marked and of medium size, so we focussed on the largest and the smallest nestlings in each nest. The largest and smallest nestlings always remained in their original brood, hence we tested the effect of enlarged or reduced brood on these unmanipulated nestlings.

All broods used in this study were first clutches and we did not observe parental desertion or mortality of parents in any nest. We studied 81 broods in total, 35 from the dry forest (reduced: 9, control: 13, enlarged: 13) and 46 from the humid forest (reduced: 13, control: 18, enlarged: 15). All them fledged at least one nestling, and mortality between the experimental manipulation and fledging (the day at which nestlings left their nests; nestling period: 20–22 days) was very low, as 91.36% (74 out of 81) of the nests fledged the same number of nestlings as were when they were 3 days old after the experimental manipulation.

Morphometric and immune parameters

When the nestlings were 13 days old, we took a 100 μL blood sample from the largest and smallest nestlings (ca. 1% of the nestling body mass, which is about 10 g) from the jugular vein using heparinized, disinfected insulin syringes following Owen (2011). This quantity of blood has negligible impacts on nestling condition and survival (Sheldon et al. 2008). We also weighed the largest and smallest nestlings with a

digital, portable scale (accuracy, 0.1 g) and measured their tarsus length with a digital calliper (accuracy, 0.01 mm). We calculated the body condition index of nestlings as the residuals of the regression (obtained by ordinary least-squares) of log-transformed tarsus length on log-transformed body mass (Labocha & Hayes 2012).

A drop of blood was smeared on a slide and air-dried following Owen (2011). The remaining blood was preserved in 1.5 mL Eppendorf vials in a portable fridge and transported to the laboratory (2 h away) where were frozen at -80 ºC (see below). Blood smears were fixed in absolute methanol for 7 min and stained with a Romanowsky stain using a commercial kit, following the manufacturer's instructions (RAL Diff-QuikTM, Siemens Healthineers). Smears were prepared with Eukitt® mounting medium and were viewed with a Leica DM 1000 LED microscope at 400× magnification. Forty fields per blood smear were photographed with a Leica ICC50 W camera connected to the microscope. Leucocytes were counted and identified following Campbell & Ellis (2007), and erythrocytes were counted using the Mizutama software (Ochoa et al. 2019). We estimated the number of heterophils, lymphocytes, and total leucocytes (sum of all types of leucocytes) for each nestling per 10,000 erythrocytes. We also estimated the heterophil-to-lymphocyte ratio (H/L ratio).

Oxidative status analyses

Blood samples preserved in vials were mixed with a cold buffered solution (20 mM Tris-HCl, 10% glycerol and 0.1% Triton X-100 (v/v), pH 8.0) in a ratio of 1:3 (v/v), frozen for 48 h at -80 ºC to break down cell membranes, and then centrifuged at 5,000 g for 10 min at 4 ºC in a Sigma 3 K30 centrifuge. The supernatant was distributed into aliquots of 50 µL (one for each biochemical assay, see below) and frozen at -80 ºC until analysis.

Because oxidative stress increases unevenly according to the characteristics of a particular reactive oxygen species (ROS) (Halliwell & Gutteridge 1995), and there is no single biomarker for oxidative stress (Monaghan et al. 2009; Hõrak & Cohen 2010), we measured the oxidative damage, total antioxidant capacity, and the activity of several antioxidant enzymes to obtain a general view of the nestling oxidative status. We quantified the oxidative damage in blood based on the levels of malondialdehyde (MDA), the main product of lipid peroxidation, and we examined the total antioxidant capacity based on the Trolox-equivalent antioxidant capacity

(TEAC), a parameter that assesses the cumulative action of all the antioxidants present in plasma. We also examined the activity of catalase (CAT), glutathione peroxidase (GPX) and glutathione S-transferase (GST). We followed well-established protocols, adapted to blue tit blood samples, to measure all these biomarkers, which were detailed elsewhere (Garrido-Bautista et al. 2021). The MDA levels and activities of these antioxidant enzymes in the blood reflect their level in other tissues, hence they are good indicators of the individual oxidative status (Margaritelis et al. 2015).

All the biochemical assays were conducted at 25 ± 0.5 ºC using a PowerWavex microplate scanning spectrophotometer (Bio-Tek Instruments, USA) in 96-well microplates (UVStar®, Greiner Bio-One, Germany). The enzymatic reactions were started by adding the supernatant, and all assays had their controls, which consisted of all components except the blood sample. The specific enzyme activity was estimated based on the haemoglobin levels. Haemoglobin concentration was determined using the Drabkin method (Spinreact, Spain), with animal-origin haemoglobin (15 g/dL) as the standard. For all the biochemical variables, two measurements were taken from each aliquot, and the average of these was used in the subsequent statistical analyses.

Parental feeding rate and nestling diet

When the nestlings were 7 days old, we placed a GoPro Hero 5 microcamera under the roof of nest boxes to record parental feeding rate and nestling diet. The microcameras were placed in the morning, at approximately 9:00 h (GMT), and recorded for approximately 120 min, always on climatologically benign days. A box of similar size and appearance was installed the previous day to ensure parental habituation. We considered the recording time in a nest from the first to the last feed (parents took several minutes to resume their feeding activity after the disturbance caused by the microcamera installation). We estimated the number of feeds and standardised the per-brood feeding rate as the number of feeds per hour in each nest, while the per-nestling feeding rate was calculated dividing the per-brood feeding rate by the brood size when the nestlings were 7 days old. A total of 40 nests were filmed (reduced: 8, control: 18, enlarged: 14). We identified prey items from a total of 2054 feeding trips, which were used for analyses of nestling diet. Food items were classified into the following prey categories given their frequency of occurrence: Lepidoptera (caterpillars and pupae), Araneae (spiders), 'other taxa' (which included Coleoptera,

Diptera, Hemiptera, Hymenoptera, Mantodea and Orthoptera) and 'not identified' items. The proportion of each prey category in the nestling diet was calculated as their numbers divided by the total number of provisioning trips. Given the differences in nutrient composition of caterpillars and spiders (Ramsay & Houston 2003; Eeva et al. 2010), we calculated the relative contribution to the nestling diet of Lepidoptera (caterpillars and pupae) and spiders, which was expressed as a ratio (Araneae/Lepidoptera ratio: proportion of spiders from combined number of Lepidoptera and Araneae). Hence, higher values denote a high proportion of spiders in the diet, while lower values denote a low proportion of spiders in the diet.

In addition, we estimated the abundance and relative proportions of leaf-consuming arthropods in the dry and humid forests following Zandt (1994). On 08 June, a date close to the nestling food peak demand (average date when the nestlings were 7 days old: 06 June ± 0.48 days), we sampled 7 randomly selected oaks in each forest type (14 oaks in total) to estimate the abundance and relative proportions of arthropods. We shot 10 times a branch in every oak, with a 2 × 2 m blanket placed in the ground under the branch, and arthropods were collected in 70%-ethanol labelled vials. Arthropods were identified to the order level and abundance expressed as number of arthropods in 4 m^2 per 10 shoots.

Statistical analyses

We used Cleveland plots to check whether the morphometric (body mass, tarsus length and body condition index), immune (number of leucocytes and H/L ratio), oxidative status (MDA, TEAC, CAT, GPX and GST), behavioural (feeding rate) and dietary (Araneae/Lepidoptera ratio) variables had outliers, and we graphically inspected these variables for normality (Zuur et al. 2010). We detected outliers for enzyme activities of three nestlings from the dry forest. The outliers were probably artefacts (e.g. small blood clots) as they far exceeded the confidence intervals at 95% for the enzyme activities (CAT: CI-95% = 6.16–8.66 U/mg Hb, n = 142, outliers = 64.26, 69.57 and 83.33 U/mg Hb; GPX: CI-95% = 7.22–11.01 mU/mg Hb, n = 130, outliers = 111.32, 112.09 and 172.51 mU/mg Hb; GST: CI-95% = 1.52–2.08 mU/mg Hb, n = 130, outliers = 17.00, 21.72 and 23.54 mU/mg Hb). An outlier was also detected for tarsus length (CI-95% = 16.32–16.54 mm, n = 149, outlier = 11.94 mm). For this reason, we performed the analyses using both the original dataset and the one

that excluded these outliers. Models including the outliers gave qualitatively the same results as those without them. Herein, we report the statistical analyses without the outliers.

Before carrying out the models, we summarized the information of the biomarkers of oxidative status (MDA, TEAC, CAT, GPX and GST) by a Principal Component Analysis (PCA). We standardized the data of the 5 biomarkers of oxidative status and obtained the correlation matrix (supplementary material; Fig. S1). We obtained two principal components with eigenvalues above 1 (Quinn & Keough 2002) that explained 70% of the variability of the original variables. The first axis explained 49.1% of the variability and was positively related with the activity of CAT, GPX and GST, while the second axis explained 20.8% of the variability and was negatively related with the MDA levels and TEAC (supplementary material; Fig. S2 and S3, Table S1). Since the first principal component summarized the information about the activity of all enzymes, which were strongly correlated, on linear combinations of the original variables, we herein refer to it as OS1. We used OS1, TEAC and MDA in the subsequent analyses.

We used the chi-square test to determine inter-forest differences in the frequency of Lepidoptera (caterpillars and pupae) and Araneae (spiders). We used the Student's *t*-test to examine differences in temperature and humidity between forest types. To determine differences between forest types in laying date, clutch size and original brood size (measured when the nestlings aged 3 days, before conducting the experimental manipulation), we used the Student's *t*-test. The Levene's test was used to check the homogeneity of variances when conducting *t*-tests. The correlation between the clutch size and laying date within each forest was established using the Pearson product-moment correlation.

We used a linear model to check whether the experimental manipulation of brood size affected the number of nestlings produced per nest. The number of nestlings measured before leaving the nest was the dependent variable, while brood size manipulation (three levels: -2, 0 and +2), forest type (two levels: dry and humid forests) and their interaction were the predictors. We used linear mixed-effects models of restricted maximum likelihood (REML-LMM) (Zuur et al. 2009) to assess the effects of the experimental manipulation of brood size on nestling condition and physiology. The immune parameters, biomarkers of oxidative status and

morphometric variables were the dependent variables in separate models. The forest type, nestling rank (two levels: largest and smallest nestling), brood size manipulation and the interaction between forest type and brood size manipulation were the fixed factors. Laying date was a covariate and nest identity was a random factor (random intercept). We included laying date as covariate in the models because delayed clutches usually produce low-quality nestlings (Barba et al. 1995; Sanz 1999).

We used a linear model to assess the effects of the brood size manipulation on parental feeding effort. The per-brood and per-nestling feeding rates were the dependent variables in separate models. The brood size manipulation, forest type and their interaction were the predictors, while laying date was a covariate. A linear model was also used to examine the effects of the brood size manipulation on nestling diet. The ratio Araneae/Lepidoptera was the dependent variable, while the brood size manipulation, forest type and their interaction were the fixed factors, and laying date was included as a covariate. We included laying date in models for feeding rate and nestling diet because prey composition and abundance typically change over the breeding season, thus influencing foraging decisions (García-Navas & Sanz 2011).

All analyses were run in the 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), 'emmeans' (Lenth 2023) and 'car' (Fox et al. 2022). The PCA was run using the package 'factoextra' (Kassambara & Mundt 2020). The performance of linear models (residual normality and homoscedasticity) was assessed using the package 'performance' (Lüdecke et al. 2023). To achieve normality of model residuals and error variance homoscedasticity, the number of leucocytes, H/L ratio and MDA level were log-transformed, while the Araneae/Lepidoptera ratio was logit-transformed. *Post hoc* analyses were used to assess between which levels of the brood size manipulation the respective dependent variables varied. We created plots using the package 'ggplot2' (Wickman 2016). The basic statistics are presented as the mean ± standard error (s.e.).

Results

Descriptive statistics of the study system

Laying occurred between 29 April and 20 May (average: 10 May ± 0.56 days), but females from the dry forest laid their eggs 3 days earlier, on average, than females from the humid forest (t_{79} = -2.84, p = 0.006). Clutch size ranged from 4 to 10 eggs (mean: 7.30 ± 0.16), and original brood size ranged from 3 to 10 (mean: 6.58 ± 0.19). Median clutch size was 7 eggs in the dry forest and 8 eggs in the humid forest. The clutch size was lower in the dry forest than in the humid forest (dry forest: 6.83 ± 0.23 eggs, humid forest: 7.65 ± 0.19 eggs; t_{79} = -2.75, p = 0.007), and was negatively correlated with laying date in both forests (dry forest: r = -0.52, p = 0.002; humid forest: r = -0.51, p < 0.001). The original brood size was also lower in the dry forest (dry forest: 6.03 ± 0.27 nestlings, humid forest: 7.00 ± 0.24 nestlings; t_{79} = -2.65, p = 0.010).

A total of 239 arthropods, comprising 12 different taxa, were collected in the two forests (supplementary material; Table S2). Lepidoptera (caterpillars and pupae) was the most common taxa found (33.47%). The frequency of spiders was higher in the humid (68.4%) than in the dry forest (31.6%; χ^2 = 6.70, p = 0.01), while the frequency of Lepidoptera tended to be higher in the humid forest (62.5% vs. 37.5%; χ^2 = 3.44, p = 0.06). The mean temperature of the dry forest (18.58 ± 0.16 ºC) was higher than the humid forest (17.36 ± 0.15 ºC; t_{1627} = -18.26, p < 0.001), while humidity was lower in the dry forest (52.08 ± 0.44 %) than in the humid forest (54.57 ± 0.43 %; t_{1627} = 14.58, p < 0.001).

Effects of brood size manipulation on nestling physiology

Brood size manipulation did not affect the number of leucocytes per 10,000 erythrocytes and H/L ratio, nor any of the biomarkers of oxidative status (Table 1). Although the immune parameters were not related to forest type, nestlings from the dry forest exhibited higher levels of lipid peroxidation than nestlings from the humid forest (dry forest: 6.95 ± 0.18 μM, humid forest: 6.49 ± 0.20; t-value = 2.42, p = 0.02; Table 1). OS1 values were higher in the dry than in the humid forest (estimate = 2.38, reference category = humid forest, t-value = 6.50, p < 0.001; Table 1).

Table 1. Results of the linear mixed-effects models for immune parameters (number of leucocytes and H/L ratio), biomarkers of oxidative status (OS1, MDA and TEAC) and morphological and condition parameters (tarsus length, body mass and body condition index) in blue tit (*Cyanistes caeruleus*) nestlings from the Sierra Nevada National Park, southeastern Spain. The statistical significance at $p < 0.05$ is marked in bold.

Dependent variable	Factor									
	Forest type		Brood size manipulation		Nestling rank		Laying date		Forest type*brood size manipulation	
	χ^2 (df = 1)	*p*-value	χ^2 (df = 2)	*p*-value	χ^2 (df = 1)	*p*-value	χ^2 (df = 1)	*p*-value	χ^2 (df = 2)	*p*-value
Number of leucocytes (per 10,000 erythrocytes)	0.23	0.63	1.19	0.55	0.64	0.42	0.26	0.61	1.28	0.53
H/L ratio	1.19	0.28	0.83	0.66	2.37	0.12	0.17	0.68	2.22	0.33
OS1 [a]	42.23	**< 0.001**	0.25	0.88	0.88	0.35	0.86	0.35	0.39	0.82
MDA (µM)	5.84	**0.01**	0.97	0.61	< 0.01	0.97	3.49	0.06	2.29	0.32
TEAC (µM eq. Trolox)	2.53	0.11	2.45	0.29	0.31	0.58	2.46	0.12	1.50	0.47
Tarsus length (mm)	0.27	0.60	2.91	0.23	3.80	0.06	< 0.01	0.99	1.85	0.39
Body mass (g)	0.20	0.66	8.31	**0.02**	42.25	**< 0.001**	2.57	0.11	1.55	0.46
Body condition index	0.04	0.85	7.59	**0.02**	35.08	**< 0.001**	3.99	**0.04**	0.53	0.76

[a] OS1 is the first component of the PCA and is positively related to the activity of catalase (CAT), glutathione peroxidase (GPX) and glutathione S-transferase (GST) (see main text).

This means that the antioxidant enzymes of nestlings from the dry forest were raised compared to the enzymes of nestlings from the humid forest: CAT (dry forest: 13.50 ± 1.15 U/mg Hb, humid forest: 3.32 ± 0.18), GPX (dry forest: 17.60 ± 2.13 mU/mg Hb, humid forest: 3.96 ± 0.36) and GST (dry forest: 3.08 ± 0.27 mU/mg Hb, humid forest: 0.97 ± 0.04).

Effects of brood size manipulation on nestling body condition

The enlarged broods resulted in more fledged nestlings than the control broods (enlarged: 8.61 ± 0.34 nestlings, control: 6.45 ± 0.29 nestlings), which were in turn more productive than the reduced broods (4.68 ± 0.38 nestlings) ($F_{2, 75} = 34.81$, $p < 0.001$; Tukey's test, $p < 0.001$ in all cases; Fig. 2). Parents from the humid forest produced more nestlings than parents from the dry forest (humid forest: 7.07 ± 0.35, dry forest: 6.26 ± 0.37; $F_{1, 75} = 6.32$, $p = 0.014$), and the effect of the brood size manipulation did not differ between the two forests (interaction forest type*brood size manipulation; $F_{2, 75} = 0.25$, $p = 0.77$; Fig. 2). The smallest nestlings reached the same structural size as the largest nestlings (tarsus length of largest nestlings: 16.51 ± 0.07 mm, tarsus length of smallest nestlings: 16.34 ± 0.09; Table 1). However, the smallest nestlings weighed less (largest nestlings: 10.39 ± 0.09 g, smallest nestlings: 9.77 ± 0.11; Table 1) and showed worse body condition index (largest nestlings: 0.024 ± 0.005, smallest nestlings: -0.025 ± 0.006; Table 1) than the largest nestlings when they were measured at 13 days. The body condition index significantly decreased with the advance of laying date (Table 1).

Parents raised lighter nestlings and with a worse body condition index when broods were experimentally enlarged with 2 extra nestlings compared to control and reduced broods (Fig. 3; Table 1; *p*-values from Tukey's test for body mass: reduced–control = 0.88, control–enlarged = 0.07, reduced–enlarged = 0.03; *p*-values from Tukey's test for body condition index: reduced–control = 0.95, control–enlarged = 0.02, reduced–enlarged = 0.06). Nestling body mass and condition did not differ between forests, and the effect of the brood size manipulation on nestling body mass and body condition index was independent from the forest type (Fig. 3; Table 1).

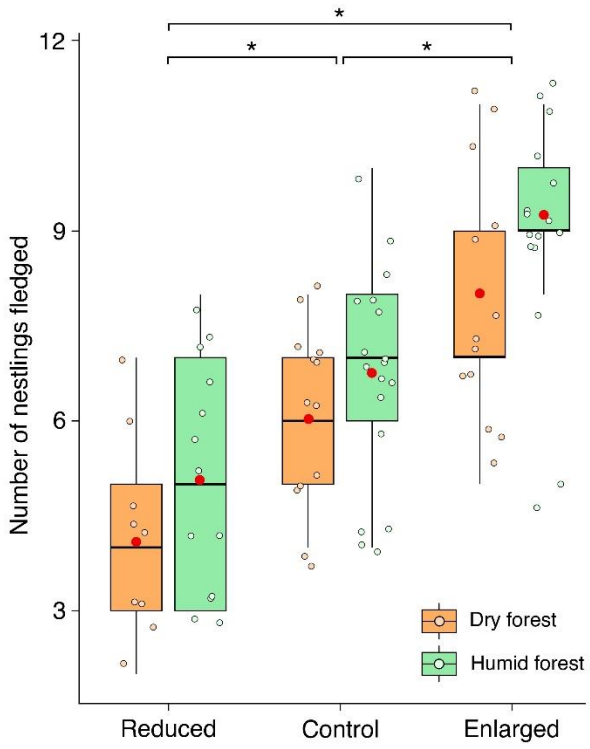

Figure 2. Variation in the number of nestlings produced per blue tit pair across the three brood size manipulation treatments (reduced: broods with -2 nestlings; control: broods with unchanged brood size; enlarged: broods with +2 nestlings) and in the two forest types (dry and humid forests). The red point shows the mean, the horizontal line the median and the boxplot represents the interquartile range. The presence of an asterisk indicates significant differences ($p < 0.05$) between the treatments.

Effects of brood size manipulation on parental feeding effort and nestling diet

Parents decreased their per-brood feeding rate when broods were experimentally reduced compared to control (Tukey's test, $p = 0.008$) and enlarged broods (Tukey's test, $p = 0.002$), but they did not increase the per-brood feeding rate when broods were experimentally enlarged compared to control broods (Tukey's test, $p = 0.83$) ($F_{2, 33} = 7.69$, $p = 0.002$; Fig. 4a). However, parents did not adjust their per-nestling feeding rate to the brood size manipulation ($F_{2, 33} = 2.33$, $p = 0.11$; Fig 4b), but they tended to decrease the per-nestling feeding rate when broods were enlarged (estimate = -0.93, reference category = control, t-value = -1.87, $p = 0.06$).

The per-brood and per-nestling feeding rates did not differ between forests ($F_{1, 33}$ = 1.68, p = 0.20 and $F_{1, 33}$ = 0.88, p = 0.35, respectively), and the effect of the brood size manipulation on per-brood and per-nestling feeding rate was independent from the forest type (interaction forest*manipulation: $F_{2, 33}$ = 1.04, p = 0.37 and $F_{2, 33}$ = 0.16, p = 0.85, respectively; Fig. 4).

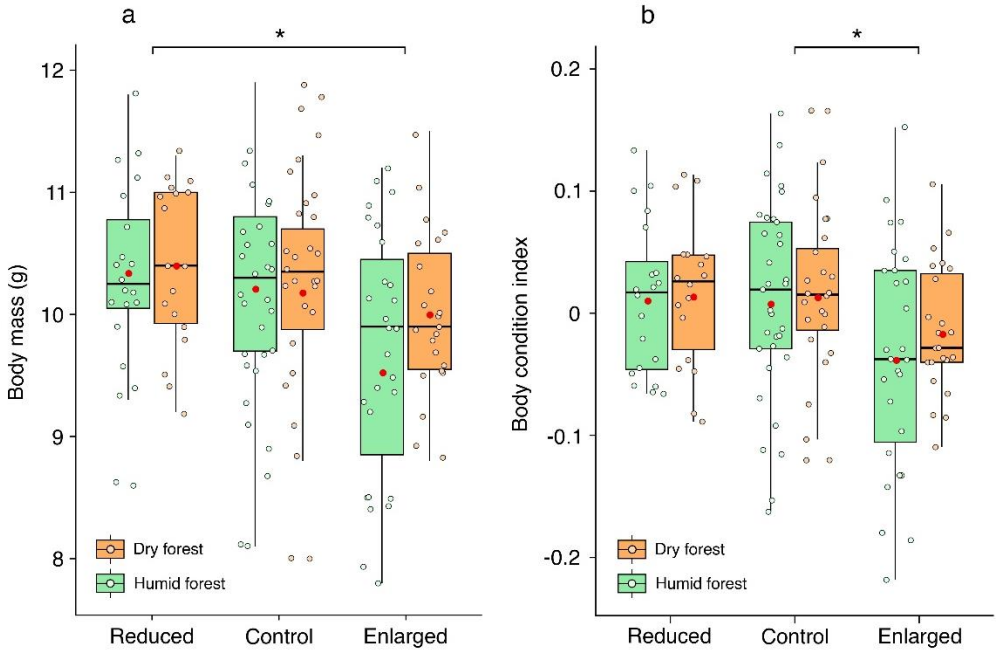

Figure 3. Variation in nestling body mass (a) and body condition index (b) across the three experimental manipulation treatments (reduced: broods with -2 nestlings; control: broods with unchanged brood size; enlarged: broods with +2 nestlings) and in the two forest types (dry and humid forests). The red point shows the mean, the horizontal line the median and the boxplot represents the interquartile range. The presence of an asterisk indicates significant differences (p < 0.05) between the treatments.

Caterpillars constituted by far the greatest proportion of prey items provided to nestlings in the two forests, followed by spiders (Table 2). The relative contribution of spiders and Lepidoptera (Araneae/Lepidoptera ratio) to the diet of nestlings did not show any relationship with forest type ($F_{1, 34}$ = 1.49, p = 0.28) nor laying date ($F_{1, 34}$ = 2.81, p = 0.10).

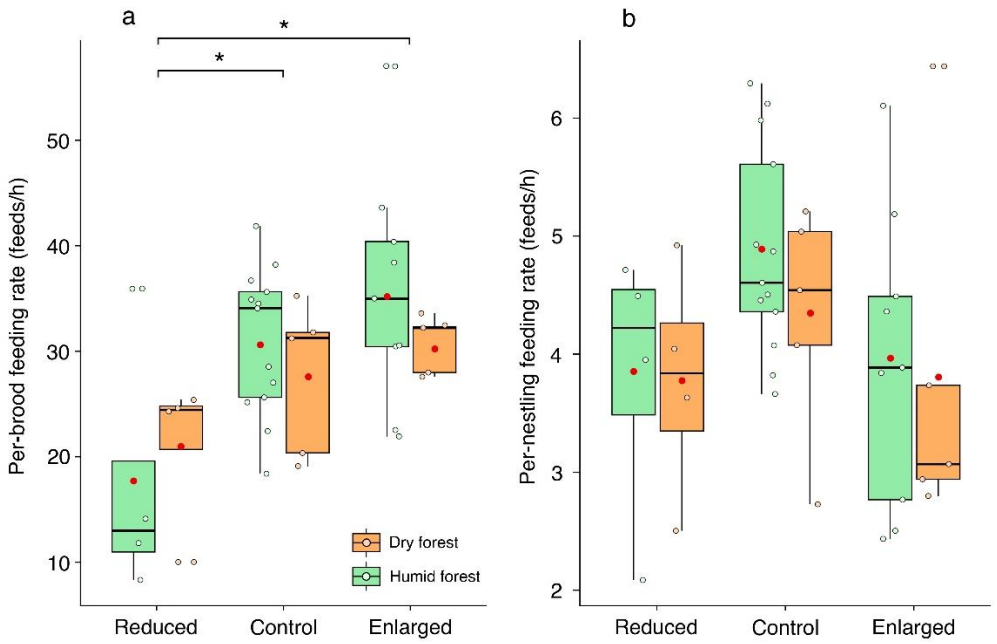

Figure 4. Variation in parental per-brood feeding rate (a) and per-nestling feeding rate (b) across the three experimental manipulation treatments (reduced: broods with -2 nestlings; control: broods with unchanged brood size; enlarged: broods with +2 nestlings) and in the two forest types (dry and humid forests). The red point shows the mean, the horizontal line the median and the boxplot represents the interquartile range. The presence of an asterisk indicates significant differences (p < 0.05) between the treatments.

Table 2. Composition of the diet (%) of blue tit (*Cyanistes caeruleus*) nestlings in the dry and humid forests from the Sierra Nevada National Park, southeastern Spain. Percentages represent average values of all nests recorded at each nest box. Confidence intervals at 95% are given in parentheses.

	Dry forest (n = 14)	Humid forest (n = 27)
Lepidoptera	73.99 (68.40 – 79.58)	71.23 (66.16 – 76.30)
Araneae	17.72 (12.60 – 22.83)	16.84 (12.73 – 20.94)
Other taxa	2.47 (0.43 – 4.51)	3.75 (1.84 – 5.67)
Not identified	5.82 (2.37 – 9.26)	8.18 (5.50 – 10.86)

Note: Lepidoptera includes larva (caterpillar) and pupa stages, while other taxa included Coleoptera, Diptera, Hemiptera (Auchenorryncha), Hymenoptera, Mantodea and Orthoptera.

Parents tended to incorporate a lower proportion of spiders (regarding Lepidoptera) in their nestling diet when the brood size was enlarged (estimate = -0.59, reference category = control, *t*-value = -1.98, $p = 0.055$), but this pattern depended on forest type since the effect was more pronounced in the humid forest (interaction forest type*brood size manipulation: $F_{2, 34} = 3.74$, $p = 0.033$; Fig. 5).

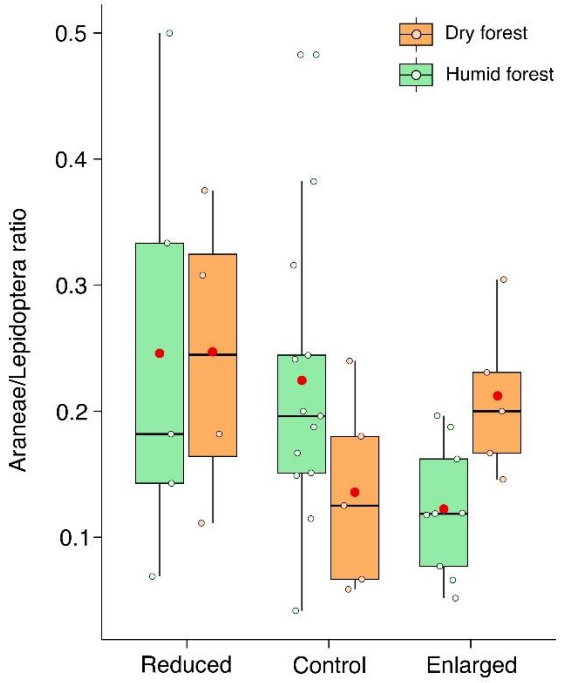

Figure 5. Variation in the relative contribution of spiders and Lepidoptera (caterpillars and pupae) across the three experimental manipulation treatments (reduced: broods with -2 nestlings; control: broods with unchanged brood size; enlarged: broods with +2 nestlings) and in the two forest types (dry and humid forests). The red point shows the mean, the horizontal line the median and the boxplot represents the interquartile range. *Note:* Higher values of Araneae/Lepidoptera ratio denote a high proportion of spiders regarding Lepidoptera in the diet, while lower values denote low proportion of spiders regarding Lepidoptera in the diet.

Discussion

The aim of this study was to test whether blue tit clutch size is optimized according to their local environmental conditions (the forest type) inside a continuous woodland

across the two slopes of a valley. The optimal clutch size was predicted to differ between the two forests since their environmental differences seem to modulate the blue tit reproductive performance (Garrido-Bautista et al. 2023). In a previous study, we found that females from the dry forest laid on average one egg less than females from the humid forest, but also that parents produced similar quality nestlings in the two habitats (Garrido-Bautista et al. 2023). These findings suggested that blue tits adjust their clutch size to an optimal level for reproductive success in each forest type, which has been experimentally demonstrated here. In the present study, females from the dry forest laid on average almost one egg less (6.83 eggs) than females from the humid forest (7.65 eggs), and parents from the two forests reared nestlings with similar body condition. The absence of a significant interaction between the brood size manipulation and forest type on nestling condition, in spite of blue tits laying one egg less in the dry forest, suggests that parents from the two forests optimized their clutch size to local circumstances. The brood enlargement reduced the nestling body mass and condition in the two slopes of the valley, whereas the reduction of brood size did not improve nestling body condition, strongly suggesting that blue tits are laying the maximal clutch size they can rear. The spatial and temporal variability in food resources in the local environment strongly influences the reproductive investment (Blondel et al. 1991; Tremblay et al. 2003; review in Grames et al. 2023), with low food availability conditions being associated to low clutch and brood sizes (e.g. Blondel et al. 1998; Xu et al. 2023). The humid forest exhibited higher food availability than the dry forest (see also Garrido-Bautista et al. 2021, 2022), and this inter-forest difference in food availability could explain why the optimal clutch size was higher in the humid forest than in the dry forest. Morevoer, it should be noted that the humid forest has a higher tree cover than the dry forest (Garrido-Bautista et al. 2023), hence absolute food availability is even higher in the humid forest than reported, as we tested the abundance of arthropods per a tree branch.

We predicted that nestling condition at fledging would be the lowest in enlarged broods because parents would be rearing a brood size different than their original clutch size. Blue tit pairs from the two forests successfully reared the extra young, indicating that parents could raise more nestlings than the eggs laid (VanderWerf 1992). However, the mean body mass and condition diminished in enlarged broods compared to control and reduced broods, a pattern previously found in several passerines, including the blue tit (Moreno et al. 1996; Fargallo & Merino 1999; Kunz

& Ekman 2000). This finding is consistent with an optimized clutch size because parents forced to rear experimentally enlarged broods could not match their performance with control broods. This would imply that the survival prospects of nestlings reared in enlarged broods will be impaired given that, in this species, body size and condition at fledging are strongly related to recruitment (Nur 1984a; Charmantier et al. 2004; Råberg et al. 2005). This optimal reproductive performance at the original clutch size has been observed in other populations. For example, great tit females with a reactive personality behave optimally by producing more and heavier fledglings at their natural brood size (Nicolaus et al. 2015), while Eurasian magpies (*Pica pica*) optimize their clutch size, in terms of production of fledglings, according to the territory quality (Högstedt 1980).

The fact that the experimental addition of offspring to broods deteriorated the nestling condition means that parents were not able to adjust their feeding effort to the new rearing conditions. As predicted, parents did not increase the per-brood feeding rate, and tended to decrease the per-nestling feeding rate, with the experimental addition of offspring to broods. Accordingly, parents reduced the per-brood feeding rate when broods were experimentally reduced. In fact, when broods are reduced, parents may be able to raise high-quality fledglings without increasing their provisioning effort (Richner et al. 1995; Sanz & Tinbergen 1999; Musgrove & Wiebe 2014; Allen et al. 2023). By reducing feeding effort, parents are expected to prevent costs in the short-term (Richner et al. 1995; Moreno et al. 1999) or in the long-term (Orell & Koivula 1988; Orell et al. 1996; Parejo & Danchin 2006), according to the cost of reproduction hypothesis (Charnov & Krebs 1974). Thus, blue tit parents from enlarged broods would ensure, by reducing the provisioning effort, their own self-maintenance and survival, in detriment to their offspring's survival (e.g. Nur 1984b, 1988; Gustafsson & Sutherland 1988; Pettifor et al. 1988; Rytkönen et al. 1996). In sum, it is likely that a trade-off between producing high-quality offspring and ensuring parental's self-maintenance could act in our blue tit population. However, we still lack data for recruitment per nest (the key measure upon which selection would act), obtained over several years of brood size manipulation experiments, to adequately test the predictions of the IOH (e.g. Pettifor 1993; Blondel et al. 1998; Pettifor et al. 2001). Thus, our conclusions remain tentative with respect to long term implications for fitness.

Surprisingly, while the brood size manipulation affected body mass and condition, it did not affect immune capacity, oxidative status, and structural body size. Given the strong links between the immune system and the oxidative status with nestling growth (Owen et al. 2010; Smith et al. 2016), we expected a variation of such mechanisms with the brood size manipulation. The experimental enlargement of broods is expected to result in nestlings suffering some developmental, physiological stress which may constraint growth. In fact, the reduced offspring quality with increasing brood size may arise from a higher immune stress and depressed immunocompetence (Saino et al. 1997, 2003; Hõrak et al. 1999; Ilmonen et al. 2003; Pap & Márkus 2003; Gil et al. 2008), also occurring in the blue tit, with experimentally brood enlargements having negative effects on nestling immune response (Cichoń & Dubiec 2005) or elevating the nestling immune stress (Bańbura et al. 2008, 2013). However, our data did not support this scenario. The reduced nestling immune capacity with increasing brood size may arise as consequence of the low amount of food resources delivered by parents, thus promoting competition for food resources. Blue tit parents tended to decrease the per-nestling feeding rate in enlarged broods, but the nestling diet composition was similar across all treatments. However, parents varied the proportion of spiders depending on forest type and brood treatment, spiders being in general relatively less abundant in the diet when broods were enlarged. This finding suggests that, when parents faced a higher feeding effort, they had problems to collect sufficient spiders, especially in the humid forest, centring their foraging effort on caterpillars, a prey easier to catch.

On the other hand, the oxidative stress suffered by nestlings (in terms of increased oxidative damage or depleted antioxidant defences) was also expected to follow the same pattern of variation along the brood size manipulation than the immune response. Correlative and experimental data have shown that the larger the brood size, the higher the oxidative stress suffered by nestlings (Costantini et al. 2006; Bourgeon et al. 2011; Reichert et al. 2015; Allen et al. 2023), probably because of sibling rivalry or competition for food resources. However, contrary to our predictions, we did not find either any effect of brood size manipulation on the oxidative status, probably because of the biased allocation of nutrients to distinct self-maintenance and growth functions (see above). Our data suggest that blue tit nestlings did not suffer oxidative costs in any of the developmental contexts (i.e. brood sizes), in contrast to other studies (Gil et al. 2019; Romero-Haro & Alonso-Alvarez 2020; Allen et al. 2023). In

contrast, nestlings inhabiting the dry forest suffered more from oxidative stress than nestlings from the humid forest, as they exhibited higher levels of lipid peroxidation and activities of antioxidant enzymes. Although differences in food availability between habitats, particularly in dietary antioxidants, may explain the inter-habitat variation in oxidative status of birds (van de Crommenacker et al. 2011; Isaksson 2013; Salmón et al. 2018), we can discard this possibility given that food availability and nestling diet composition was similar in the two forests. Nest-dwelling ectoparasites may also impose oxidative costs to nestlings (Hanssen et al. 2013; López-Arrabé et al. 2015; but see Maronde et al. 2018). However, inter-habitat variation in parasite prevalence cannot be responsible for the pattern observed, given that ectoparasites are indeed more frequent in nests from the humid forest (Garrido-Bautista et al. 2023; also see Garrido-Bautista et al. 2021). In contrast, differences in ambient temperature between the two forests could be responsible for the variation in nestling oxidative stress (Andersson et al. 2018; Zhu et al. 2020). Overall, the findings suggest that physiological responses are invariant with brood size except perhaps under extreme brood sizes. Nevertheless, we cannot rule out that other physiological variables not measured here may be affected by rearing conditions (e.g. metabolic enzymes, macronutrients concentration, or telomere length; Burness et al. 2000; Bańbura et al. 2013; Reichert et al. 2015; Gil et al. 2019). Still, taken together, our results do not support the view that stress-induced reduction in immune function or changes in oxidative status are important pathways mediating the trade-off between the number and quality of offspring.

Conclusions

Our findings suggest that the blue tit clutch size depends on habitat quality at the fine-scale: the clutch size was on average one egg less in the dry forest than the humid forest (separated by less than 1 km, but connected by a pine forest inhabited by blue tits) and we experimentally showed that this variation in clutch size was habitat-dependent optimized. Nestlings showed worse body condition in experimentally enlarged broods than in control and reduced broods, the latter two showing non-significant differences, but the effect of the experiment was similar in the two forests. The brood size manipulation negatively affected nestling mass gaining without of an apparent physiological cost, neither at the immune nor oxidative level, while the structural growth was not affected, either. Moreover, parents failed to increase their

provisioning effort when the broods were experimentally enlarged, also supporting the view that parents adjust their clutch size to the maximum number of nestlings they can successfully rear. Overall, these results, together with previous studies, are consistent with a fine-scale and habitat-dependent local adaptation process for clutch size: blue tits from the dry and humid forests form two differentiated genetic clusters, with natural selection favouring different clutch sizes between these genetic clusters (Garrido-Bautista et al. submitted).

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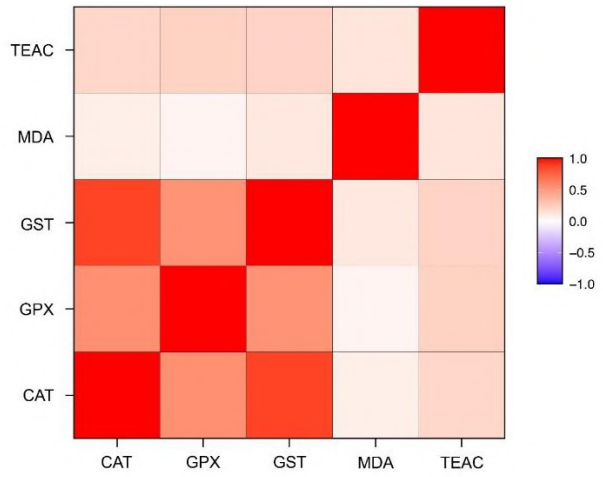

Figure S1. Correlation matrix of the standardized values of the five biomarkers of oxidative status measured in blue tit (*Cyanistes caeruleus*) nestlings. Abbreviations: catalase (CAT), glutathione peroxidase (GPX), glutathione S-transferase (GST), malondialdehyde (MDA), Trolox-equivalent antioxidant capacity (TEAC).

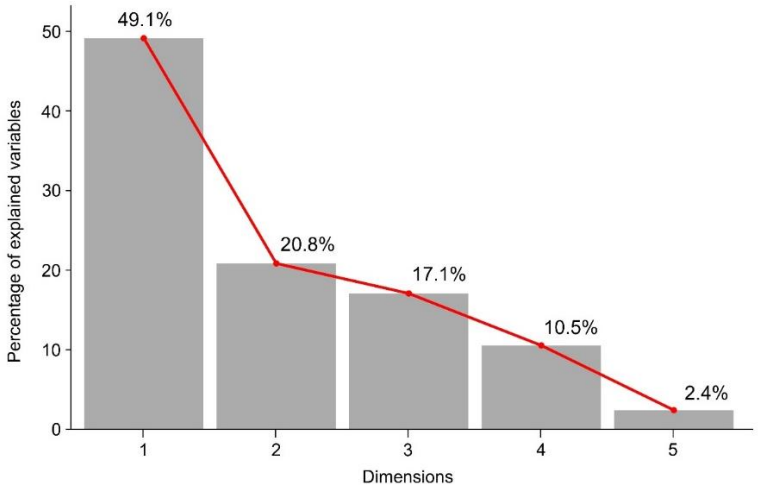

Figure S2. Scree plot of the PCA of the five biomarkers of oxidative status (TEAC, MDA, CAT, GPX and GST) measured in blue tit (*Cyanistes caeruleus*) nestlings. The first dimension was retained for the analyses, which explained approximately the 50% of the variability of the original variables.

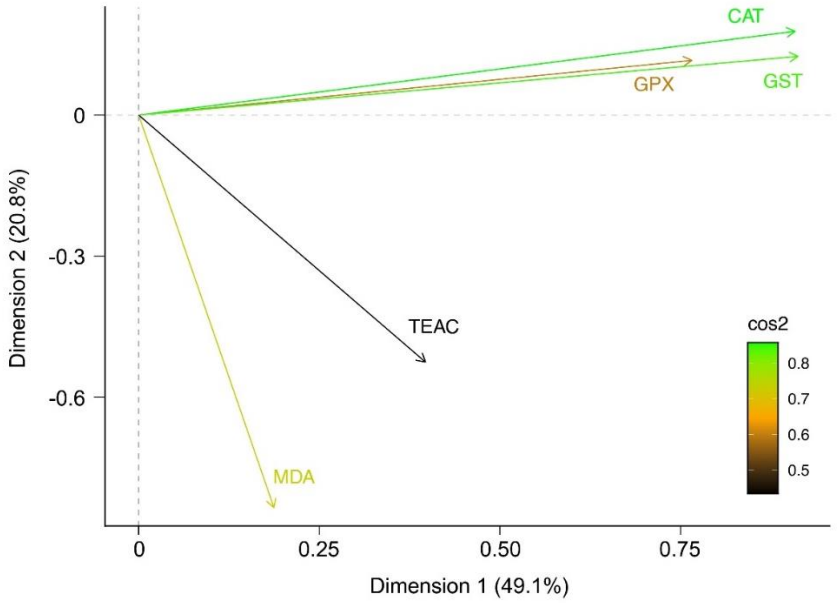

Figure S3. Loadings and quality of representation of each biomarker of oxidative status measured in blue tit (*Cyanistes caeruleus*) nestlings on the two principal components. The first axis (Dimension 1) explained 49.1% of the variability and was positively related to the activity of CAT, GPX and GST. The second axis (Dimension 2) explained 20.8% of the variability and was negatively related to the MDA levels and TEAC.

Table S1. Eigenvalues of the two first dimensions from the PCA considering the five biomarkers of oxidative status (TEAC, MDA, CAT, GPX and GST) measured in blue tit (*Cyanistes caeruleus*) nestlings.

	TEAC (µM eq. Trolox)	MDA (µM)	CAT (U/mg Hb)	GPX (mU/mg Hb)	GST (mU/mg Hb)
Dimension 1	0.254	0.120	0.582	0.490	0.585
Dimension 2	-0.517	-0.821	0.175	0.114	0.123

Table S2. Number of arthropods (number of arthropods in 4 m^2 per 10 shoots) collected in the dry and humid forests. The relative proportion (percentage over the total) is shown in parentheses.

	Dry forest	Humid forest	Total
Araneae	6 (6.5)	13 (8.8)	19 (7.9)
Blattodea	2 (2.2)	1 (0.7)	3 (1.3)
Coleoptera	10 (10.9)	7 (4.8)	17 (7.1)
Dermaptera	1 (1.1)	0 (0)	1 (0.4)
Diptera	0 (0)	2 (1.4)	2 (0.8)
Ixodida	1 (1.1)	0 (0)	1 (0.4)
Hemiptera (Auchenorryncha)	1 (1.1)	3 (2.0)	4 (1.7)
Heteroptera	9 (9.8)	35 (23.8)	44 (18.4)
Hymenoptera	30 (32.6)	35 (23.8)	65 (27.2)
Lepidoptera	30 (32.6)	50 (34.0)	80 (33.5)
Raphidioptera	1 (1.1)	0 (0)	1 (0.4)
Thysanoptera	1 (1.1)	1 (0.7)	2 (0.8)
Total	92	147	239

GENERAL DISCUSSION

Part I. Coping with habitat heterogeneity at the small scale

This thesis documents how the small scale environmental heterogeneity promotes the adaptive divergence within a blue tit (*Cyanistes caeruleus*) population and the evolution of a habitat-dependent optimal clutch size. The first part of the thesis examines how habitat heterogeneity influences the offspring's phenotype, while the second part of the thesis demonstrates how such habitat heterogeneity has promoted the genotypic and phenotypic divergence between blue tit populations, as well as the evolution of fine scale variation in optimal clutch size. Each of the eight chapters of this thesis provides a discussion of the relevant issues, so this final section aims to provide a synthetic view to integrate the main findings of this thesis in an ecological (part I) and evolutionary (part II) framework.

The studied blue tit population reproduced in a small, continuous Mediterranean woodland located along a valley and formed by different forest formations that were characterised by several habitat-specific factors. Mediterranean environments are typically characterised by high spatial and temporal heterogeneity, creating a wide range of selective pressures in small areas (Blondel et al. 2010). Such spatial heterogeneity at the small scale was observed in the study area. The west-facing slope of the valley, composed by Scots pines (*Pinus sylvestris*) and a humid forest of Pyrenean oaks (*Quercus pyrenaica*), received less solar radiation than the east-facing slope of the valley, which was composed by Holm oaks (*Quercus ilex*) and a dry forest of Pyrenean oaks. The west-facing slope of the valley thus provided a more humid and productive environment for breeding blue tits, given its higher humidity and greater percentage of dense tree cover and food availability compared to the east-facing slope. The study area's topography and vegetation structure create a microscale geographic variation similar to the evolution canyons described by Nevo (2006, 2009, 2012), in which opposite, yet closely neighbouring, slopes display marked microclimatic (e.g. temperature, humidity) and biotic contrasts (e.g. presence of parasites, food availability) with potentially different eco-evolutionary processes developing on each slope of the valley.

The environmental differences between habitats determined the presence of parasites infesting blue tit nests. Environmental factors, including ambient temperature and humidity, play an important role in the presence of nest-dwelling ectoparasites in nests within a given habitat (Heeb et al. 2000; Castaño-Vázquez et al.

2018, 2021; Mennerat et al. 2021). Accordingly, nests occupying the west-facing slope of the valley were more frequently infested by nest-dwelling ectoparasites, such as blowflies (*Protocalliphora azurea*) and hen fleas (*Ceratophyllus gallinae*), than those on the east-facing slope, as the latter produced a drier environment for ectoparasites. In addition, parasitoids (*Nasonia vitripennis*) of blowfly pupae were more common in nests located in the west-facing slope of the valley (Chapter 1). *Nasonia vitripennis* parasitoids can potentially regulate blowfly populations (Bennett & Whitworth 1991; Grillenberger et al. 2008, 2009; Daoust et al. 2012), and their high rate of parasitoidism achieved in the west-facing slope could explain why the numbers of blowflies are similar in infested-nests from the two slopes of the valley (Chapter 6).

Parasites are important selective agents for hosts because they reduce host fitness (Schmid-Hempel 2011). The blood-sucking activity of nest-dwelling ectoparasites is well-known to impact blood and cell-blood physiology, body size and body condition of nestlings (Richner et al. 1993; Merino & Potti 1995; Christe et al. 1996; Hurtrez-Boussès et al. 1997; Puchala 2004; Simon et al. 2004; Brommer et al. 2011; López-Arrabé et al. 2015), sometimes even provoking nestling death (Møller et al. 2009). Blue tit nestlings from the studied population did not suffer from any ectoparasite-induced physiological costs, neither in terms of oxidative status (Chapter 4), nor to the immune system (Chapter 5). Nest-dwelling ectoparasites either did not affect nestling body size or short term survival (Chapter 6), although the presence of fleas in nests was associated to a lower body mass for small, marginal nestlings (Chapter 5; see Christe et al. 1998). Moreover, nest-dwelling ectoparasites may also impact several adult reproductive parameters, such as egg size (Potti 2008), number of nestlings produced (Lemoine et al. 2012) or lifetime reproductive success (Fitze et al. 2004). No evidence for such detrimental effects of nest-dwelling ectoparasites on blue tit reproduction was observed in the studied population (Chapter 6), although nests housing more nestlings were more frequently parasitised by fleas, as observed in other parasite–host systems (Hurtrez-Boussès et al. 1999; Arriero et al. 2008).

On the other hand, although there was no variation in the prevalence per nest of flying ectoparasites (*Culicoides* biting midges and *Simulium* black flies) between forests, the abundance of biting midges in blue tit nests was higher in the east-facing than in the west-facing slope of the valley (Chapter 2). The habitat type and environmental conditions have an effect on biting flies breeding in an area (Rivero de

Aguilar et al. 2018; van Hoesel et al. 2019), ultimately determining the abundance of flying ectoparasites captured in avian nests (Tomás et al. 2008, 2020; Martínez-de la Puente et al. 2009, 2010). These blood-sucking flying insects, similarly to nest-dwelling ectoparasites, may provoke detrimental effects on nestling condition (Tomás et al. 2008; Martínez-de la Puente et al. 2010), and, indeed, a negative correlation between biting midges in blue tit nests and nestling body mass was found (Chapter 6). However, no associations were found between the presence of flying ectoparasites and blue tit reproductive parameters, probably because of their weak evolutionary influence on avian reproduction in contrast to other parasites (Martin et al. 2001). The females of both biting midges and black flies are well-known vectors of several pathogens and parasites, including avian trypanosomes (Svobodová et al. 2017), avian malaria and malaria-like haemosporidians (Valkiūnas 2005; Martínez-de la Puente et al. 2011) and filarial worms (Bain & Babayan 2003; Bartlett 2008). The probability of infection by microfilariae in adult blue tits was similar across the woodland, and the presence of these blood parasites in the bloodstream of adult blue tits was slightly and negatively associated with host body mass (Chapter 3), as observed in other parasite–host systems (Atawal et al. 2019; de la Torre et al. 2020; but see Sehgal et al. 2005). No habitat-dependent probability of infection by microfilaria was found, in contrast to previous studies which revealed the importance of habitat characteristics in determining microfilaria prevalence (Savage et al. 2009; Siers et al. 2010; Clark et al. 2016), mainly because blood parasites are strongly driven by environmental factors which limit vector distribution (Ferraguti et al. 2018).

Overall, the findings of the first part of the thesis suggest that nestling blue tits were subjected to negligible, or even absent, parasite-imposed costs across the woodland. Blue tit nestlings may have displayed a wide variety of behavioural defence mechanisms (review in Bush & Clayton 2018), while, in a not-mutually exclusive process, parents could have increased their provisioning (Johnson & Albrecht 1993; Christe et al. 1996; Tripet & Richner 1997) or cleaning (Bush & Clayton 2018) effort in infested nests. This could explain, at least in part (see the next section), why blue tit parents produced similar quality offspring – in terms of nestling body size and body condition – across the woodland (Chapter 6), despite the role of forest type in shaping the antioxidant (Chapter 4) and immune (Chapter 5) systems of blue tit nestlings. The first part of the thesis also shows that habitat heterogeneity is the main responsible for the variation in the immune system and the expression of certain components of

the antioxidant system of blue tit nestlings. In fact, habitat quality has a strong influence in the variation of several components of the antioxidant (van de Crommenacker et al. 2011; Isaksson 2013; Salmón et al. 2018) and immune (Hoi-Leitner et al. 2001; Soursa et al. 2004; Bańbura et al. 2013) systems of birds, mainly because of the inter-habitat variation in food quantity and quality (Bańbura et al. 1995; Marciniak et al. 2007). Nevertheless, blue tit parents were able to raise similar quality offspring across the woodland (Chapters 4 and 6), despite the inter-habitat variation in nestling physiology. Given that food availability was higher in the humid Pyrenean oak forest on the west-facing slope than the Pyrenean oak forest on the east-facing slope, a potential solution to be adopted by blue tit parents from the east-facing slope of the valley to maintain their offspring condition is to reduce the clutch size. This pattern has been observed in other bird populations (Blondel et al. 1991, 1998; Xu et al. 2023). Thus, by reducing the clutch size, parents from the east-facing slope of the valley would produce a similar quality offspring (in terms of body condition) than parents from the opposite slope. The second part of this thesis demonstrates that this is the most probable evolutionary scenario underlying the clutch size variation.

Part II. Population divergence and evolution of optimal clutch size

The infer-forest environmental differences modulated the blue tit breeding performance (Chapter 6). Females started laying eggs earlier in deciduous (dry and humid Pyrenean oak forests) forests than in conniferous and evergreen (Scots pine and Holm oak forests) forests, probably because spring development of caterpillars occurs later in evergreen forests (Blondel et al. 1999; Tremblay et al. 2003). The clutch size was the lowest in the Scots pine forest – probably because conniferous forests support low caterpillar populations (Tremblay et al. 2003) – and the highest in the humid Pyrenean oak forest, with intermediate values in the Holm oak and dry Pyrenean oak forests. This pattern agrees with previous evidence, since female blue tits lay larger clutches in deciduous than in evergreen forests (Blondel et al. 1987, 1991; Dias et al. 1994; Fargallo & Johnston 1997; Tremblay et al. 2003, 2005; Lambrechts et al. 2004; Blondel 2007). However, the aforementioned studies examined the habitat-dependent breeding performance at the macro- and micro-scale, usually comparing reproductive parameters between distant geographical regions or forest patches and fragments. To the best of my knowledge, there is no evidence on how the blue tit adjusts its breeding performance at fine scale throughout continuous and

heterogeneous woodlands. Thus, this thesis documents for the first time that a fine scale adjustment of breeding performance may indeed occur along continuous systems (Chapter 6).

Moreover, given the absence of habitat-dependent differences in hatching success and fledgling success, the clutch size determined the number of offspring a blue tit pair raised per reproductive event. For example, female blue tits from the dry Pyrenean oak forest on the east-facing slope laid on average one egg less than females from the humid Pyrenean oak forest on the west-facing slope (Chapters 6 and 8). Accordingly, blue tit pairs from the dry Pyrenean oak forest produced on average one fledgling less than their counterparts from the humid Pyrenean oak forest on the opposite slope of the valley (Chapter 6). Nestling body mass and tarsus length, predictors of blue tit nestling survival and recruitment (Nur 1984; Blondel et al. 1998; Charmantier et al. 2004), did not differ between forest types (Chapters 6 and 8), which suggests that the blue tits produced similar quality fledglings along the valley. Hence, blue tits from the two opposite slopes of the valley adjusted their clutch size to local environmental conditions in order to maintain their offspring quality.

As seen above, blue tits appeared to adapt to different environmental conditions across the continuous woodland by adjusting clutch size to an optimal level for reproductive success in each habitat. But what is the process underlying this clutch size variation among the two slopes of the valley? Chapters 7 and 8 tried to answer this question using different approaches. Chapter 7 used natal dispersal, genotypic and phenotypic data to test if population genetic differentiation and phenotypic adaptive divergence could be responsible for the observed habitat-dependent variation in clutch size. For its part, Chapter 8 used experimental data to test if the different clutch sizes laid by females from the east-facing and west-facing slopes of the valley were optimal (i.e. if the clutch size laid in each habitat maximized the quantity and quality of offspring).

Most of first-year blue tits were recaptured as reproductive adults in their natal slope of the valley. Accordingly, microsatellite-based genetic analyses revealed the presence of two genetic clusters along the woodland, with blue tits of each genetic cluster associated with the slope they inhabit. Although the studied population showed some gene flow between the two genetic clusters, the genetic differentiation between the two slopes of the valley was still significant (Chapter 7). Despite their

potential high dispersal capacity, blue tits disperse over short distances, typically less than one km from their natal territory (Matthysen et al. 2005; Foerster et al. 2006; Parejo et al. 2007; Ortego et al. 2011; García-Navas et al. 2014). This limited and non-random (i.e. philopatric behaviour) dispersion contributes to reducing gene flow between close blue tit populations and enhancing genetic differentiation (Ortego et al. 2011; Porlier et al. 2012; Ferrer et al. 2016). Moreover, blue tits generally inbreed with related partners and females show less promiscuity with genetically distant males (Foerster et al. 2006), thus enhancing the genetic population differentiation.

According to the results of the P_{ST}–F_{ST} comparison, the two genetic clusters showed divergence in clutch size exceeding the level of differentiation expected based on genetic drift, hence suggesting divergent selection on clutch size, with blue tits being locally adapted to the conditions of the slope they inhabit (Chapter 7). Clutch size has a heritable component in natural bird populations (review in Postma & van Noordwijk 2005a), hence allowing the clutch size to provide responses for selection to act on. In fact, literature shows several examples of small-scale local adaptation for clutch size. For example: (1) strong selection against immigrant genes is the responsible to maintain small and locally adapted clutches in an insular population of great tits (*Parus major*) (Postma & van Noordwijk 2005b; Postma et al. 2009); (2) divergent selection causes local adaptation for clutch size in two continental populations of blue tits separated by few kilometres (García-Navas et al. 2014); and (3) insular populations of blue tits inhabiting close forest patches exhibit locally adapted clutches due to non-random dispersal and the existence of selective barriers (Blondel et al. 2006; Charmantier et al. 2016). Overall, blue tits from the opposite slopes of the valley form two differentiated genetic clusters, with selection favouring different clutch sizes between these clusters. These different clutch sizes appear to be adjusted to an optimal level for reproductive success in each habitat (Chapter 6). However, an experimental approach is necessary to validate and identify the optimal clutch size for each slope of the valley, given the caveats of the P_{ST}–F_{ST} comparison (Pujol et al. 2008; Santure et al. 2010; Kekkonen et al. 2012).

The findings from the brood size manipulation experiment, conducted in the dry and humid Pyrenean oak forests, suggest that blue tit parents from the two slopes of the valley optimized their clutch size to local circumstances. Specifically, the brood enlargement reduced the nestling condition in the two slopes of the valley, whereas

the reduction of brood size did not improve nestling condition (a predictor of blue tit nestling rectuitment; Nur 1984; Charmantier et al. 2004; Råberg et al. 2005), mainly because parents failed to increase their provisioning effort when broods were enlarged (Chapter 8). Thus, blue tits were laying the maximal clutch size they can rear. The optimal clutch size is defined as that which maximizes the fitness (Charnov & Krebs 1974) and is expected to differ between ecologically different habitats given that individuals tend to adjust their clutch size to local circumstances (environmental variation: Perrins & Moss 1975; territory quality: Högstedt 1980; food availability: Török et al. 2004). This would lead to local adaptation for this trait (Postma & van Noordwijk 2005b; Blondel et al. 2006; Postma et al. 2009; García-Navas et al. 2014; Charmantier et al. 2016). Experimental research shows several examples of clutch size optimization in terms of offspring recruitment (Perrins & Moss 1975; Gustafsson & Sutherland 1988; Pettifor et al. 1988, 2001; Tinbergen & Daan 1990; Pettifor 1993), with control broods showing the highest recruitment. The findings from the brood size manipulation experiment are consistent with an optimized clutch size because parents forced to rear experimentally enlarged broods could not match their performance with control broods, thus producing low-quality fledglings which are expected to be impaired in terms of survival (Nur 1984; Charmantier et al. 2004; Råberg et al. 2005).

On the other hand, the gene flow between close and adjacent populations with different optima may preclude the optimization of clutch size (Dhondt et al. 1990; Blondel et al. 1998; Postma & van Noordwijk 2005b), which could explain why optimization has not been found in a number of studies (Nur 1984; Dhondt et al. 1990; Blondel et al. 1998; Sanz & Tinbergen 1999; Tinbergen & Both, 1999; Rytkönen & Orell 2001; Tinbergen & Sanz 2004; Török et al. 2004). However, this evolutionary scenario can be discarded for my studied system given that: (1) blue tits from the dry and humid Pyrenean oak forests form two differentiated genetic clusters (Chapter 7); (2) divergent selection on clutch size explains the local adaptation to the conditions of the slope the blue tits inhabit (Chapter 7); and (3) the effect of the brood size manipulation experiment was similar in the dry and humid Pyrenean oak forests (Chapter 8).

Overall, the results of the second part of this thesis demonstrate a fine-scale and habitat-dependent local adaptation process for clutch size. The clutch size is on average one egg less in the dry Pyrenean oak forest than in the humid Pyrenean oak forest (located in the east-facing and west-facing slopes of the valley, respectively),

this variation being maintained across years. Blue tits from the opposite slopes form two differentiated genetic clusters, mainly as a consequence of restricted dispersal, and selection seems to favour different clutch sizes between these clusters, suggesting the existence of local adaptation for this trait. Accordingly, a brood size manipulation experiment showed that this variation in clutch size was habitat-dependent optimized: nestlings showed worse body condition in experimentally enlarged broods than in control and reduced broods in a similar manner in the dry and humid Pyrenean oak forests. In sum, blue tits from the two slopes of the valley form two differentiated genetic clusters which exhibit different optimal and locally adapted clutch sizes as a consequence of non-random dispersal and divergent selection. The findings of this thesis challenge the general view that extensive dispersal and gene flow prevent small-scale divergence, highlighting that habitat heterogeneity at extremely small spatial scales (inside a continuous woodland) can generate genotypic and phenotypic adaptive divergence (different optimal clutch sizes) even in an animal with potential high dispersal capacity.

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CONCLUSIONS

Conclusions

1. Blue tits (*Cyanistes caeruleus*) breeding in the continuous and heterogeneous woodland located along a valley faced different selective pressures. The west-facing slope of the valley provided a more humid and productive environment, with a higher food availability and prevalence of nest-dwelling ectoparasites, than the east-facing slope of the valley.

2. Blue tits adjusted their reproductive effort to the forest type where they bred. Females laid larger clutches in the humid forest from the west-facing slope than in the dry forest from the east-facing slope of the valley, leading to the same pattern of variation for the number of nestlings produced per reproductive event.

3. Blue tits adopted this forest-dependent variation in clutch size in order to maintain their offspring quality, in terms of body mass and body condition, despite the influence of forest type on offspring physiology.

4. In general, both blue tit parents and nestlings did not suffer from habitat-dependent parasite-imposed costs to reproduction and physiology, respectively. However, fleas and biting midges negatively affected nestling body mass, while haemoparasites were negatively associated with parent body mass.

5. Blue tits showed a strong philopatry, with first-year blue tits being recaptured as reproductive adults in their natal slope of the valley.

6. Microsatellite analyses showed a significant degree of genetic differentiation between blue tits from the two slopes of the valley, thus revealing congruence between genetic and dispersal data and suggesting relatively low gene flow between the two genetic populations.

7. The two genetic populations showed divergence in clutch size, exceeding the level of differentiation expected based on genetic drift, hence indicating that natural selection has favoured different clutch sizes in the two slopes of the valley.

8. A brood size manipulation experiment demonstrated that the different clutch sizes observed in the two slopes of the valley were optimal. The experimental increase of the brood size provoked a decrease in the offspring condition, in terms of mean body

mass and body condition, in the dry and humid forests, mainly because parents did not increase their feeding effort.

Conclusiones

1. Los herrerillos (*Cyanistes caeruleus*) que se reprodujeron en las formaciones boscosas adyacentes que forman un continuo de hábitats y se sitúan a lo largo de un valle se enfrentaron a diferentes presiones selectivas. La loma este del valle mostró un ambiente más húmedo y productivo, con una mayor disponibilidad de alimento y prevalencia de ectoparásitos, que la loma oeste del valle.

2. Los herrerillos ajustaron su esfuerzo reproductivo al tipo de bosque donde se reprodujeron. Las hembras pusieron puestas mayores en el bosque húmedo de la loma este que las hembras del bosque seco de la loma oeste del valle, provocando el mismo patrón de variación para el número de descendientes por evento reproductivo.

3. Los herrerillos adoptaron dicha variación en el tamaño de puesta para mantener la calidad de su descendencia, en términos de masa corporal y condición física, a pesar de la influencia del tipo de bosque en la fisiología de la descendencia.

4. Los herrerillos parentales y volantones, en general, no sufrieron costes derivados del parasitismo en términos de reproducción y fisiología, respectivamente. Sin embargo, las pulgas y los ceratopogónidos afectaron negativamente a la masa corporal de la descendencia, mientras que las microfilarias estuvieron asociados negativamente a la masa corporal de los parentales.

5. Los datos de dispersión natal mostraron una fuerte filopatría de los herrerillos, con los volantones siendo anillados en su primer año como reproductores en la misma loma del valle donde nacieron.

6. Los análisis de microsatélites mostraron un nivel de diferenciación genética significativo entre los herrerillos de las dos lomas del valle, revelando así una congruencia entre los datos genéticos y de dispersión, y sugiriendo un flujo génico bajo entre las dos poblaciones genéticas.

7. Las dos poblaciones genéticas mostraron una divergencia en el tamaño de puesta mayor de la que se esperaría según la deriva genética, indicando así que la selección natural ha favorecido distintos tamaños de puesta en las dos lomas del valle.

8. Un experimento de alteración del tamaño de nidada demostró que los diferentes tamaños de puesta observados en las dos lomas del valle son óptimos. El incremento

experimental del tamaño de nidada causó una disminución de la condición de la descendencia, en términos de masa corporal y condición física, en los bosques húmedo y seco, principalmente porque los parentales no fueron capaces de incrementar su esfuerzo de ceba.

APPENDIX

Appendix

Other publications not included in this thesis

During the course of this thesis other articles have been published (or are in a stage near final publication) in peer-reviewed journals, but they were not included as chapters because their scope was far from the main content of the thesis. These articles are the result of several international stays and collaborations carried out during the course of the thesis.

1. Jiménez-Nájar P., Garrido-Bautista J., Tarifa R., Rivas J. M. & Moreno-Rueda G. 2021. Diet of sympatric barn owls *Tyto alba* and short-eared owls *Asio flammeus* in an agricultural landscape in south-east Spain. Ornis Svecica 31: 139–150. Doi: 10.34080/os.v31.23108.

2. Garrido-Bautista J., Santos-Baena C., Ramos J. A., Moreno-Rueda G. & Norte A. C. 2022. A mixed brood of coal tits *Periparus ater* and blue tits *Cyanistes caeruleus* in Central Portugal. Ardea 110: 239–242. Doi: 10.5253/arde.2022.a4.

3. Garrido-Bautista J., Moreno-Rueda G., Nunes M., Ramos J. A. & Norte A. C. 2023. Nestling growth pattern and breeding biology in the Eurasian nuthatch *Sitta europea*. Ardea 111: 1–10. Doi: 10.5253/arde.2022.a36.

4. Nadal-Jiménez P., Frost C. L., Norte A. C., Garrido-Bautista J., Wilkes T. E., Connell R., Rice A., Krams I., Eeva T., Christe P., Moreno-Rueda G. & Hurst G. D. D. 2023. The son-killer microbe *Arsenophonus nasoniae* is a widespread associate of the parasitic wasp *Nasonia vitripennis* in Europe. Journal of Invertebrate Pathology 199: 107947. Doi: 10.1016/j.jip.2023.107947.

5. Melero-Romero P., Garrido-Bautista J., Pérez-Rodríguez L., Ramos J. A., Norte A. C. & Moreno-Rueda G. 2023. Begging calls and mouth colouration as predictors of breeding success in blue tits. Journal of Zoology (in second review).

6. Garrido-Bautista J., Ramos J. A., Arce S. I., Melero-Romero P., Ferrerira R., Santos-Baena C., Guímaro H. R., Martín-Villegas C., Moreno-Rueda G. & Norte A. C. 2023. Is there a role for aromatic plants in blue tit (*Cyanistes caeruleus*) nests? Results from a correlational and an experimental study. Behavioral Ecology and Sociobiology (in second review).

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Agradecimientos

Tras más de trescientas páginas escritas sobre ciencia (en concreto, sobre herrerillos y evolución), henos aquí. En el que, sin duda alguna, es el apartado más difícil de escribir. Sobre todo para los científicos, esos seres cuadriculados y carentes de emociones y sentimientos más allá de los que profesan a la ciencia –por favor, nótese el sarcasmo y la ironía que gobernarán las siguientes páginas y que estuvieron ausentes, por su naturaleza subjetiva, en los apartados anteriores–. Digo difícil de escribir porque el científico se ha construido, en el sentido no utilitario ni determinista de la palabra, para escribir textos objetivos, concisos y metodológicos. De hecho, este apartado, el de los agradecimientos, es el único rincón de expresión para el científico en esta etapa, la predoctoral. No hay reglas, métodos, directices ni extensión máxima (¿o sí?). Entonces, ¿cómo empezar este apartado? ¿Qué incluir en él? Llevo días pensándolo y en qué expresar en estas páginas. Como no hay reglas establecidas, propondré las mías propias y expresaré –a veces con ironía y sarcasmo, ya lo advertí– los agradecmientos y algunas opiniones subyacentes a dichos agradecimientos que, creo, han servido para llevar a buen término esta tesis doctoral.

Empezaré por agradecer, como ya habrá intuido incorrectamente el lector (quien quiera que sea), a los diferentes organismos públicos que, con sus proyectos de investigación, han sufragado esta tesis doctoral y han financiado mi contrato laboral, que no beca –con su correspondiente cotización a la seguridad social que me permitirá tener derecho a solicitar la prestación por desempleo en un par de meses desde que escribo estas palabras–. En concreto, agradecería al Ministerio de Educación y Formación Profesional y al Ministerio de Ciencia, Innovación y Universidades. Pero, tras unas cuantas repeticiones electorales, inestabilidades y crispaciones políticas varias, una pandemia global y una invasión militar en el este de Europa, ambos ministerios han cambiado tanto de nombre (y ministros, he perdido la cuenta de cuántas dimisiones y reemplazos han ocurrido en solo cuatro años) que ya no recuerdo a cuál debería agradecer. Creo que, a septiembre de 2023, los agradecimientos deberían ir al Ministerio de Universidades y al Ministerio de Ciencia e Innovación. Me abstengo de toda responsabilidad si esta información no llegara a ser correcta. Quizás el lector se pregunte el por qué agradecer a estos organismos institucionales en una tesis doctoral, pero créame si le digo que la mayoría de las tesis doctorales se abandonarían en ausencia de un contrato laboral o subvención económica de

cualquier otro tipo (por no hablar de la flagrante violación de los derechos laborales que supondría dicha situación, pero ese es otro tema).

Financiación aparte, que no menos importante para la realización de una tesis doctoral (experimental), los mayores agradecimientos (ahora sí, como intuirá el lector) van para mi director de tesis, Gregorio. Esta tesis no hubiera existido sin él. No solo por el hecho evidente de dirigir el trabajo de cuatro años, programar los plazos y experimentos, corregir todos y cada uno de los capítulos, y un largo y redundante etcétera. Me parece que agradecer esto es obvio, cualquier buen director lo cumple (por desgracia, esto no es así para muchos compatriotas doctorandos, quienes son dirigidos por seres autócratas, a veces narcisistas, expertos en demoler o robar –según se considere oportuno– ideas ajenas). En este sentido, Gregorio no ha sido un buen director, ha sido un excelente director. No creo que pudiera haber tenido otro director mejor; me parece inverosímil el mero hecho de pensarlo. Él me ha enseñado cómo se hace, y no se hace, la buena ciencia, pero también a cómo cultivar una mente científica, crítica, capaz de cuestionarse hasta los hallazgos más triviales. Esto puede parecer irrelevante, pero no lo es. Paradójicamente, ahora que estoy dentro del mundo científico, me *creo* menos ciertos artículos científicos (sobre todo cuando conoces la forma de trabajar tan sincera y honrada de los autores) que cuando los leía en mi época de estudiante. No estoy defendiendo una crisis de creencia en la ciencia, sino en el sistema científico y, en general, muchas personas que hacen ciencia –como casos recientes, el lector puede buscar las retracciones de artículos científicos ocurridas durante el *boom* de publicación relacionado con el Covid-19 o la subordinación de ciertos científicos a Arabia Saudí a cambio de dinero–. Espero que el lector entienda que aquí hablo en términos de probabilidad gaussiana. Es evidente que no todos los científicos hacen mala praxis en ciencia, al igual que no todas las hembras de la loma este del valle ponen ocho huevos. Creo compartir con Frans de Waal su diferenciación entre lo que es la ciencia y lo que son los científicos: "*la ciencia es una empresa colectiva con reglas de compromiso que permiten al conjunto hacer progresos aunque sus componentes arrastren los pies*". Aquí es donde yace la clave para determinar si una mente científica es lo suficientemente crítica como para poder separar lo que es el cuerpo teórico y consolidado de un hallazgo de los pies que lo arrastran.

En definitiva, agradezco a mi director Gregorio, entre otras muchas cosas puramente académicas, el poder despejar el lodo que inunda la ciénaga de la ciencia para acercarse a la verdad. Sin olvidarme de la libertad que me ha dado, siempre respetando los derechos laborales –algo que otros directores pobremente cumplen– y los tiempos y plazos de la tesis doctoral, para llevar a cabo ideas propias (que, espero, acabarán publicándose a lo largo de los siguientes meses o años) y colaboraciones (algunas de las cuales pueden verse en el anexo de esta tesis doctoral, mientras que otras, espero, acabarán por publicarse en los siguientes meses). El aprendizaje y el enriquecimiento científico han sido impecables, y no hay otro responsable que mi director de tesis.

Toda tesis doctoral debe agradecer a las personas que han hecho posible, en mayor o menor medida, su culminación. Y esta no iba a ser una excepción. A Abelardo, Eliana, José Luis, Mar, Nicola, Paula y Zaida agradezco su enorme esfuerzo realizado en las campañas de campo con los herrerillos realizadas en el área de estudio (ignore el lector el orden de los nombres, pues es puramente alfabético según las normas que acabo de adoptar y no se deben a su grado de implicación en el trabajo realizado –a diferencia de los artículos científicos–). Cualquiera que investigue usando animales silvestres sabe lo intenso (sobre todo en un equipo de trabajo de tres personas controlando 150 nidos), y satisfactorio, que es trabajar durante casi tres meses para muestrear toda una población y recolectar todos los datos programados. A Amalia, Cristina y Eva agradezco su inestimable ayuda e implicación en todos los análisis de laboratorio llevados a cabo, los cuales han conformado una parte sustancial de esta tesis doctoral –aunque otros, que lamentablemente no llegaron a buen término, dieron más quebraderos de cabeza que soluciones–. No me olvido de los alumnos e investigadores (Alberto, Antonio y Laura) que han ayudado en la ardua tarea de medir la actividad de unas cuantas enzimas y la cantidad de oxidantes y antioxidantes en una maratón de dos días al año, que, en teoría, no hace daño (¿o quizás sí? Ya lo dirá el estrés oxidativo sufrido por nuestras células).

Casi la práctica totalidad del capítulo 7 de esta tesis doctoral se debe a Mohammed Bakkali. Él es la verdadera cabeza pensante tras los resultados que se pueden observar ahí. Agradezco a Mohammed su labor de mentor, a todas las habilidades adquiridas gracias a su conocimiento y a la ingente cantidad de reuniones abordadas desde el inicio, cuando aún no teníamos ni el primer kit de polimerasa ni la lista definitiva de

microsatélites a usar (llevaba sin pisar un laboratorio desde 2018. Ahora creo saber desenvolverme, casi sin torpezas, en él). A Laura deben ir unos agradecimientos especiales por su incansable labor para mantener operativo el laboratorio. Perdóname por dejarte 20 cajas de puntas al día para autoclavar durante más de dos meses. Creo que no volvería a repetir un genotipado de casi 200 individuos con 28 marcadores, aunque el tiempo dirá. Espero (no) equivocarme. Mike, a quien agradezo enormemente su ayuda en los análisis de genética de poblaciones, estará conmigo en este punto. Alejandro, Ana, Carlos, Gema, Mode, Tati y muchos otros nombres más también se merecen un agradecimiento al acompañarme, bien charlando bien viendo *Juego de Tronos*, en mi paso por el laboratorio de genética.

Y hablando de laboratorios. Muchos otros agradecimientos deben ir a las personas con las que trabajé en mi paso por las instalaciones del Instituto Konrad Lorenz de Etología. El lector –si es pretendidamente culto y no biólogo– quizás se haya sorprendido al leer este nombre. Yo también me sorprendí la primera vez que estuve frente a las puertas de este Intituto. Una placa y un busto del ilustre etólogo austríaco, y premio Nobel de Fisiología o Medicina (paradójicamente el comportamiento no era fisiología ni medicina en 1973), daban la bienvenida al Instituto. Allí me pregunté: *"¿cómo pollas ha llegado un güeteño –proveniente de un insignifante municipio apenas conocido por sus vinos capaces de tumbar al mayor veterano de guerra– a estar en el mayor centro de investigación en etología del mundo?"* A septiembre de 2023 me lo sigo preguntando. Dustin, a pesar de sus ausencias por motivos que no expondré aquí, fue un gran mentor durante los tres meses que estuve trabajando allí. Al igual que mi director, me dio la libertad para llevar mi ritmo de trabajo y colaborar con diferentes investigadores de Viena (el capítulo 3 es una muestra de ello). A Balint, Franz, Gopi, Hans-Peter, Josef, Sarah y Steve agradezco enormemente su ayuda con las tareas del laboratorio, las reuniones de grupo y las discusiones (en el sentido académico y no de *barra de bar* de la palabra) para solucionar los problemas que iban surgiendo. Y, por supuesto, al resto de integrantes de los grupos de investigación del Instituto, aunque la lista es demasiada larga como para exponerla aquí. Lo único que lamento es no haber tenido el tiempo suficiente tras mi regreso para incluir los resultados principales, aunque espero que vean la luz en los próximos meses.

A Jesús, Josué y Mario agradezco muchas cosas: su ayuda en la elaboración de protocolos de trabajo, la identificación de los *Culicoides* y otros insectos hematófagos,

y su enorme conocimiento para adentrarme un poco más en este apasionante mundo de la ecología de las relaciones parásito–hospedador. Estoy seguro de que a partir de este momento surgirán muchas más colaboraciones y buenos momentos. No me olvido de Stanislav y Miguel, a quienes agradezco la identificación taxonómica, que no menos importante, de los parásitos que han jugado un enorme papel en esta tesis doctoral. A todos los alumnos que han pasado por el grupo de investigación durante estos cuatro años y he tenido la oportunidad de trabajar, de una forma u otra, con ellos: África, Albert, Jesús, Pablo y Rosa. A Arturo, Blanca, Carlos, David, Jaime, José Luis y Miguel por su importante labor en la recolección de datos de campo y que han ayudado, sin duda alguna, a la culminación del primer capítulo de esta tesis y a mi *entrada* en el mundo científico. Entrada que, por cierto, fue algo traumática e inesperada. Imagine el lector entrar por primera vez a trabajar a un grupo de investigación y que su primera labor sea cortar pupas de mosca conservadas en etanol por años y rellenas de otras tantas larvas diminutas, todo emitiendo un olor indescriptiblemente horrible al que, muy probablemente, todo taxónomo haya hecho (desgraciadamente) su olfato.

Los halagos hacia mi director bien podrían aplicarse a mi supervisora, Cláudia, durante mi estancia en Coímbra. No tengo queja alguna, ha sido una supervisora excelente. Con ella he aprendido a liderar una campaña de campo entera y a sacarle el máximo jugo a los recursos (recuerde el lector que la inversión en ciencia en España y Portugal está algo por encima de Bulgaria, Chipre o Turquía) y a los datos (los artículos incluidos en el apéndice podrían ser una muestra de ello). Agradecimientos académicos aparte, Cláudia también me ha enseñado sobre los *males de la ciencia*, como lo llaman Juan Ignacio Pérez y Joaquín Sevilla, pero también sobre –aquí me permitiré el lujo de introducir un nuevo concepto– la *maldición de la ciencia*. Jamás se me olvidarán sus palabras durante una de las tantas pausas que hacíamos para tomar café expresso en el laboratorio: *"A veces, cuando veo a mi hijo interesarse por algún escarabajo u otro insecto que encuentra por el camino, tengo miedo de incularle tal pasión por los animales que le lleven en un futuro a ser investigador"*. Seguramente al lector alejado del mundo académico le parezcan irrelevantes estas palabras. Créame si le digo que a más de un científico le harán pensar. Y mucho. Baste con que el lector no académico se informe mínimamente sobre las dignas condiciones laborales de los científicos, la estabilidad laboral (y emocional) de este trabajo, las cuantiosas nóminas recibidas en proporción a la labor y grado de responsabilidad

social del científico o a la facilidad que ofrece el sistema para la reconciliación familiar, entre otros muchos ejemplos de excelente praxis en el marco de los derechos laborales que no expondré aquí para ahorrar espacio. Durante mi paso por Coímbra también he de agradecer la compañía, y la labor durante la campaña de campo, de multitud de personas: Carolina, Ivo, Jaime, Jorge, José, Lara, Nathalie, Sara y Vitor, gracias por hacerme más amena mi estancia en tan bella ciudad.

No podían faltar los agradecimientos a todos los compañeros doctorandos y técnicos del Departamento de Zoología. Andrea, Anna, Carlos, Dani, Darío, José Ignacio, Laura, Lucía, Mar y Mari Carmen, mil gracias por acompañarme en esta etapa compartiendo momentos excelentes, la mayoría de las veces en la propia Facultad de Ciencias (debido, para la mayoría de nosotros, a nuestro horario reducido de mañana). Habéis sido los mejores compañeros de tesis que se puede tener; los cuatro años se me han pasado volados. A Laura por sus cafés y relatos de viajes alrededor del mundo; a Sofía por estar al otro lado del teléfono y contarnos nuestro paso por la (ajetreada) ciencia. A mis colegas de Granada, Gabri, Jesús (o *chiqui*, como lo conocemos desde hace una eternidad) y José Ignacio (o *pepe nacho*, como también lo conocemos desde hace otra eternidad). Los agradecimientos para vosotros van más por nuestras quedadas alrededor de jarras de cerveza y conversaciones trascendetales, y no tan trascendentales, que por otra cosa. No miento si afirmo que parte de lo expuesto en esta tesis está en parte motivado por nuestras discusiones sobre teología natural, filosofía de la naturaleza y biología evolutiva (en esencia, para desbancar cualquier intencionalidad aparente en el trascurso evolutivo). Lo siento *pepe nacho* –quien desde hace varios años se encuentra formándose como teólogo tomista y filósofo de la naturaleza en Montefiascone–, pero el reloj de Paley y Tomás de Aquino obedece más a un relojero ciego que al más artesano y experto relojero omnisciente. No encuentro una explicación más plausible y parsimoniosa que el resultado de un proceso natural y ciego al porvenir de los acontecimientos para explicar por qué las hembras de la loma este del valle iban a poner un huevo más que las hembras de la loma de enfrente. Y en caso contrario, no comprendo por qué una deidad iba a crear dicho patrón y para qué.

Estamos llegando al final (al fin). A Carmen, gracias por tanto, por tu amor, por ser como eres y por haber estado conmigo durante todo este largo viaje, incluso en los momentos de mayor dificultad. Siempre has estado ahí, mimándome, y lo agradeceré

eternamente. A mi familia, mis padres y hermanos (y Rulo), por estar también siempre ahí, apoyándome, aunque no entiendan muy bien a lo que me dedico –durante la tesis doctoral he impartido clases, que no recibido clases. Espero, aunque albergo mis dudas, que este concepto acabe por implantarse en el acervo de conocimiento de la población no académica–. Debo agradecer especialmente a mis padres el no haberme educado (adoctrinado) en cualquier creencia religiosa –que muy probablemente habría sido el catolicismo–, lo que, casi con total seguridad, habría impedido siquiera en que creyera a día de hoy en la evolución por selección natural. ¿O sí? Aquí he de hacer un pequeño inciso. Sea consciente el lector de que, por inverosímil que parezca (al menos a mí me lo parece), hay muchos biólogos creyentes entre las filas de la ciencia, algunos incluso practicantes y con creencias aférrimas al clero (esto sí que me parece inverosímil y aún no consigo encontrar una explicación a por qué estas personas no sufren algún grado de disonancia cognitiva). Menciono esto porque quizás, si hubiera sido adoctrinado, habría llegado a este mismo punto –a las puertas del título de doctor en biología evolutiva– siendo creyente. ¿Qué pensaría entonces? ¿Que todo en lo que he investigado por años es producto de un diseñador inteligente que ha dirigido la evolución? O peor aún, ¿que, quizás siendo creacionista, la divergencia genética y fenotípica que ocurre entre poblaciones de una misma especie, supuestamente inmutable, fuera una prueba de fé de alguna deidad? Puedo entender que este tipo de creencias afloren en la senectud –como ha ocurrido con varios biólogos o naturalistas ilustres, como Alfred Russel Wallace, Francis Crick o el actual Francis Collins entre otros, que recurrieron al misticismo o la religión en sus últimos años de vida–, al igual que ocurre con el conformismo político y la pérdida del espíritu revolucionario. Al fin y al cabo, ya no tienes que rendir cuentas a nadie y tu principal preocupación es muy probable que sea la pérdida de neguentropía que te mantiene con vida. No entretengo más al lector. Agradezco la oportunidad que se me ha dado para estudiar algunos versos de esa *poesía de la realidad* de la que habla Dawkins.

Granada, a 6 de septiembre de 2023

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