BEHAVIOURAL ADAPTATIONS OF CAVITY NESTING BIRDS

ALEJANDRO CANTARERO CARMONA

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BEHAVIOURAL ADAPTATIONS OF CAVITY NESTING BIRDS

Memoria presentada por el Licenciado D. Alejandro Cantarero Carmona para optar al grado de Doctor en Biología, dentro del programa de doctorado "Comportamiento Animal" de la Universidad de Córdoba, dirigida por el Dr. Juan Moreno Klemming del Museo Nacional de Ciencias Naturales – CSIC y el Dr. Alberto José Redondo Villa de la Facultad de Ciencias de la Universidad Córdoba.

Córdoba, 2015

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TÍTULO DE LA TESIS: BEHAVIOURAL ADAPTATIONS OF CAVITY NESTING BIRDS

DOCTORANDO/A: ALEJANDRO CANTARERO CARMONA

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

La Tesis Doctoral denominada “Behavioural Adaptations of Cavity Nesting Birds”, realizada bajo nuestra dirección por Alejandro Cantarero Carmona continúa la línea de investigación iniciada por el doctorando en su Trabajo Fin de Máster. El presente trabajo comenzó a raíz de la realización de un trabajo en vídeo de divulgación científica para la serie de televisión “Discovering Animal Behaviour” de la que Alberto J. Redondo es director. En el capítulo tercero trataba los trabajos sobre papamoscas cerrojillo (*Ficedula hypoleuca*) del Dr. Juan Moreno Klemming. Para la filmación de esta película desarrollamos una serie de técnicas que permitían grabar con una gran calidad en el interior de las cajas nido.

Esta vez la divulgación científica supuso un camino de ida y vuelta, ofreciendo una herramienta para avanzar en el descubrimiento científico de la biología reproductora de las aves que anidan en cavidades.

El doctorando Alejandro Cantarero ha realizado un excelente trabajo aprovechando al máximo esta técnica, complementándola con numerosas mejoras, de forma que ha podido ser testigo de cientos de horas de intimidad en la vida de las aves. Su capacidad, su dedicación y su excelente trabajo bajo la experta dirección del Dr. Juan Moreno han dado como resultado un magnífico trabajo que aporta numerosos avances en el conocimiento del comportamiento reproductor de estas aves y sus implicaciones evolutivas.

Los objetivos planteados durante el desarrollo de la Tesis se han cumplido con éxito. Los experimentos planteados han sido satisfactorios encontrándose resultados muy interesantes que abren nuevas fronteras en la investigación del comportamiento animal. Fruto de la gran calidad de este trabajo son las 5 publicaciones en las más destacadas revistas científicas indexadas en el Journal Citation Report que tratan sobre este campo:

- CANTARERO, A., LÓPEZ-ARRABÉ, J. y MORENO, J. 2015. Selection of nest-site and nesting material in the Eurasian Nuthatch *Sitta europaea*. Ardea (in press).

- CANTARERO, A., LÓPEZ-ARRABÉ, J., SAAVEDRA-GARCÉS, I., RODRÍGUEZ-GARCÍA, V., PALMA, A. y MORENO, J. 2014. The significance of nest structure and nesting material for hole-nesting passerines: an experimental study with Nuthatches *Sitta europaea*. Acta Ornithologica 49: 143-155.
- CANTARERO, A., LÓPEZ-ARRABÉ, J., PALMA, A., REDONDO, A. J. y MORENO, J. 2014. Males respond to female begging signals of need: a handicapping experiment in the Pied flycatcher *Ficedula hypoleuca*. Animal Behaviour 94: 167–173.
- CANTARERO, A., LÓPEZ-ARRABÉ, J., REDONDO, A. J. y MORENO, J. 2013. Behavioural responses to ectoparasites in Pied Flycatchers *Ficedula hypoleuca*: an experimental study. Journal of Avian Biology 44: 591-599.
- CANTARERO, A., LÓPEZ-ARRABÉ, J., RODRÍGUEZ-GARCÍA, v., GONZÁLEZ-BRAOJOS, S., RUIZ-DE-CASTAÑEDA, R., REDONDO, A. J. y MORENO, J. 2013. Factors affecting the presence and abundance of generalist ectoparasites in nests of three sympatric hole-nesting bird species. Acta Ornithologica 48: 39-54.

Por lo tanto, consideramos que la Tesis cumple todos los requisitos de calidad para su presentación.

Córdoba, 2 de Marzo de 2015

Firma del/de los director/es

Albert

Fdo.: Juan Moreno Klemming

Fdo.: Alberto José Redondo Villa

TÍTULO DE LA TESIS: BEHAVIOURAL ADAPTATIONS OF CAVITY NESTING BIRDS

DOCTORANDO/A: ALEJANDRO CANTARERO CARMONA

ESCRITO RAZONADO DEL RESPONSABLE DE LA LÍNEA DE INVESTIGACIÓN

El doctorando Alejandro Cantarero Carmona, ha demostrado una alta capacidad y dedicación en su trabajo investigación, habiendo publicado ya 5 artículos en revistas indexadas en el Journal Citation Report del tema de su Tesis doctoral, además de algunas otras publicaciones dentro de la línea de Ecología Evolutiva y de las Estrategias Vitales en Aves.

El grupo de investigación donde está desarrollando su labor de investigación es puntero a nivel internacional y de reconocido prestigio, lo que asegura una muy fructífera carrera investigadora del doctorando.

Por lo tanto, considero que la Tesis cumple todos los requisitos de calidad para su presentación.

Córdoba, 2 de Marzo de 2015

Firma del responsable de línea de investigación

Fdo.: Luis Arias de Reyna Martínez

This thesis has been presented as a compendium of publications. The following works have been published in journals included in the three first quartiles according to the Journal Citation Reports:

1. **Title:** Behavioural responses to ectoparasites in Pied Flycatchers *Ficedula hypoleuca*: an experimental study

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2. **Title:** Factors affecting the presence and abundance of generalist ectoparasites in nests of three sympatric hole-nesting bird species

   Authors: Cantarero, A., López-Arrabé, J., Rodríguez-García, V., González-Braojos, S., Ruiz-de-Castañeda, R., Redondo, A. J. & Moreno, J.

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3. **Title:** Males respond to female begging signals of need: a handicapping experiment in the Pied flycatcher *Ficedula hypoleuca*

   Authors: Cantarero, A., López-Arrabé, J., Palma, A., Redondo, A. J. & Moreno, J.

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   Reference: Cantarero, A., López-Arrabé, J., Palma, A., Redondo, A. J. & Moreno, J. 2014. Males respond to female begging signals of need: a handicapping experiment in the Pied flycatcher *Ficedula hypoleuca*. Animal Behaviour, 94, 167-173.

4. **Title:** The significance of nest structure and nesting material for hole-nesting passerines: an experimental study with Nuthatches *Sitta europaea*

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5. **Title:** Selection of nest-site and nesting material are affected by nest construction costs in the Eurasian Nuthatch *Sitta europaea*

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A Pepe

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ABSTRACT

Nesting cavities offer conditions of relatively constant temperature and humidity as well as protection from rain, solar radiation and. Availability of nesting cavities, thermal properties of the nests and ectoparasites loads are among the main factors implicated in the evolution of cavity nesting behaviour. The main goal of this thesis is increased understanding from an evolutionary perspective of the behavioural strategies arising during several stages of the reproductive cycle in small altricial cavity-nesting birds such as the Pied Flycatcher *Ficedula hypoleuca*, Eurasian Nuthatch *Sitta europaea* and Blue Tit *Cyanistes caeruleus*. This PhD thesis involves descriptive studies and field experiments aimed at understanding the mechanisms underlying adaptations for nest site selection and defense, for determining nest structure and composition, for defense against ectoparasites and for female nutrition during the incubation stage in their natural habitats.

Given that nesting cavities are a scarce resource, there may be strong competition over them. We found that levels of testosterone differ between populations of the same species, being higher in populations where the likelihood of nest-site usurpation by intruders is greater. We also found that the level of female aggressiveness against intruders decrease with higher T levels in high density areas. After obtaining a nest cavity or nest-box, nest building begins. Some species such as Nuthatches show clear preferences for certain nesting materials and cavities for breeding. The selection of nesting material and the amount of mud in Nuthatch nest-boxes can be explained as a trade-off between requirements for nest construction and availability as a function of transport distance.

Nesting cavities constitute micro-environments very likely to be colonized by ectoparasites and certain nesting materials may be used due to their insecticidal properties. Additionally, nests without any structure such as those built by Nuthatches may offer fewer opportunities for hiding to ectoparasites and nest composition may affect ectoparasite development through the effects of microclimatic conditions associated with different nest materials. Here we show that the replacement of unstructured nests by structured nests did not result in changes in ectoparasite loads for Nuthatches, which suggests that the preferences for nest materials in Nuthatches are unrelated to ectoparasitism. We suggest that Nuthatch nests contribute to reduce the thermal loss for nestlings and possibly eggs during female absences by remaining buried into loose and heat-producing bark flakes.

Generalist ectoparasites infest nests of avian cavity-nesting passerines as a response to different factors exhibited by host species. Differences in nest composition among host species are not the main factor explaining ectoparasite prevalences and abundances, while nest size, breeding phenology, brood size and nest-cavity micro-climate may all affect levels of infestation in different ways for each host-parasite association. Given the negative impact of ectoparasites on nestlings there will be selection on hosts to reduce parasite infestations through behavioural means. We found that frequency and intensity of female anti-parasite behaviours during the incubation and nestling periods decreased as a consequence of the experimental reduction of ectoparasite infestation and these behaviours were more intense in the host species with highest infestation levels.

As a response of higher nutritional needs arising from higher ectoparasite loads we showed that nestlings begged more intensely. While begging by nestlings has received a fair deal of attention as an honest system of communication, begging between mates has received scant attention. We tested if female begging during incubation is an honest signal of energetic need and found that experimentally handicapping female pied flycatchers intensified begging displays arising from condition impairment and that males were able to respond by increasing their feeding rates to females.

RESUMEN

Las cavidades de nidificación ofrecen unas condiciones de humedad y temperatura relativamente constantes así como protección de la lluvia, la radiación solar y los depredadores. La disponibilidad de dichas cavidades, las propiedades térmicas de los nidos y la carga de ectoparásitos son los principales factores implicados en la evolución del comportamiento de las aves que las utilizan para su reproducción. El objetivo principal de esta tesis es incrementar el conocimiento desde una perspectiva evolutiva de las estrategias de comportamiento que surgen durante diversas etapas del ciclo reproductivo en aves paseriformes que anidan en cavidades, tales como el Papamoscas Cerrojillo *Ficedula hypoleuca*, el Trepador Azul *Sitta europaea* y el Herrerillo Común *Cyanistes caeruleus*. Esta tesis incluye estudios descriptivos y experimentos de campo destinados a comprender los mecanismos subyacentes en la adaptación a la selección y defensa de sitios de nidificación, en la determinación de la estructura y composición del nido, en las respuestas a ectoparásitos y en la comunicación de señales de necesidad entre la pareja reproductora durante la incubación.

Dado que las cavidades de nidificación son un recurso escaso hay una fuerte competencia sobre ellas. Se encontró que los niveles de testosterona en hembras de papamoscas difieren entre poblaciones de la misma especie, siendo más elevados en poblaciones donde la probabilidad de usurpación de nidos por intrusos es mayor. Además, se encontró que el nivel de agresividad contra intrusos de hembras que criaron en zonas de alta densidad disminuye con altos niveles de testosterona. Tras la elección de un sitio de nidificación comienza la construcción del nido. Algunas especies como los trepadores mostraron patrones claros en selección de cajas nido y en la preferencia de ciertos materiales de nidificación. La selección del material de nidificación y la cantidad de barro que utilizan los trepadores puede explicarse como un compromiso entre la necesidad de su utilización y la disponibilidad de los mismos en función de la distancia de transporte.

Las cavidades de nidificación constituyen microambientes muy propensos a ser colonizados por ectoparásitos y ciertos materiales de nidificación pueden ser

utilizados por sus propiedades insecticidas. Además, los nidos desestructurados pueden ofrecer menos oportunidades de ocultación a los ectoparásitos y la composición del nido puede afectar al desarrollo de los ectoparásitos como consecuencia de las condiciones microclimáticas asociadas a diferentes materiales de nidificación. Se mostró que la sustitución de los nidos desestructurados por nidos estructurados no dio lugar a cambios en la carga de ectoparásitos, lo que sugiere que la preferencia de ciertos materiales no está relacionada con el ectoparasitismo. Sugerimos que los trepadores construyen nidos formados por material suelto para reducir la pérdida de calor de los pollos al poder permanecer enterrados en cortezas que podrían generar calor.

Los ectoparásitos generalistas infestan nidos de aves como respuesta a diferentes factores exhibidos por las especies huésped. Las diferencias en la composición del nido entre especies no son el principal factor que explica la prevalencia y abundancia de ectoparásitos, mientras que el tamaño del nido, la fenología reproductiva, el tamaño de la nidada y el microclima del nido pueden afectar a los niveles de infestación de diferentes formas. Dado el impacto negativo de ectoparásitos sobre los pollos, habrá una selección en los hospedadores para reducir dichos efectos a través de diversos mecanismos de comportamiento. Se encontró que la frecuencia y la intensidad de los comportamientos anti-parásitos de las hembras durante varias etapas del ciclo disminuyeron como consecuencia de la reducción experimental de la infestación de ectoparásitos y estos comportamientos fueron más intensos en las especies hospedadoras que presentaban los niveles de infestación más altos.

Como respuesta a necesidades nutricionales producidas por los parásitos, los pollos solicitaron comida más intensamente. Mientras que la petición de los pollos se ha considerado un sistema honesto de comunicación, la petición entre machos y hembras ha recibido escasa atención. Hemos probado si la petición femenina durante la incubación es una señal honesta de necesidad energética y encontramos que hembras de papamoscas dificultadas para el vuelo experimentalmente intensificaron sus señales de petición y que los machos fueron capaces de responder a dicha petición incrementando su tasa de cebas.

INTRODUCTION

Bird nests are structures constructed by reproducing adults for developing eggs and chicks (Collias and Collias 1984; Hansell 2000). Bird nests are considered extended phenotypes (Dawkins 1982) whose main function is to increase the probability of offspring survival. Nest building is an energetically costly and time demanding activity (Hansell 2000) that may increase detectability by potential predators of both builders and developing offspring inside nests (Collias & Collias 1984), so selection pressures exerted by predators must have influenced the evolution of nest characteristics. Nest predation is usually the greatest cause of nest loss for nesting birds (Lack 1954; Nice 1957; Ricklefs 1971). The properties that contribute to a predation-resistant structure may also contribute to maintaining an optimal microclimate (Rhodes et al. 2009). Thus, nest predation and thermal conditions may be the main factors implicated in the evolution of cavity nesting behaviour (Hansell 2000). Nesting cavities offer conditions of relatively constant temperature and humidity as well as protection from rain, solar radiation and predators (Hansell 2000; Nilsson 1984), and therefore the relative breeding success of cavity-nesting bird species is higher than that of open-nesting species (Lack 1954; Nice 1957). However, cavity nesting may require specific adaptations given the presumably limited availability of adequate nest holes and the sanitary and microclimatic implications of enclosed breeding.

The present PhD thesis is an approach to understanding from an evolutionary perspective the behavioural strategies arising at several stages of the reproductive cycle in cavity-nesting birds such as the Pied Flycatcher *Ficedula hypoleuca*, Eurasian Nuthatch *Sitta europaea* and Blue Tit *Cyanistes caeruleus.*

1. ADAPTATIONS FOR NEST DEFENSE

Natural selection favors individuals that choose resources that enhance breeding success, but limited availability of such resources can limit the number of individuals that breed (Li and Martin 1991). For obligate secondary cavity nesters, unable to excavate their own cavity, nest holes constitute a scarce resource that may limit the availability of breeding opportunities and there inducing a strong competition over them (Ingold 1994; Leffelaar and Robertson 1985; Li and Martin 1991; Nilsson 1984). Thus, Eurasian Nuthatches narrow the entrance of cavities by plastering mud on the outside (Matthysen 1998), thereby making the entrance narrower in order to avoid nest site competitors such as the European Starling *Sturnus vulgaris* (Collias and Collias 1984). Losing the nest site would be one of the worst scenarios for the breeding pair (Rätti 2000). Some cavity nesters have become accustomed to use artificial nest-boxes , and populations of some of these species may be increased dramatically in this way (Collias and Collias 1984). Competition for nest-boxes takes place between individuals of different or of the same species (Collias and Collias 1984) and may favour aggressive nest defense behaviours (Bentz et al. 2013). Males are typically considered the more aggressive sex (Kokko 1999; Lewis et al. 2001; Wiley and Poston 1996; Wittenberger 1981), although females may display aggressive behaviours towards conspecifics in some of the same contexts as males such as the defence of their nest site (Karlsen and Slagsvold 1997; Kral et al. 1996; Male et al. 2006; Sandell 2007; Slagsvold et al. 1992).

Aggressiveness is partly determined by hormonal status (Moss et al. 1994). Many aspects of male reproduction in vertebrates are influenced or controlled by the steroid hormone testosterone (Smith et al. 2005). Testosterone (T) levels are generally lower in females than in males (Moreno et al. 2014; Silverin and Wingfield 1982), and there is mixed evidence concerning the importance of T for female social aggressiveness. Some studies have shown that circulating T concentrations in females are associated with aggressive behaviour (Gill et al. 2007; Lahaye et al. 2012; Moss et al. 1994; Sandell 2007; Veiga and Polo 2008), while others have not (Hau et al. 2004; Jawor et al. 2006). Territorial exclusion of female intruders may be especially necessary during the initial stages of reproduction when nests-sites may be taken over (Gowaty and Wagner 1988; Rosvall 2011). Competition among females for breeding sites can be rough and even lethal, especially during the nest-building period (Morales et al. 2014), where it is known that T levels can be increased rapidly in response to territorial intrusions (Silverin 1993).

2. ADAPTATIONS IN NEST STRUCTURE AND NESTING MATERIAL

Hole-nesting bird species vary in the characteristics of their nest sites (Li and Martin 1991) and build their own characteristic nests (Hansell 2000). Birds typically make use of local materials but some birds are nest material specialists (Hansell 2000). However, for many species the functional properties of their nests remain unknown. Nest construction may be influenced by factors such as the availability of nest materials (Moreno et al. 2009) and may involve a large expenditure of time and energy (Moreno et al. 2008) mainly due to the costs of transporting material to the nest site (Putnam 1949). These costs may be reduced by using old nest material (Nores and Nores 1994) and/or if nesting material is located close to the nest site (Collias and Collias 1984). Availability may play an important role in the selection of nest materials.

The physical structure of the nest is determined by the degree of cohesion between the different materials used and may influence embryo development and chick growth, so that nest quality may have important consequences for the condition and reproductive success of parents (Álvarez and Barba 2011; Dawson et al. 2011; Lambrechts et al. 2012; Lombardo et al. 1995; Moreno et al. 2010b). Nest construction from a thermal aspect represents a compromise between heat conservation, heat dissipation, and protection from external heat sources (Heenan and Seymour 2011). It is reasonable that heat loss can be minimized by optimizing the physical structure of the nest (Heenan and Seymour 2011; Hilton et al. 2004; Reid et al. 2000) or by choosing a suitable nesting material (Álvarez et al. 2013). Consequently, we expect birds to adjust their nest characteristics in response to environmental conditions (Deeming 2011; Hansell 2000). Moreover, the regulation of thermal conditions within acceptable limits may be energetically costly for parents (Nord and Nilsson 2012; Williams 1996). The structure of nests may mitigate this energetic demand on parents (Hansell 2000). Thus, building a thermally favorable nest saves parental energy by reducing heat loss from attended and non-attended clutches (Heenan and Seymour 2011; Moreno et al. 2010b).

Nesting cavities are not always waterproof (Wesolowski et al. 2002). The insulation quality of nests is dependent on several factors, such as nest structure

(McGowan et al. 2004), thickness, height and volume (Alabrudzińska et al. 2003; Grubbauer and Hoi 1996), nest material quality (Mertens 1977) and moisture content (Deeming 2011; Pinowski et al. 2006). Large amounts of nest material, although of benefit to reduce incubation costs (Moreno et al. 2010a), may collect and retain humidity above optimal levels. The risk of the nests getting wet could be reduced by incorporating more hydrophobic material such as bark flakes (Wesolowski and Rowiński 2004). Furthermore, bark flakes could help to stabilize thermal fluctuations in the nesting cavity by conserving heat during the cooler hours of the day. Dense accumulations of bark flakes may produce heat due to microbiological activity as observed in composting (Collias and Collias 1984). Other birds like megapodes also use the heat of accumulated vegetal material to keep their eggs warm. Heat production may be especially noteworthy during the night when nest materials cool down after heating up during the day.

Incubation behaviour may be affected by nest microclimate and structure (Álvarez and Barba 2009). Nuthatches *Sitta* spp. use nests made of loose heaps of bark flakes without any structure or nest cup to contain eggs and nestlings (Matthysen 1998). Eggs and nestlings are found buried in the loose material. When the Nuthatch female returns to the nest, she lowers herself on the clutch and turns about in half-circles until the eggs are free from nest material (Matthysen 1998). This may reduce hatching success in unstructured nests by losing contact with some eggs within the nest material. The same may occur when nestlings are small. Moreover, the dispersion of the nestlings within the nest resulting from the lack of a structured nest cup may reduce contact among nestlings and thereby heat exchange, an important factor during periods of parental inattention (Webb 1993). Heat loss can be reduced by decreasing the area exposed through postural changes of nestlings or huddling (Webb 1993). Nuthatch nestlings may show no tendency for huddling due to the difficulty in keeping together during female absences in unstructured nests made of loose bark flakes. Moreover, nestlings may experience lower thermoregulatory costs while buried in the insulating nest material.

3. ADAPTATIONS TO ECTOPARASITES

Given relatively constant environmental conditions offered, nesting cavities constitute micro-environments very likely to be colonized by bacteria, decomposers and detritivores due to the presence of faeces and food remains of breeding birds, and by ectoparasites that feed on blood, skin and feathers of avian hosts (Collias and Collias 1984; Mazgajski 2007). Nest ectoparasites feeding on the blood of nestlings and adults constitute an important selective force affecting avian life history evolution as they remove nutritional and energy resources from hosts that could otherwise be used for growth, maintenance, or reproduction (Møller 1993). Accordingly, ectoparasite presence and abundance in nesting cavities may have constituted an additional important evolutionary factor modulating adaptations of hole-nesting birds (Heeb et al. 2000; Tripet et al. 2002).

Ectoparasites cause removal of nutritional and energy resources from hosts that could otherwise be used for growth, maintenance, or reproduction (Møller 1993). They may also induce costly immune and inflammatory responses (Møller et al. 2005). Moreover, the immature immune systems of altricial nestlings may result in stronger direct impacts from ectoparasitism due to the need to assign sufficient nutritional resources to growth (Saino et al. 1998; Szep and Møller 1999). Given the negative impact of ectoparasites on host fitness, there will be selection on hosts to avoid parasite infestations through behavioural, physiological and immunological responses (Hart 1992; Hart 1997; Heeb et al. 1998). All these responses are complementary and may be induced in adults, nestlings or both (Hart 1992; Keymer and Read 1991; Simon et al. 2005). Nesting adults may avoid nest sites with high ectoparasite loads (Moore 2002) due to the association between old nest material and higher abundance of certain types of ectoparasites (López-Arrabé et al. 2012; Mazgajski 2007) and bacteria (González-Braojos et al. 2012). Adults may also take measures to indirectly minimize the effects of nest parasites through incorporation of fresh plant material containing compounds that either directly affect the development of parasites (Clark and Mason 1988; Lafuma et al. 2001; Malan et al. 2002) or stimulate elements of the immune system of chicks that help them to cope better with the harmful activities of ectoparasites (Mennerat et al. 2009). Nevertheless, given counter-adaptations in ectoparasites, adult cavity-nesting birds are faced with their presence, and may have evolved a suite of behaviours directed a minimizing their impact (Hart 1992; Keymer and Read 1991; Loye and Zuk 1991).

Avian hosts may try to compensate for the deleterious effects of ectoparasitism through behavioural modifications (Hart 1992; Keymer and Read 1991; Loye and Zuk 1991; Simon et al. 2005). Hosts can increase their provisioning rates to the offspring (Tripet and Richner 1997b) which may affect their current and future reproduction (Richner and Tripet 1999). Given potential fitness costs, they can evolve behavioural responses to minimize ectoparasite loads (Christe et al. 1996; Tripet et al. 2002; Waite et al. 2012). The main behavioural defenses against ectoparasites are grooming and nest sanitation (Christe et al. 1996). Grooming behaviour may be operationally defined as manipulation of the plumage with the bill (Murray 1990; Nelson et al. 1977). One of its functions may be to dislodge ectoparasites hiding or residing among feathers (Cotgreave and Clayton 1994; Waite et al. 2012). Thus both adults and nestlings may groom themselves in the presence of ectoparasites (O'Connor et al. 2010). Nest sanitation (Welty 1982) refers to behaviours by parents in altricial species tending to remove ectoparasites on nestlings or nest material (Hurtrez-Boussès et al. 2000), removing from the nest both these as well as eggshells (Montevecchi 1974), fecal material (Blair 1941) or dead nestlings (Skutch 1976). Parents are expected to allocate time to nest sanitation in order to control the load of harmful ectoparasites in the nest materials and on the nestlings. Such anti-parasite behaviours may be time-consuming (Cotgreave and Clayton 1994) and therefore may reduce the time that a parent bird can devote to foraging and provisioning offspring. Behavioural adaptations to control and reduce ectoparasite impacts may mainly be detected in host populations where ectoparasites have important effects on reproductive success. That nest sanitation may be important is suggested by the fact that the condition and health of breeding females can determine the rates of ectoparasite infestation (López-Arrabé et al. 2012; Tomás et al. 2005; Tomás et al. 2007a; Tomás et al. 2007b).

For some hole-nesting passerines, fleas *Ceratophyllus gallinae*, blowflies *Protocalliphora azurea* and mites *Dermanyssus gallinoides* constitute the most important groups of nest-dwelling ectoparasites (Merino et al. 1998; Merino and

Potti 1995; Moreno et al. 2009; Rendell and Verbeek 1996). Species of these arthropod taxa are usually not host-specific (López-Arrabé et al. 2012; Moreno et al. 2009; Tripet and Richner 1997a). Their relative abundance differs according to host species even in conditions of strict sympatry (Bauchau 1998; Bennett and Whitworth 1991; Moreno et al. 2009). Nuthatches, Pied Flycatchers and Tits *Paridae* coexist frequently in European deciduous woodlands and present different prevalences and intensities of infestation by the different ectoparasite taxa (Bauchau 1998; Moreno et al. 2009). Matthyssen (1998) found that Nuthatch nests contained fewer fleas than Great Tit *Parus major* nests in similar nest-boxes and habitats, and that more fleas were found in nests built of leaves instead of pine bark. Nuthatches may prefer pine bark as nest material because it contains toxic secondary compounds that may have insecticidal properties, in particular the monoterpene limonene (Carroll 1994). Limonene (and other plant compounds such as hydrocyanic acid) repel northern fowl mites (*Ornithonyssus sylviarum*), an ectoparasitic mite (Carroll 1994). Bauchau (1998) found that Great Tit nests showed higher abundances of mites, fleas and blowflies than Pied Flycatcher nests in the Netherlands. One of the factors suggested to explain differences in ectoparasite loads between species is nest design and composition (Bauchau 1998; Moreno et al. 2009; Remeš and Krist 2005). Unstructured nests like those of Nuthatches offer fewer opportunities for hiding to ectoparasites, and nest composition may affect ectoparasite development through the effects of microclimatic conditions associated with different nest materials (Heeb et al. 2000). Large amounts of nest material, although of benefit to reduce incubation costs (Moreno et al. 2010a), may collect and retain humidity above optimal levels and attract parasitic arthropods and pathogenic bacteria (Moreno 2012). Thus, interspecific differences in ectoparasite abundances could be explained by interspecific differences in nest composition and structure.

4. ADAPTATIONS FOR INCUBATION

Males of many avian species in which only the female incubates provision their mates during the incubation period (Lifjeld and Slagsvold 1986; Lyon and Montgomerie 1985; Ricklefs 1974). In some species, for example hornbills, females are totally dependent on males for food during the incubation stage (Poonswad et al. 2004). In a variety of other species it is more common for incubating females to receive only some of their food from their mates, although they also leave the nest to forage in order to sustain their energy requirements (Poonswad et al. 2004). Mate feeding may have evolved as a behavioural strategy to compensate for energetically costly activities for the female during reproduction (Galván and Sanz 2011), which may include the posthatching stage.

Food provided by males during incubation has been proposed to be an important energy source for females, a proposal termed the 'female nutrition hypothesis' (Niebuhr 1981). In fact, several studies have demonstrated that higher rates of male incubation feeding to their mates can improve female body condition (Lifjeld and Slagsvold 1986) and increase nest attentiveness by reducing the amount of time the female spends foraging off the nest (Boulton et al. 2010; Halupka 1994; Leclaire et al. 2011; but see Lifjeld and Slagsvold 1989; Matysioková and Remeš 2010; Matysioková and Remeš 2011; Moreno and Carlson 1989; Pearse et al. 2004; Smith et al. 1989; Stein et al. 2010) and thereby help to advance hatching (Lyon and Montgomerie 1985; Nilsson and Smith 1988), improve hatching success (Galván and Sanz 2011; Lyon and Montgomerie 1985; Nilsson and Smith 1988) or improve fledgling condition (Lifjeld and Slagsvold 1986; Røskaft 1983). This suggests that incubation feeding has evolved as a behavioural strategy to partly compensate for the energetic limitations of females while incubating (Galván and Sanz 2011). Although there are probable fitness advantages for the breeding pair derived from male incubation feeding, there may also be costs for males induced by intensified foraging activity at an early stage of the season (Leclaire et al. 2011; Lifjeld and Slagsvold 1986; Smith et al. 1989). Thus males may experience a trade-off between provisioning their mate and feeding themselves (Lifjeld and Slagsvold 1986; Lifjeld et al. 1987; Lyon and Montgomerie 1985; Moreno et al. 2011). They may also allocate more or less effort to finding and copulating with extrapair mates (Hill et al. 2011; Wagner 1992). Male incubation feeding intensity could thus be more a product of differences in male age, condition and mating strategy than of female nutritional needs (Lifjeld and Slagsvold 1986; Lifjeld and Slagsvold 1989; Lifjeld et al. 1987).

To distinguish between the 'female nutrition' and alternative scenarios it is necessary to experimentally manipulate female condition and study male responses, as males may adjust their feeding activity to the optimal level of attendance at each nest in a non experimental situation (Moreno et al. 2011). Only according to the 'female nutrition' hypothesis would we expect a direct male response by either increasing (experimentally reduced female condition) or reducing (experimentally increased female condition) his provisioning rate. Both experimental approaches have provided support for the female nutrition hypothesis (reduced condition: Moreno et al. 2011; Paillisson et al. 2007; improved condition: Smith et al. 1989; Wright and Cuthill 1989; Wright and Cuthill 1990a; Wright and Cuthill 1990b).

To improve their condition during incubation, females should be able to communicate their needs to mates. As both sexes have at least partially overlapping reproductive interests (Moore and Rohwer 2012), communication between incubating females and their mates should be reliable (Searcy and Nowicki 2005). Begging by nestlings has received a fair amount of attention as an honest system of communication (Cotton et al. 1996; Mock et al. 2011; Wright and Leonard 2002), whereas begging between mates has received scant attention. Females beg to their mates in courtship contexts (Clancy 2005; East 1981; Ellis 2008; Otter et al. 2007; Tobias and Seddon 2002), while incubating (Ellis 2008; Moore and Rohwer 2012; Tobias and Seddon 2002) and also during the nestling feeding phase before apportioning food to the nestlings (Clancy 2005). Female begging displays include loud vocalizations, body postures and wing fluttering, which closely resemble the begging displays of older nestlings (Ellis et al. 2009; Godfray 1991; Harper 1986). The striking similarity of female and nestling begging displays suggests the retention into adulthood in females of typically juvenile behaviours (Moore and Rohwer 2012). Otter et al. (2007) manipulated the hunger levels during egg laying of black-capped chickadee, *Poecile atricapillus*, females and showed no effect on male provisioning, even finding a decrease in female food solicitation. Furthermore, Moore and Rohwer (2012) found a correlation between begging displays of incubating yellow warbler, *Setophaga petechia*, females and mate provisioning rate in relation to environmental conditions. However, to our knowledge, it has never been confirmed experimentally that males adjust incubation feeding effort to female begging intensity. To test this link, begging intensity could be manipulated directly although this is difficult. Several behavioural components (posture, vocalizations, wing fluttering) presumably contribute to begging behaviour but the information content expressed in each component is still unknown. Alternatively, begging behaviour may be manipulated through hunger. Hunger depends on energy balance which may be experimentally altered through either food supplementation or handicapping (see above).

AIMS AND HYPOTHESES

This thesis is framed within the scientific field of Evolutionary Ecology and the main goal is increase understanding from an evolutionary perspective of the behavioural strategies arising during several stages of the reproductive cycle in small altricial cavity-nesting birds such as the Pied Flycatcher, Eurasian Nuthatch and Blue Tit. This PhD thesis involves descriptive studies and field experiments aimed at understanding the mechanisms underlying adaptations for nest site selection and defense, for determining nest structure and composition, for defense against ectoparasites and for female nutrition during the incubation stage in their natural habitats.

- **Objective I.** Explore the implications of the steroid hormone testosterone in relation to female-female competition for breeding resources by studying natural levels of aggressiveness between three geographically separated populations of pied flycatchers (Chapter I).

- **Objective II.** Elucidate if selection of nesting material and the amount of mud in Nuthatch nest-boxes can be explained as a trade-off between requirements for nest construction and availability as a function of transport distance (Chapter II).

- **Objective III.** Understand through a field experiment the implications of unstructured bark flake nests in Nuthatches for microclimatic conditions in the nest, ectoparasite infestation, parental care and nestling begging (Chapter III).

- **Objective IV.** Explore experimentally whether the ectoparasite loads lead to change the frequency and duration of anti-parasite behaviours by adult hosts, as well as whether such anti-parasite behaviours are able to compensate for the deleterious effects that parasites may have on nestlings (Chapter IV).

- **Objective V.** Study the implications of ectoparasitism in three sympatric avian cavity-nesters, namely Pied Flycatchers, Blue Tits and Nuthatches, to explore if differences in prevalence and abundance of generalist ectoparasites (blowflies, fleas and mites) can be related to interspecific differences in their nest size, nest composition and cavity microclimate. Furthermore, we have aimed at detecting if interspecific variation in the incidence and intensity of anti-parasite behaviours is a consequence of the abundance of ectoparasites in these three species (Chapter V).

- **Objective VI.** Test experimentally whether female begging during incubation is an honest signal of energetic need and whether mates respond to it (Chapter VI).

To answer these aims, this thesis combines information obtained during intense fieldwork campaigns from observations and diverse experiments. The thesis is divided into six chapters, whose development has been linked to different aspects of the reproductive cycle of the cavity-nesting birds.

1. ADAPTATIONS FOR NEST DEFENSE

CHAPTER I. Nesting holes are a scarce resource for cavity nesting birds and the need to occupy them may be an important selective force for the evolution of aggressive female behaviours, which may be mediated by testosterone (T) levels. The aim of this study was to explore the variation in circulating T levels of females between three geographically separated populations of pied flycatchers. We exposed female pied flycatchers from two of these populations to simulated territorial intrusions using a stuffed female when nest construction was almost complete. We also collected female blood samples to measure T levels in all populations. The purpose of the study was to explore the implications of T levels for female-female competition by studying natural levels of aggressiveness towards intruders in two populations with a marked difference in female plumage phenotype, a southern population in Valsaín (central Spain) and a northern population on the island of Ruissalo (Finland). We also collected female blood samples from a high density population located near the village of Lozoya (central

Spain) to further explore density-related variation in T levels. Furthermore, we aimed to detect if variation of T levels may explain female incubation attendance. We predicted that if T level is a proximate mechanism regulating female behaviour in the early stages of breeding, then:

1. T levels should be related to female aggressiveness towards female decoys.
2. T levels should be higher in females from populations more exposed to aggressive territorial interactions.
3. T levels should be higher when the availability of nest-boxes for breeding is lower later in the season.
4. Incubation attendance should be lower when T levels are higher.

2. ADAPTATIONS IN NEST STRUCTURE AND NESTING MATERIAL

CHAPTER II. Selection of nest sites and nesting material may have important implications for avian reproductive behaviour and performance. Nest construction may involve costs arising of transporting material that may be reduced considerably if nest materials are located close to the nest-site. Nuthatch nests in our nest-box study area are mainly composed of pine bark flakes or alternatively of strips of bark of the widespread shrub *Cistus laurifolius*, with variable amounts of mud used for plastering the entrance. In the present study we have attempted to elucidate if selection of nesting material and the amount of mud in Nuthatch nest-boxes can be explained as a trade-off between requirements for nest construction and availability as a function of transport distance. We have explored whether:

1. The selection of nest-boxes by Nuthatches depends on the distance to streams where mud can be collected;
2. This relation is stronger for Nuthatches than for another sympatric cavity-nester, the Pied Flycatcher;
3. Selection of Nuthatch nesting material (pine or *Cistus* bark) depends on the availability of pine, in particular the distance to the nearest pine;

4. The amount of mud in Nuthatch nest-boxes is negatively associated with the distance to streams.

CHAPTER III. Nest structure and nesting material may have important consequences for avian reproductive behaviour and performance. Nuthatches *Sitta* spp. build nests made of loose bark flakes without any structure or nest cup to contain eggs and nestlings. We have aimed at understanding the implications of unstructured bark flake nests in Nuthatches for microclimatic conditions in the nest, ectoparasite infestation, parental care and nestling begging through a nest exchange experiment. To that end, we have experimentally replaced natural bark nests of Nuthatches by structured moss nests built at the same time by Great Tits for some pairs and compared their ectoparasite abundances, nest microclimate variables and the behaviour of nestlings and parents with those in natural Nuthatch nests. We have predicted that the experimental nest replacements would affect ectoparasite abundance and nest microclimate and possibly the behaviour of nestlings and parents depending on the magnitude and sign of effects on ectoparasites and cavity microclimate. This in turn could affect nestling growth and reproductive success.

3. ADAPTATIONS TO ECTOPARASITES

CHAPTER IV. Nests of cavity-nesting birds usually harbor some species of haematophagous ectoparasites that feed on the incubating adults and nestlings. Given the negative impact of ectoparasites on nestlings there will be selection on hosts to reduce parasite infestations through behavioural means. We have experimentally reduced the abundance of all ectoparasites in nests of pied flycatchers *Ficedula hypoleuca* to explore both whether there are changes in the frequency and duration of putative anti-parasite behaviours by tending adults, as well as whether such anti-parasite behaviours are able to compensate for the deleterious effects that parasites may have on nestlings. Our objectives were to explore changes in the frequency and duration of parental grooming and nest sanitation behaviours as a consequence of the abundance of ectoparasites, and to examine the impacts of these behaviours of adult birds. We have hypothesized that:

1. Behavioural responses to ectoparasites should be more frequent in control nests than in experimental nests. This pattern should occur during both the incubation and nestling periods;
2. There should be a trade-off between brooding nestlings and nest sanitation behaviours at the early nestling stage;
3. Nestlings should beg more intensely in control nests due to the increased food demand induced by ectoparasites;
4. Parents should respond to higher begging levels in control nests by increasing provisioning rates only if time consumed by anti-parasite behaviours does not compromise that available for foraging.

CHAPTER V. Nesting cavities constitute micro-environments very likely to be colonized by ectoparasites which feed on blood of the incubating female and the nestlings. Given the negative impact of ectoparasites on nestlings there will be selection on hosts to minimize ectoparasite loads through behavioural defenses. We have addressed the implications of ectoparasitism in three sympatric avian cavity-nesters, namely Pied Flycatchers, Blue Tits and Nuthatches, to explore if differences in prevalence and abundance of generalist ectoparasites (blowflies, fleas and mites) can be related to interspecific differences in their nest size, nest composition and cavity microclimate. Furthermore, we have aimed at detecting if interspecific variation in the incidence and intensity of anti-parasite behaviours is a consequence of the abundance of ectoparasites. We have explored if:

1. Variation in ectoparasite abundance between host species is associated with interspecific differences in nest size and composition;
2. Avian hosts using pine bark as nest building material (Nuthatches and some Pied Flycatchers) show lower prevalence and abundances of some ectoparasites;
3. Cavity microclimate affects ectoparasite abundance;

4. Behavioural responses to ectoparasites are more frequent in avian hosts with higher infestations. This pattern should occur during both the incubation and nestling periods;
5. There is a trade-off in time allocation between brooding nestlings and nest sanitation behaviours during the early nestling stage.

4. ADAPTATIONS FOR INCUBATION

CHAPTER VI. The 'female nutrition' hypothesis proposes that food provided by males during incubation is an important energy source for females in bird species in which females alone incubate. Females should be able to communicate their needs through begging signals to mates and males may compensate for the energetic limitations of females through their feeding visits, owing to their overlapping reproductive interests. To test whether female begging during incubation is an honest signal of energetic need and whether mates respond to it we experimentally handicapped female pied flycatchers at the beginning of incubation by clipping two primary flight feathers on each wing. We assumed that clipping should increase the female's flight costs and therefore her energy requirements during incubation (Matysioková and Remeš 2011; Pennycuick 1982). We then compared control and experimental females by video filming their behaviour within the nest-box during incubation (before and after female manipulation) and at two stages of the nestling period (3 and 9 days of age). We predicted following the 'female nutrition hypothesis' that impaired flight ability caused by handicapping would (1) lead to increase female begging displays during incubation because of the manipulation of female condition and hunger and (2) induce more male incubation feeding in response to female needs. Depending on the effects of the experiment on male incubation feeding rate, we might or might not expect changes in female incubation behaviour and in body mass loss between incubation and the nestling phase.

STUDY SITE AND STUDY SPECIES

The general methods of this thesis are focused on the study site and study species. A more detailed description of the methods used in each experiment will be found in each chapter.

1. STUDY SITE

Picture of the study site (Valsaín, Segovia).

The studies presented in this thesis were conducted during the springs of 2011, 2012, 2013 and 2014. The study site is a montane forest of Pyrenean oak, *Quercus pyrenaica*, at 1200 m.a.s.l. in Valsaín, central Spain (40˚ 54' N, 4˚ 01' W) where passerines birds breeding in nest-boxes have been studied since 1991 (see Sanz et al. 2003 for general description). The mean annual temperature in this area is 10-11º C with a mean rainfall of 650-1000 mm. Scattered pines *Pinus sylvestris* are found among the oaks while the shrub layer consists mainly of *Cistus laurifolius* (Moreno et al. 2009, Cantarero et al. 2013). The study area is crossed by some seasonal watercourses.

There are 570 nest-boxes erected in the study area placed hanging from a branch attached to a metal hook (see Lambrechts et al. 2010 for dimensions, structure and placement of nest-boxes) and they are occupied mainly by Pied Flycatchers, Great tits, Nuthatches, Blue tits and Rock sparrows *Petronia petronia*. The use of such artificial cavities in avian research has greatly advanced our understanding of breeding behaviour in cavity-nesting species. To characterize the study area each nest-box location is identified with a Global Positioning System (GPS) waypoint. Breeding activities are followed routinely every year and laying and hatching dates and brood sizes at hatching and fledging are determined. All the nest-boxes are cleaned every year after the breeding season.

2. STUDY SPECIES

a. Pied Flycatcher *Ficedula hypoleuca*

The Pied Flycatcher is a small (12-13g g) passerine bird, which breeds in many forested areas of the Palaearctic region (Lundberg and Alatalo 1992). It only stays in the north for the spring and summer, spending the rest of the year on migration or in the wintering areas in tropical West Africa (Lundberg and Alatalo 1992). The Pied Flycatcher breeds naturally in hole in trees but it adapts readily to breeding in nest-boxes. This is one of the main reasons why it has become such a popular object of research. Pied Flycatchers are easily catchable in nest-boxes, which is an enormous advantage in behavioural studies.

In Central Spain, the first males usually start to arrive at the middle of April and, as in most migratory passerines, males arrive ahead of females. Nest building is performed mainly by the female although males also provide materials (Martínez-de la Puente et al. 2009). Egg laying in the population under study typically begins in late May, and clutch sizes range from 4 to 7 eggs. In our study area Pied Flycatchers incorporate strips of bark of *Cistus laurifolius*, pine *Pinus sylvestris* bark and dry grass as nest material (Moreno et al. 2009). The length of the incubation period is about 13-16 days (Lundberg and Alatalo 1992), wherein the female incubates alone and receives part of her food from her mate (Moreno et al. 2011). Both male and female contribute to feeding the nestlings. The mean nestling period varies between 13 and16 days (Järvinen 1990). From the first egg, the mean duration of the breeding cycle is about 36 days.

European Pied Flycatcher male (left image) and female (right image).

b. Eurasian Nuthatch *Sitta europaea*

The Nuthatch is a small (23 g) cavity-nesting bird which breeds mainly in mature deciduous or mixed woodland with large, old trees, preferably oaks (Matthysen 1998). Pairs hold permanent territories and they prefer to build their nests in existing cavities in trees but can also use nest-boxes for breeding.

The foundation of the nest consists of pieces or rotten wood or bark and on the top of this comes a layer of lining material, typically bark flakes of Scots pine or other trees (Matthysen 1998), in our study area mainly pine bark flakes and strips of bark of *Cistus laurifolius.* Besides bark flakes, Nuthatches use mud in nest building by narrowing the entrance of cavities (Matthysen 1998) in order to avoid nest site competitors (Collias and Collias 1984). The total amount of mud may weigh up to 1 - 2 kg and is collected by the female in small pellets and carried to the nest (Matthysen 1998). Egg laying in central and western Europe typically occurs during the second half of April, and clutch sizes range from 5 to 9 eggs (Matthysen 1998). The length of incubation period is about 13-18 days (Löhrl 1958), wherein the female incubates alone and receives part of her food from her mate (Matthysen 1998). In our population, females always cover the eggs with flakes of bark before leaving the nest during incubation. Young Nuthatches develop more slowly than other passerines of comparable size (Löhrl 1958) such as Great Tit *Parus major*. Both sexes feed the young (Matthysen 1998). The mean nestling period varies between 23-27 days (Järvinen 1990). From the first egg, the mean duration of the breeding cycle is 48 days.

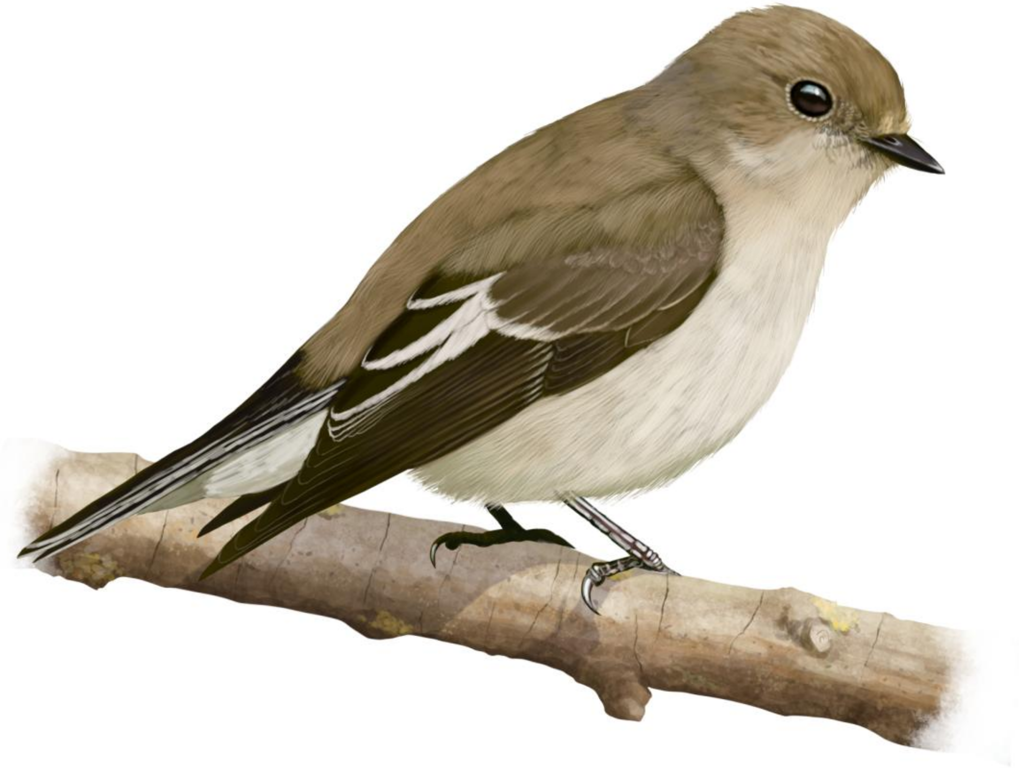
Eurasian Nuthatch

c. Blue Tit *Cyanistes caeruleus*

The Blue Tit is a small (10 g) hole-nesting passerine of European woodlands, which breeds mainly in deciduous forests (Cramp and Perrins 1993). It is a resident bird, which adapts readily to breeding in nest-boxes. Blue Tits build their nests mainly of moss and hair. Egg laying in central Spain typically begins in the second half of April, and clutch sizes range from 4 to 14 eggs (Fargallo 2004), and the number of fledglings averages 7 (Fargallo and Johnston 1997). Females incubate and brood the chicks alone, receiving part of their food from their mates, and both sexes feed the young (Fargallo and Johnston 1997; Moreno et al. 1996). Both male and female provision the nestlings. Young fledge within 17–20 days of hatching (Cramp and Perrins 1993). From laying of the first egg, the mean duration of the breeding cycle is 42 days.

Blue Tit

CHAPTER I



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CHAPTER I

Nest defense behaviour and testosterone levels in female Pied Flycatchers

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ABSTRACT. Nesting holes are a scarce resource for cavity nesting birds and an important selective force for the evolution of aggressive female behaviours, which may be mediated by testosterone (T) levels. We studied the implications of T levels for female-female competition by comparing natural levels of aggressiveness towards simulated female intruders (decoys) in two populations of the pied flycatcher (*Ficedula hypoleuca*) with a marked difference in breeding density (high density population in Finland, low density population in Spain). We also related aggressiveness to T levels in both populations. Another high density population in Spain was used to estimate T levels but without decoy tests. To this end, we exposed free-living females to simulated territorial intrusions during 30 min when nest construction was almost complete. T levels of females were measured at the beginning of incubation. Furthermore, we aimed at detecting if variation of T levels may explain female incubation attendance. Females showed higher T levels in the populations where pied flycatchers were exposed to a higher likelihood of conspecific interactions (high breeding density) than in the population with low breeding density. Female territorial presence, vigilance at the nest-box and proximity to decoys were negatively related to circulating T levels in the population where the females showed high T levels, but not in the low density population. Differences in T levels between populations did not result in differences in female incubation attendance, but T levels were negatively related to the incubation attendance in females from the population showing high T levels. T levels in females prior to laying reflect the need to defend nesting cavities which is higher at high breeding density and in subdominant females. High T levels are costly in terms of incubation attendance.

INTRODUCTION

Females in many vertebrate species express competitive traits (Cain & Ketterson 2012; LeBas 2006). Ornaments, weapons and aggressive behaviours may be selected by strong social competition for ecological resources other than mates, such as food, protection, territories or breeding cavities (Tobias et al. 2012; West-Eberhard 1979; 1983). Females are expected to compete over resources that directly influence breeding success (Rosvall 2011). Although this competition is often assumed to be subtle and inconspicuous, females do behave aggressively in some of the same contexts as males (Stockley & Campbell 2013). Several hypotheses have been proposed to explain female aggression towards conspecifics during the breeding season (Gill et al. 2007). Female aggression towards conspecific females is expected to be intense if it can prevent or delay the opportunity for males to attract additional females (Slagsvold & Lifjeld 1994). The obvious benefit to be gained from aggression is that females may thereby be able to monopolize male parental care by forcing the intruder to occupy a more distant nest site or delaying her breeding onset (Lifjeld & Slagsvold 1989; Slagsvold *et al.* 1992; Slagsvold & Lifjeld 1994).

Females may also defend their nest site or food resources in their territory (Karlsen & Slagsvold 1997; Kral et al. 1996; Male et al. 2006; Sandell 2007; Slagsvold et al. 1992). Aggressive females are more likely to acquire resources important for breeding such as a nesting cavities (Sandell & Smith 1997). Nest holes are a scarce resource for cavity nesting birds and there is a strong competition over them in some species (Dale et al. 1992; Dale & Slagsvold 1995; Leffelaar & Robertson 1985). Social selection for aggressive competition among females may be particularly important in obligate secondary cavity nesters (Breiehagen & Slagsvold 1988; Rosvall 2008), since losing the nest site would be disastrous for the breeding female (Rätti 2000).

In territorial species, nesting in areas with high breeding density may mean more conspecific aggressive interactions (Alonso-Alvarez & Velando 2001; Male et al. 2006; Mitchell & Robertson 1996). Rosvall (2008) modified the levels of competition for female tree swallows (*Tachycineta bicolor*) by experimentally reducing cavity availability and showed that more aggressive females were more likely to obtain nesting cavities. Furthermore, Bentz et al. (2013) found that tree swallow females breeding in high-density areas experienced a higher number of aggressive interactions and that their eggs had higher testosterone concentrations when breeding density was experimentally increased. Lower breeding density could reduce competition due to the higher probability of obtaining resources (Dunn & Winkler 2010).

The aggressiveness of an individual is partially determined by its hormonal status (Moss et al. 1994). Many aspects of male reproduction in vertebrates are influenced or controlled by the steroid hormone testosterone (Smith et al. 2005). Testosterone (T) levels are generally lower in females than in males (Moreno et al. 2014; Silverin & Wingfield 1982) and these levels are higher in females of colonial species than in solitary species (Møller et al. 2005). There is mixed evidence for the importance of T for female social aggressiveness. While some studies have found a positive association between female aggressive behaviour and endogenous (Cain & Ketterson 2012; Elekonich & Wingfield 2000; Gill et al. 2007) or manipulated (Lahaye et al. 2012; Moss et al. 1994; Sandell 2007; Veiga & Polo 2008) T levels, others have not found this association (Jawor et al. 2006). It is known that during periods of intense intrasexual competition such as territorial establishment, T levels are seasonally elevated in both males (Gowaty 1981; Silverin 1993; Wingfield et al. 2001) and females (Gowaty 1981; Kral et al. 1996; Lahaye et al. 2012; Sandell 2007). Territorial exclusion of female intruders may be especially necessary during these initial stages of reproduction when nests-sites may be taken over (Gowaty & Wagner 1988; Rosvall 2011). There might, however, also be selective forces that act against higher T levels: previous experimental and correlative studies have shown that higher T levels could also inhibit parental care (Alonso-Alvarez 2001; Cain & Ketterson 2013; De Ridder et al. 2000; O'Neal et al. 2008; Oring et al. 1989; Pinxten et al. 2007) or reduce reproductive performance (Gerlach & Ketterson 2013; López-Rull & Gil 2009; Martínez-Padilla et al. 2014).

In many passerines like the pied flycatcher *Ficedula hypoleuca*, females are highly aggressive towards intruding females during initial breeding stages and therefore female-female aggression has been well studied in this species (Breiehagen & Slagsvold 1988; Dale & Slagsvold 1995; Karlsen & Slagsvold 1997;

Morales et al. 2014; Rätti et al. 1995; Slagsvold et al. 1992). The availability of nesting holes in most areas is probably the main factor limiting population density in this species (von Haartman 1956). Competition among females for breeding sites can be rough and even lethal, especially during the nest-building period (Morales et al. 2014), where it is known that T levels can be increased rapidly by territorial intrusions in males (Silverin 1993).

The aim of this study was to explore the variation in circulating T levels of females between three geographically separated populations of pied flycatchers. We exposed female pied flycatchers from two of these populations to simulated territorial intrusions using a stuffed female when nest construction was almost complete. We also collected female blood samples to measure T levels in all populations. The purpose of the study was to explore the implications of T levels for female-female competition by studying natural levels of aggressiveness towards intruders in two populations with a marked difference in female plumage phenotype, a southern population in Valsaín (central Spain) and a northern population on the island of Ruissalo (Finland). Only females in Iberian populations present a white forehead patch as in males (Morales et al. 2014; Potti 1993), while white wing patches are also larger in these populations than in populations further north (Cantarero et al. in prep). Plumage phenotype has been linked to T levels in the species (Moreno et al. 2014). We also collected female blood samples from a high density population located near the village of Lozoya (central Spain) to further explore density-related variation in T levels. Furthermore, we aimed to detect if variation of T levels may explain female incubation attendance. We predicted that if T level is a proximate mechanism regulating female behaviour in the early stages of breeding, then:

(1) T levels should be related to female aggressiveness towards female decoys.
(2) T levels should be higher in females from populations more exposed to aggressive territorial interactions.
(3) T levels should be higher when the availability of nest-boxes for breeding is lower later in the season.
(4) Incubation attendance should be lower when T levels are higher.

MATERIAL AND METHODS

General field methods

The study was conducted during the spring of 2014 in three study areas. The Valsaín (40˚ 54’ N, 4˚ 01’ W, 1300 m altitude) and Lozoya (40º 58’ N, 3º 48’ W, 1400 m altitude) study areas in central Spain had 570 and 100 nest-boxes respectively erected in montane forests of Pyrenean oak, *Quercus pyrenaica*. From Valsaín we used in this study a subpopulation composed of 270 nest-boxes. The Ruissalo study area in Turku, Finland (60˚ 35′ N, 27˚ 09′ S), had 436 nest-boxes erected in forests dominated by oak (*Quercus robur*) and Scots pine (*Pinus sylvestris*). We used a Ruissalo subpopulation composed of 270 nest-boxes. In all areas, breeding activities of pied flycatchers are followed routinely every year and laying and hatching dates, as well as brood sizes at hatching and fledging are determined. The breeding density of pied flycatchers and the occupancy rate of nest-boxes, by pied flycatchers and other cavity-nesting birds, were different between areas (see Table 1). To characterize each study area we identified each nest-box location with a Global Positioning System (GPS) waypoint and calculated density using the outermost boxes as the boundaries of the area.

Simulated territorial intrusions and video recordings

Nest-boxes were checked every few days (3-4 days) to detect the initiation and progress of nest construction. When nest construction was complete (presence of a nest cup), we simulated territorial intrusions in Valsaín and Ruissalo by placing a pied flycatcher female decoy on top of the nest box. Following Morales et al. (2014), we used as decoys two stuffed females that were found naturally dead in the population in previous years and thereafter preserved frozen at -20 °C until stuffing. We used two different stuffed females in each area, selecting one randomly for each nest as commonly done in other avian territorial intrusion tests (e.g., Morales et al. 2014; Moreno et al. 2014; Vergara et al. 2007). We included 32 nests in Valsaín (all pied flycatcher nests from this subpopulation) and 30 randomly selected nests in Ruissalo. We recorded pied flycatcher activity near the decoy and the space surrounding the nest- boxes for about 30 min (32.33±SE 5.5 min) with digital video cameras placed 10-20 m away from the nest-box tree.

Five days after clutch completion (day 6 of incubation), nest boxes were again filmed (94.77±SE 13.3 min) with digital video cameras placed at least 10 m from the nest and covering the front of the nest-box and its immediate surroundings. Because of technical problems, we failed to record the behaviour of one nest in Ruissalo. All films were recorded at 8:00-15:00 h, and we found no significant effects of the time of day on behavioural variables (effect of hour $P>0.10$).

Female capture

Seven days after clutch completion, incubating females from all study sites were captured by hand while they were at the nest-box during daytime. They were ringed if necessary or identified by their ring. Within 2 min after capture, a blood sample (about 50-150 µl) was collected from the brachial vein in heparinized microcapillaries and stored in eppendorf tubes in an ice-box until returning to the lab in the same day where blood was centrifuged at 1200 rpm for ten minutes. Plasma was then separated, collected in eppendorf tubes and frozen at -20 ºC until analysis. Most females continued incubating immediately after being placed back on the nest. No female deserted the nest after capture.

Testosterone analysis

To determine plasma concentration of T, volumes of 10-50 µl of plasma were transferred to labelled glass tubes and steroid extracted by adding 3 ml of diethyl-ether to the tubes, vortexing for 2 min, and centrifuging at 100x for 5 min in a cooled centrifuge (4ºC). The tubes were snap-frozen in a bath of ethanol with dry ice, and the supernatant transferred to a new clean tube where they were dried by gently warming them in a water bath. Extractions were resuspended with 150 µl of steroid buffer (Cayman Chemical, Ann Arbor, Michigan, USA) and vigorously mixed.

Assays were conducted in duplicate (intra-assay CV = 7.2%) in a single enzyme immunoassay kit (ref. 582701, Cayman Chemical, Ann Arbor, Michigan, USA) following kit recommendations. Serial dilutions of a pool (from 1: 1 to 1:16) showed an excellent dilution pattern not different from expectations, suggesting antibody specificity and lack of matrix interferences. The standard curve provided a very good fit to standard ($r^2 > 0.92$). The detection limit of the assay (80% maximum binding) was found at 6.27 pg/ml and none of the samples fell below this limit. Since volumes of 50 µl of resuspended extractions were used in the assay, final calculations took into account sample-specific dilution coefficients. Because the plasma volume was insufficient, we failed to measure levels of 13 different females, 8 from the Finnish population and 5 from the Spanish population (4 from Valsaín and 1 from Lozoya).

We have validated the assumption that T levels show individual consistency throughout the breeding cycle. Of the sample of 22 females from Lozoya for which we obtained data on incubation T levels, 18 were captured also on day 7 of the nestling period. T levels during the nestling stage were related consistently to T levels at the beginning of incubation more than 3 weeks earlier ($F_{1,17}$=5.22, P=0.037, adjusted R^2=0.32). Given this individual consistency, we assume that female T levels measured during incubation are significantly associated with female T levels when decoy presentations were conducted (approx. 2 weeks earlier). We did not try to sample females before full incubation as they are very sensitive to capture at this stage and may desert the nest site.

Behavioural data analysis

Video recordings were watched using VLC Media Player software. From recordings taken when nest construction was almost complete (decoy tests) we obtained the following information: appearance on film or not (presence), time until the female appears (latency), proportion of test time appearing on film (vigilance), proportion of time appearing on film at less than 30 cm from the decoy (proximity), number of physical contacts with decoy by female and male per min (attack rate) and number of visits to the nest per min (nest attentiveness). Proximity is considered an index of aggressive disposition (Rätti 2000).

From recordings taken during incubation we estimated the proportion of time spent by the female inside the nest-box (“egg attendance”), which includes the time allocated to incubating and turning the eggs, and the mean duration of incubation sessions and recesses (periods when the nest was unattended). In addition, we also counted the number of incubation feedings by males.

Statistical analyses

The differences in circulating T levels of females between populations were analyzed with general linear models (GLM) with population as explanatory factor. T-levels were log-transformed for the analysis for normalizing the distribution.

We performed GLMs to test the association between T levels and female behaviour during the simulated territorial intrusions for each study population using date and time of filming as continuous predictors. We analyzed both the full data set and observations when females appeared on film.

Female incubation behaviour variables were normally distributed (Kolmogorov-Smirnov, $P>0.20$) and were therefore analyzed with GLM models assuming a normal error with population as explanatory factor and T levels, date and time of filming as continuous predictors. Furthermore, we used GLM to explore relationships between female behaviours within each population.

RESULTS

The distance to the nearest-neighbour as an index of breeding density was significantly different between the three populations ($F_{1,160}=6.80$, $P=0.001$), being shorter in Lozoya (38.36±6.88 m) than in Ruissalo (46.14±17.08 m) and in Valsaín (61.82±56.01 m) populations. The high-density study areas (Lozoya and Ruissalo) showed no significant difference in the distance to the nearest-neighbour (Fisher's LSD post hoc test, $P=0.128$) but the distance was shorter in both of them than in the low-density study area (Valsaín, Fisher's LSD, $P<0.001$ in both cases).

There were differences in circulating T levels between females from the three populations ($F_{1,67}=10.63$, $P<0.001$, Fig. 1). Regarding the populations subjected to simulated territorial intrusions, females from the high density population had significantly higher T levels than females from the low density population during the incubation stage (500.31±472.01 vs. 188.11±216.12 pg/ml; $F_{1,47}=6.13$, $P=0.017$). The females from Lozoya (high density population) showed higher circulating T levels (601.18±299.74 pg/ml, range 25.13-1219.60 pg/ml) than females from Ruissalo ($F_{1,39}=2.17$, $P=0.036$) and Valsaín ($F_{1,47}=5.45$, $P<0.001$)

during incubation. Laying date showed no association with the T levels of females in the Valsaín population (Spearman correlation: r_s=-0.047, P=0.811), but later-laying females tended to have higher T in Ruissalo (r_s=0.429, P=0.059).

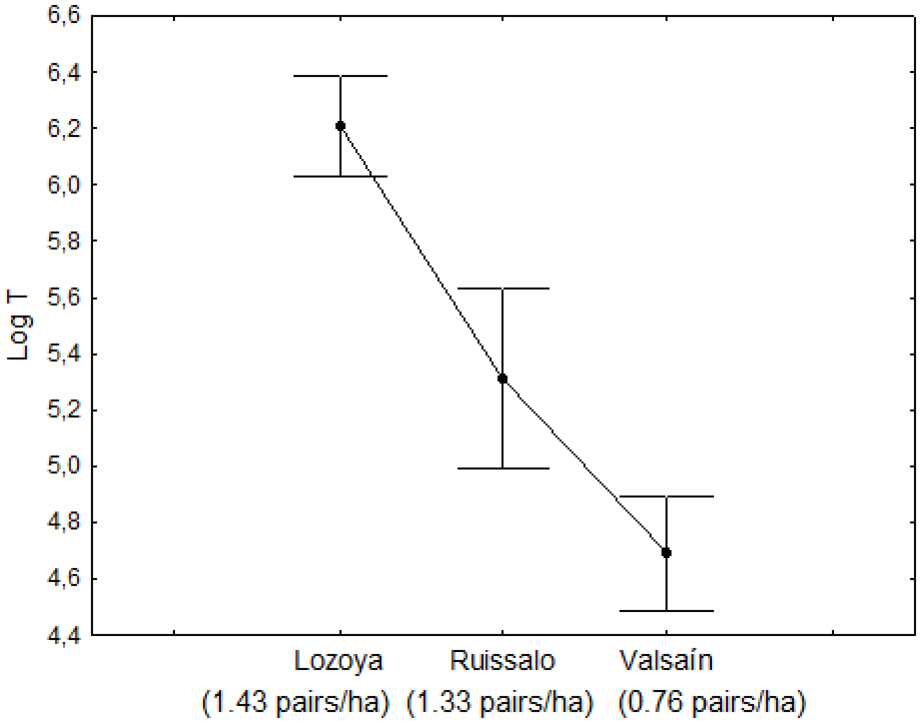

Figure 1. Differences in T levels of Pied Flycatcher incubating females between the three populations. Means ± SE are shown for each study area.

During the simulated intrusions test a slightly higher proportion of females were present around the nest-box in the high density population (22 of 30 females, 73.34 %) than in the low density population (20 of 32 females, 62.50 %). There were differences in circulating T levels of incubating females in relation to the presence (Yes) or absence (No) of the female during the simulated territorial intrusions for each population. Females from the high density population population that were present during the decoy tests showed lower T levels than females who were absent during these tests (Fig. 2; $F_{1,21}$=8.87, P<0.001). These T levels were similar between the females in the low density population (Fig. 2; $F_{1,21}$=1.40, P=0.623).

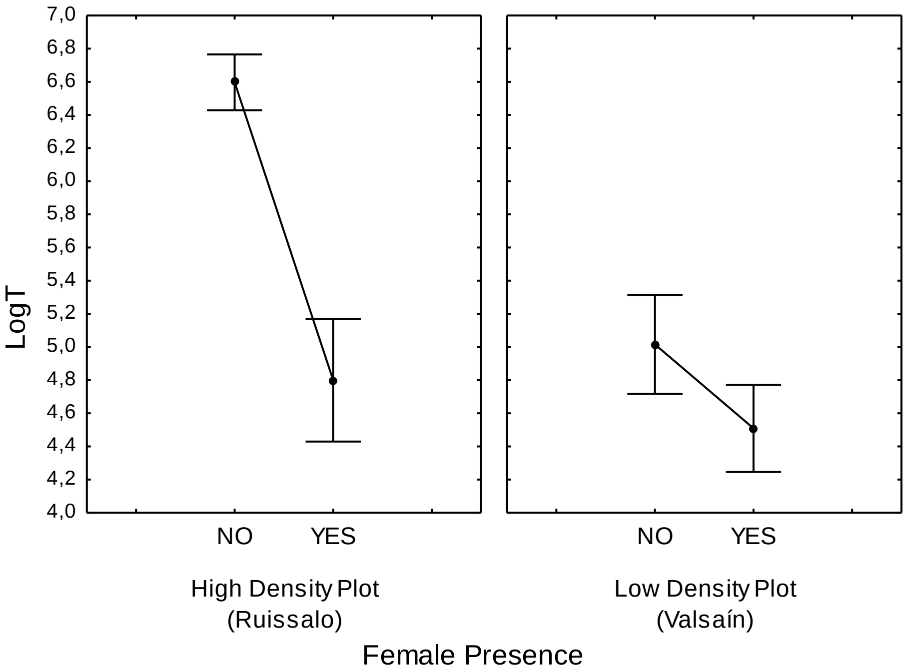

Figure 2. Differences in T levels of Pied Flycatcher incubating females in relation to the presence (Yes) or absence (No) of the female during the simulated territorial intrusions for Ruissalo and Valsaín populations.

While territorial vigilance was negatively related to T level in the high-density population, this was not the case in the low-density population, both when analyzing the whole data set and only observations with presence of the female (Fig. 3A and Fig. 3B). In the high-density population, there was a negative association between T and female proximity (Table 2). None of the populations showed relationships between T levels and latency time, attacks rate or nest attentiveness (Table 2, all $P>0.05$). The two populations showed a strong positive correlation between the presence of the female and the presence of the male in the film during the simulated territorial intrusions ($r_S=0.525$, $P<0.001$).

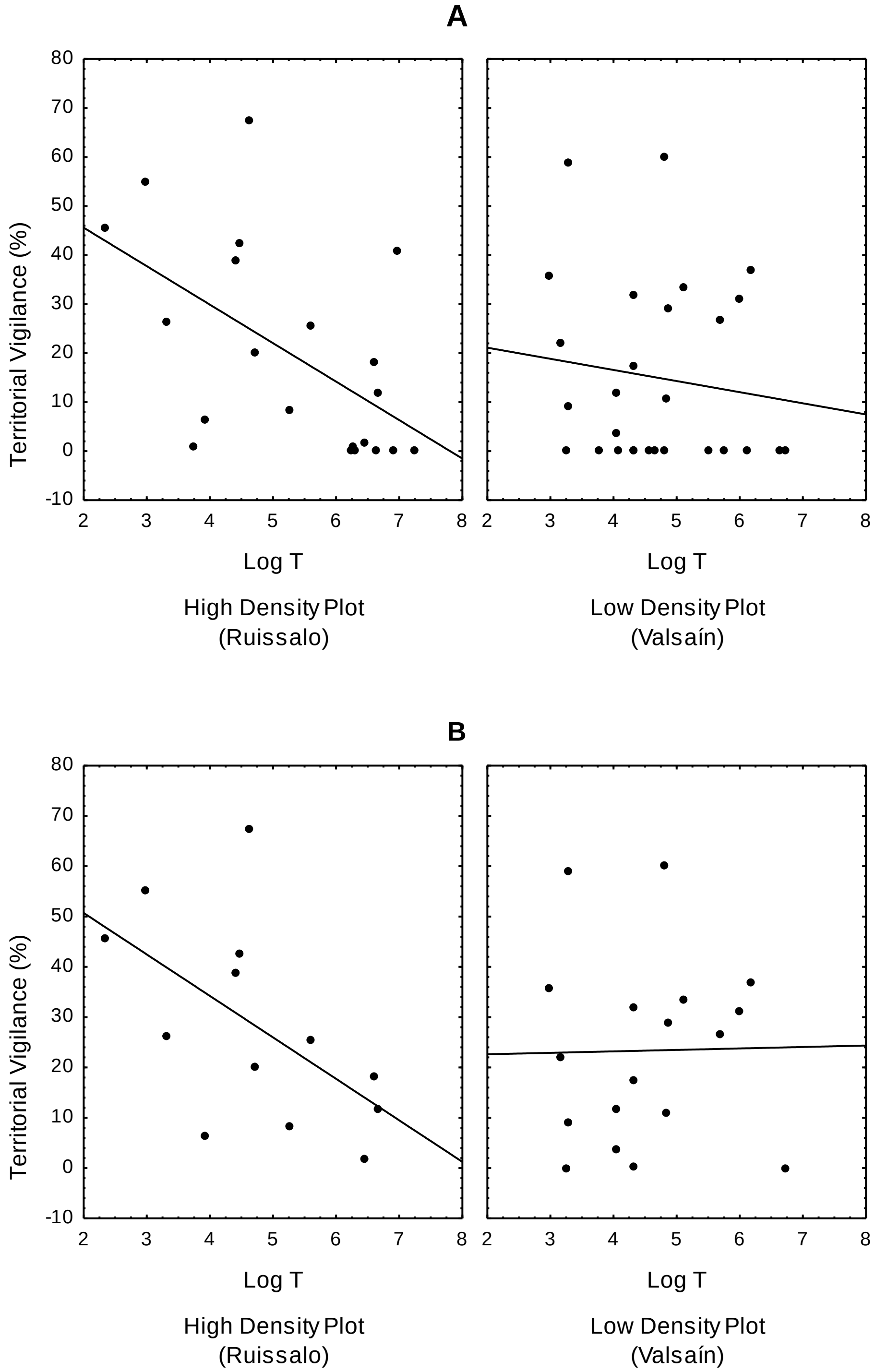

Figure 3. Association between T levels of females and female territorial vigilance for (A) all females (Ruissalo: *F*=8.14, *P*=0.010; Valsaín: *F*=0.45, *P*=0.507) and (B) only for females present during decoy trials (Ruissalo: *F*=5.20, *P*=0.045; Valsaín: *F*=0.01, *P*=0.945) for each population.

Study site	**Location**	**Nesting density (pairs/ha)**	**Occupancy rate (%)**	**Females (n)**	**Testosterone analysis (n)**	**Decoy test (n)**	**Incubation behaviour (n)**
Ruissalo	Finland	1.33	86.09	30	22	30	29
Valsaín	Spain	0.76	32.22	32	28	32	32
Lozoya	Spain	1.43	95	22	21	-	-

Table 1. Study area description and sample size (number of females) included in each part of the study.

	High density area (Ruissalo)						**Low density area (Valsaín)**					
	All females			**Excluding absent females**			**All females**			**Excluding absent females**		
	β	***F***	***p***	***β***	***F***	***p***	***β***	***F***	***p***	***β***	***F***	***p***
Latency time (min)	-	-	-	-0.141	0.265	0.614	-	-	-	0.178	0.521	0.480
Proximity (% time < 30 cm)	-0.511	6.347	**0.021**	-0.619	5.599	**0.042**	-0.325	3.068	0.092	-0.328	1.934	0.183
Attacks rate (attacks/min)	-0.191	0.719	0.407	-0.116	0.178	0.679	-0.342	3.314	0.081	-0.381	2.551	0.131
Attentiveness (visits/h)	-0.133	0.324	0.576	-0.374	1.957	0.187	-0.162	0.701	0.409	-0.122	0.245	0.627

Table 2. Results of GLM analyses (significant p-values in bold) for association between female behaviour during the simulated territorial intrusions and circulating T levels for the two study population with presence/absence of the female

We found no differences in incubation attendance (% incubation time) or the mean incubation session or recess durations of females between populations (all $P>0.10$). Male incubation feeding rates were similar in the two study sites (high density area: 2.2±2.86 times per hour; low density area: 3.22±3.42 times per hour; $F_{1,59}=8.87$, $P=0.343$). Patterns of female incubation in the high density area where characterized by females with higher T levels having shorter incubation sessions (Table 3). There was a positive association between T levels of females breeding in the low density area and the mean duration of recesses (Table 3).

	High density area (Ruissalo)			**Low density area (Valsaín)**		
	β	***F***	***p***	***β***	***F***	***p***
Egg attendance (%)	0.111	0.238	0.631	-0.137	0.504	0.484
Mean session (min)	-0.625	10.909	**0.004**	0.316	2.881	0.106
Mean recess (min)	-0.235	1.109	0.305	0.455	6.771	**0.015**

Table 3. Results of GLM analyses (significant p-values in bold) for association between female behaviour on incubation stage and circulating T levels for the two study population

DISCUSSION

Our results indicate that circulating T levels of female pied flycatchers differ between the three studied populations and are also differently related to nest defense in two of the populations. In the high density population, T levels showed a negative relationship with the availability of potential nest-boxes for breeding. Also in the high density population, the level of female aggressiveness against intruders decreased with higher T levels. Moreover, females that were absent during the simulated intrusions had higher T levels than those that were present during tests. Differences in T levels between populations did not result in differences in female incubation attendance, but in the high density population, females spent less time incubating when T levels were higher.

High population density causes increased aggression among individuals in many vertebrate species (Pilz & Smith 2004; Vergara et al. 2007). The crowded conditions imposed by breeding at high densities may lead to social instability associated with an increased number of territorial aggressions (Lacava et al. 2011). Following this, it is very plausible that the behaviour towards decoys shown by the females in the high density population is ultimately a result of competition for nesting-sites among females. Circulating T levels of high density population females during incubation were negatively related to female aggressiveness at the nest-building stage. This seems at first counterintuitive, but it may be that females with higher T levels may not consider the presence of female intruders as risky and may therefore tend to ignore potential competitors. Veiga et al. (2004) found that female spotless starling *Sturnus unicolor* tended to be more successful in acquiring a nest box when T levels were increased experimentally. The lower T levels observed in females who were present versus females who were absent during the simulated intrusions in the high density population may be a result of their non-dominant social status. Given the higher probability of usurpation due to the lower availability of cavities for female intruders in this population, females with low T levels may be forced to stay more time closer to the nest-box to confront conspecific intruders. It is a common observation in other species that the more dominant individuals are not necessarily the most aggressive ones (Beaugrand & Zayan 1985; Bekoff 1977; Higley 2003; Pérez-Guisado & Muñoz-Serrano 2009). Aggressiveness as measured in some studies may not relate to the capacity to dominate other individuals, so we should try to relate this capacity to T-levels in further studies. If nesting sites are not limiting, as in the low density population, females should not defend so intensely the cavity and could allocate more time to other activities which would limit any association between T level and behaviour. The strong correlation between the presence of both pair members during the simulated territorial intrusions suggest that the female response may serve to signal her mated status to the female intruder (Pärn et al. 2008; Slagsvold et al. 1992; Slagsvold & Lifjeld 1994) or that males are guarding their mate given their fertile state (Canal et al. 2012) and/or are attracted by the presence of other females in order to try to copulate with them (Dickinson 1997; 2001; Morales et al. 2014).

We found that pied flycatcher females breeding in Lozoya and Ruissalo showed higher circulating T levels and that these populations also showed high breeding density. The number of nesting holes is in most areas the main factor limiting population density of this species (von Haartman 1956) and poses an important selective force determining the strength of inter- and intraspecific competition (Jacot et al. 2009). The higher proportion of occupied nest-boxes for breeding in some areas denotes the capacity of these areas to attract a higher number of individuals trying to breed. A higher number of females trying to obtain a nesting cavity may induce stiffer competition with territory holders, both for males and for females. The more intense competition for nest-boxes may induce females from these populations to invest more energy and time in cavity defense and may lead to increased T circulation (O'Neal et al. 2008; Wingfield 1994). Similar results were reported by Silverin (1998) when comparing the territorial behaviour and T levels of male pied flycatchers breeding in optimal and suboptimal habitats.

High T levels may be adaptive when nesting-sites are limited because they prepare the individuals for intense competition with conspecifics (Moss et al. 1994). In many bird species, some females are present on the breeding sites but the intense competition among females for breeding opportunities may exclude them from breeding (Stutchbury & Robertson 1985; Stutchbury & Robertson 1987). These females are referred to as female floaters (Brown 1969; Smith 1978) and may replace a resident female that has died or deserted. If there are no opportunities of this kind, or the nest sites are limited as in the high density populations, their only option is to try to take over nest sites by force. Given the higher probability of usurpation, differences in aggressions and T levels may be caused by differences in the value of the nest-boxes and through an additional effect of population density on intrusion rates (Silverin 1998). Furthermore, because the breeding season is shorter in the north than in the south, females cannot wait very long to decide how much resources must be invested in reproduction (Järvinen 1989). This can lead to a higher level of competition for breeding sites, especially if these are scarce. If breeding sites are not limiting, as in low density populations, the selective pressures to obtain a nest-site may be weaker.

We found a weak marginally significant positive association between T level and breeding date in the high density population. When pied flycatcher females arrive to the breeding areas, they pair up and sequentially occupy nest-boxes defended by males. Early breeding increases the chances for both males and females to secure suitable nesting-sites (Slagsvold 1976). Thus, the availability of potential mates and nest-sites decreases as the season progresses. When nest-boxes are a scarce resource for breeding at high densities, aggressive interactions between females may be expected to be higher since the availability of male-defended nest-boxes would be lower.

High T levels in females in the high density area were associated with low intensity of egg attendance, as predicted by the negative interaction between T and prolactin levels in birds (Lormee et al. 2000; Oring et al. 1989; Schoech et al. 1998; Van Roo et al. 2003; Vleck et al. 2000). Several studies have shown trade-offs between investment in reproductive competition and parental care (Alonso-Alvarez 2001; Cain & Ketterson 2013; Moreno et al. 1999), suggesting an important cost of high T levels in terms of egg or nestling attendance (Cain & Ketterson 2013; De Ridder et al. 2000). Rosvall (2013) increased experimentally T levels in female tree swallows and showed a decline in incubation attendance. Similar results were reported in spotless starlings (De Ridder *et al.* 2000; Veiga & Polo 2008), zebra finches *Taeniopygia guttata* (Rutkowska et al. 2005) and rufous whistlers *Pachycephala rufiventris* (McDonald et al. 2001). These findings imply that high T levels may disrupt the expression of normal incubation behaviour (Oring et al. 1989; Schwagmeyer et al. 2005). This cost may constrain competition-induced increases in female T levels.

Our study shows that the relationship between T and competitive behaviour in females can be complex and differ between populations. High T may be related to dominance and make aggression unnecessary also in songbirds. Competitive capacity and aggressiveness should be separated in future studies. We furthermore suggest that the population differences detected in T levels of females reflect the need to defend nesting cavities and that this need is stronger where the likelihood of usurpation by intruders is greater. Further experimental studies are needed to establish this potential relationship between T levels and breeding density.

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CHAPTER II

This chapter reproduces entirely the manuscript:

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CHAPTER II

Selection of nest-site and nesting material in the Eurasian Nuthatch *Sitta europaea*

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ABSTRACT. Selection of nest sites and nesting material may have important implications for avian reproductive behaviour and performance. Nest construction may involve costs arising from transporting material that may be reduced considerably if nest materials are located close to the nest-site. Nuthatch *Sitta europaea* nests in our nest-box study area are mainly composed of pine bark flakes or alternatively of strips of bark of the widespread shrub *Cistus laurifolius*, with variable amounts of mud being used for plastering the entrance. Several small streams run through the area, an oak *Quercus pyrenaica* forest with a few scattered pines *Pinus sylvestris*. Here we show that nuthatches collected pine bark only when nest-sites were situated close to pines, used more mud when breeding close to streams, and selected nest-sites closer to streams than a sympatric species not using mud, the Pied Flycatcher *Ficedula hypoleuca.* Nuthatches used pine bark only when there was a pine-tree less than 100 m away from the nest-box and selected *Cistus* bark when transport distance is greater. We suggest that the selection of nest sites and nest materials in this species may be constrained by costs of transport of nest material.

INTRODUCTION

Hole-nesting bird species vary in the characteristics of their nest sites (Li & Martin 1991) and build their own characteristic nests (Hansell 2000). Nest construction may be influenced by factors such as the availability of nest materials (Moreno *et al.* 2009) and involves a large expenditure of time and energy (Collias & Collias 1984) due to the costs of transporting material to the nest site (Putnam 1949). These costs may be reduced by using old nest material (Nores & Nores 1994) and/or if nesting material is located close to the nest site (Collias & Collias 1984). Availability plays an important role in the selection of nest materials. Birds typically make use of local materials but some birds are nest material specialists (Hansell 2000), although for many species the functional properties of nests remain unknown.

Nuthatches *Sitta* spp. use nests made of loose material without any structure (Matthysen 1998). The base of the nest consists of pieces or rotten wood or bark covered by a layer of lining material, typically bark flakes of Scots pine or other trees (Matthysen 1998). Nuthatch *Sitta europaea* nests were composed in our study area mainly of pine *Pinus sylvestris* bark flakes or strips of bark of *Cistus laurifolius* (Cantarero *et al.* 2013). In several cases, Nutchaches are known to travel more than 100 m to obtain this material (Löhrl 1958, Bohr 1962). One nest in a large cavity contained no fewer than 11440 barks fragments (Olsson 1957).

Besides bark flakes, Nuthatches use mud in nest building by narrowing the entrance of cavities (Matthysen 1998) in order to exclude nest site competitors (Collias & Collias 1984). The total amount of mud may weigh up to 1 - 2 kg and is collected by the female in small pellets (Matthysen 1998).

In the present study we have attempted to elucidate if selection of nesting material and the amount of mud in Nuthatch nest-boxes can be explained as a trade-off between requirements for nest construction and availability as a function of transport distance. We have explored whether:

(1) The selection of nest-boxes by Nuthatches depends on the distance to streams where mud can be collected;

(2) This relation is stronger for Nuthatches than for another sympatric cavity-nesters, the Pied Flycatcher *Ficedula hypoleuca*;

(3) Selection of Nuthatch nesting material (pine or *Cistus* bark) depends on the availability of pine, in particular the distance to the nearest pine;

(4) The amount of mud in Nuthatch nest-boxes is negatively associated with the distance to streams.

MATERIAL AND METHODS

Study area and species

We conducted the study during the springs of 2011, 2012 and 2013 in a population of Nuthatches and Pied Flycatchers breeding in artificial nest-boxes in a montane forest of Pyrenean oak, *Quercus pyrenaica*, at 1200 m.a.s.l. in Valsaín, central Spain (40° 54’ N, 4° 01’ W). Scattered pines are found among the oaks while the shrub layer consists mainly of *Cistus laurifolius* (Moreno *et al.* 2009). Several seasonal streams cross the study area.

Breeding activities in nest-boxes are followed routinely every year. There are 300 nest-boxes erected in the study area, each hanging from a branch attached to a metal hook. Pied Flycatchers occupied 73 nest-boxes in 2011, 61 nest-boxes in 2012 and 66 nest-boxes in 2013. Nuthatches occupied 13 nest-boxes in 2011, 21 nest-boxes in 2012 and 17 nest-boxes in 2013.

The Nuthatch is a small cavity-nesting woodland bird that prefers to build its nest in existing cavities in trees, but it may also use nest-boxes for breeding. Nuthatches are territorial in pairs throughout the year (Matthysen 1998). Nuthatches are the first species to breed and to occupy nest-boxes in the spring, and are dominant over all other species using nest-boxes in our study area.

The Pied Flycatcher is a small hole-nesting passerine of European woodlands (Lundberg & Alatalo 1992), whose nest is composed mainly of strips of bark of *Cistus laurifolius* in our study area (Moreno *et al.* 2009).

To characterize the study area we marked each nest-box location with a Global Positioning System (GPS) waypoint, as well as the scattered pines and the permanent streams. The nearest distance between any two features was calculated using ArcGIS10 Desktop software (Redlands, CA: ESRI). From these distances we calculated the average distance between the streams and all nest-boxes, the nest-boxes occupied by Nuthatches and the nest-boxes occupied by Pied Flycatchers. Furthermore, we have calculated the shortest distance between the nest-boxes occupied by Nuthatches and the nearest pine.

Differences in nesting material and amount of mud

All Nuthatch nests were collected after the end of breeding and subsequently disassembled into different components. Nest composition was defined as the nesting material occupying at least 80-90% of the total volume of the nest: pine bark flakes or strips of bark of *Cistus*.

In 2012 and 2013, the intensity of plastering with mud was assessed by visual inspection of the nest-box and classified on a binary scale: 0 = low amount of mud, mainly on the inside upper rim of the entrance tunnel; or 1 = abundant mud clearly visible on the outside (Figure 1).

Figure 1. Examples of the intensity of plastering of the nest-box with mud: 0 = low amount of mud on left image, and 1 = abundant mud clearly visible on right image.

Statistical analyses

The distance to the nearest stream for the occupied nest-boxes was normally distributed and was therefore analyzed with GLMM models (SAS 9.1, SAS Institute) assuming a normal error distribution with species as explanatory factor and nest and year as random factor.

We used generalized linear mixed models (Glimmix procedure) with nest and year as random factor to test whether nest composition (*Cistus* or pine bark) could be explained from the distance to the nearest pines and whether the amount of mud on nest-boxes (low or abundant) could be explained from distance to the streams.

RESULTS

The distance to streams between all the nest-boxes and nest-boxes occupied by Pied Flycatchers was similar while Nuthatches occupied nest-boxes significantly closer to streams (F1,471=7.12, P<0.001, Fig. 2).

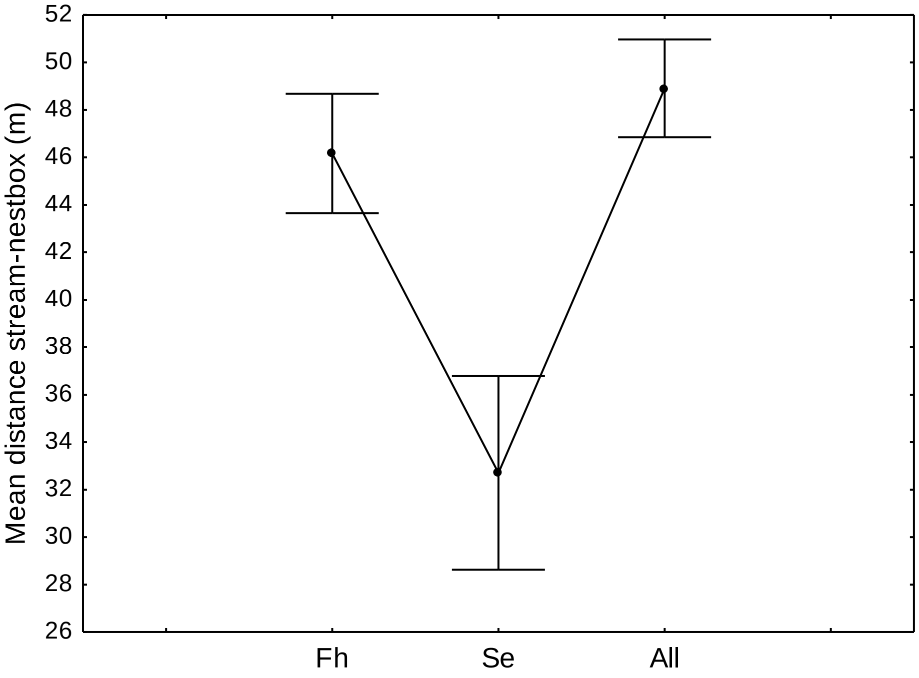

Figure 2. Mean (±SE) distance (m) between the nearest stream and nest-boxes occupied by Pied Flycatchers (Fh, n=200), and nest-boxes occupied by Nuthatches (Se, n=51) and all the nest-boxes available in the study area (All, n=300).

Nuthatches used pine bark as nesting material when there was a pine close to the nest-box (average distance to nearest pine of these nests 63±44 m) and used *Cistus* barks when the distance to the nearest pine was long (average distance to nearest pine of these nests 222±109 m; $F_{1,47}$=17.1, P<0.001). The maximum distance of a nest built of pine bark (n=27) to the nearest pine was 104 meters and the minimum distance of a nest built of *Cistus* bark (n=24) to the nearest pine was 106 meters

Nuthatch nests used more mud as nesting material (n=20) when there was a stream close to the nest-box (average distance to streams of nests with mud 23±21 m) and used lower amounts of mud no mud (n=19) when the distance to the nearest stream was long (average distance to streams of nests without mud 44±35 m; $F_{1,35}$=4.03, P=0.005).

DISCUSSION

This study shows some clear patterns of nesting material and nest-box selection in Nuthatches. We found that Nuthatches used pine bark when there was a pine available within 100 m and selected Cistus bark when this distance is greater. We also found that Nuthatches selected nest-boxes closer to streams than Pied Flycatchers. The distance to the streams also explained the amount of mud used by Nuthatches for plastering the nest-boxes.

Nest building is one of the better-studied elements in the natural history of Nuthatches (Matthysen 1998), but the information about nesting material selection is limited. Do Nuthatches have preferences for particular nesting materials? While some studies found that certain bird species may have a preference for a particular nesting material, despite a high cost of collecting (Putnam 1949), other studies found that birds may simply use the first type of suitable material that they encounter (Surgey et al. 2012). The availability of nesting material and the distances travelled to collect them can impose a significant energetic cost and may also increase the risk of predation while the adult is searching for material (Hansell 2000).

Nuthatch nests in our study area were composed mainly of pine bark flakes or strips of bark of Cistus shrubs (Cantarero et al. 2013). Cistus bark was abundantly available throughout the study area, but this nesting material was replaced by pine bark when pines were available close to the nesting site. Several studies found that Nuthatches may leave the territory in search of pine trees (Löhrl

1958, Bohr 1962), travelling hundreds of meters, but the reason why Nuthatches prefer this nesting material remains unknown (Cantarero et al. 2013). Variation in the type of bark used for nest construction shows that Nuthatches are flexible in their choice of nesting material; particular materials may be preferable but may be hard to find or costly to transport. Flight is an energy demanding activity that imposes several physiological challenges on birds (e.g. Costantini et al. 2008). Before egg-laying, Nuthatch females spend 10-20 % of their time nest building and reduce resting time while devoting more time to foraging than males (Enoksson 1990). Obtaining pine bark from far away could lengthen the period of nest building, a stage that typically takes a few weeks (Matthysen 1998). Females may enhance their own fitness by reducing their effort on nest construction (Moreno et al. 2010). Furthermore, given the strictly territorial character of Nuthatches (Matthysen 1998), intruding into a foreign territory to find adequate nesting material may increase the risk of becoming involved in territorial interactions with neighbours.

To conclude, we found significant differences in the distance to streams of occupied nest-boxes by Nuthatches and Pied Flycatchers. Availability of nesting materials may act as a constraint on nest site selection (Hansell 2000). By selecting nest-boxes near streams, Nuthatches may reduce considerably the energetic costs of nest-building (Matthysen 1998). The higher amount of mud from nest sites near streams supports this suggestion, though based on this study we cannot exclude the possibility that other factors than transport costs contribute to the observed nesting patterns as well. Pied Flycatchers do not use mud as nesting material, which may explain why we found no preference for nesting near streams in this species.

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CHAPTER III

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CHAPTER III

The significance of nest structure and nesting material for hole-nesting passerines: an experimental study with Nuthatches Sitta europaea

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ABSTRACT. Nest structure and nesting material may have important consequences for avian reproductive behaviour and performance. Nuthatches *Sitta* spp. build nests made of loose bark flakes without any structure or nest cup to contain eggs and nestlings. We have aimed at understanding the implications of unstructured bark flake nests in Nuthatches for microclimatic conditions in the nest, ectoparasite infestation, parental care and nestling begging through a nest exchange experiment. To that end, we have experimentally replaced natural bark nests of Nuthatches *Sitta europaea* by structured moss nests built at the same time by Great Tits *Parus major* for some pairs and compared their ectoparasite abundances, nest microclimate variables and the behaviour of nestlings and parents with those in natural Nuthatch nests. The experimental treatment did not affect ectoparasite loads. Nest-boxes containing structured nests made with moss showed higher and more variable temperatures, higher thermal maxima and less variable humidity conditions than unstructured control nests made by bark flakes. However, bark flakes conserve heat better than moss during the night and morning hours, which may be transmitted to buried eggs and nestlings and reduce incubation and brooding costs for females. This may explain why females remained out for longer during incubation recesses at natural nests. Nestlings of 9 days in natural nests rested further apart than nestlings in structured experimental nests although there were no differences with respect to begging intensity between the two treatments. Hatching and fledging success was similar in both groups but experimental nests resulted in nestlings with shorter tarsi and wings before fledging. The poorer nestling growth in experimental nests cannot be explained by effects of ectoparasites, nestling aggregation or nestling begging or parental care. Adaptations for remaining buried in the nest material and the heat-conserving properties of loose bark flakes may reduce energy costs for nestlings during female absences.

INTRODUCTION

The majority of bird species build their own characteristic nests (Álvarez et al. 2013; Collias and Collias 1984; Hansell 2000). The physical structure of the nest is determined by the degree of cohesion between the different materials used and may influence embryo development and chick growth so that nest quality may have important consequences for the condition and the reproductive success of parents (Álvarez and Barba 2011; Dawson et al. 2011; Lambrechts et al. 2012; Lombardo et al. 1995). Nest construction from a thermal aspect represents a compromise between heat conservation, heat dissipation, and protection from external heat sources (Heenan and Seymour 2011). It is reasonable that heat loss can be minimized by optimizing the physical structure of the nest (Heenan and Seymour 2011; Hilton et al. 2004; Reid et al. 2000) or by choosing a suitable nesting material (Álvarez et al. 2013). Consequently, we expect birds to adjust their nest characteristics in response to environmental conditions (Deeming 2011; Hansell 2000). Moreover, the regulation of thermal conditions within acceptable limits may be energetically costly for parents (Nord and Nilsson 2012; Williams 1996). The structure of nests may mitigate this energetic demand on parents (Hansell 2000). Thus, building a thermally favorable nest saves parental energy by reducing heat loss from attended and non-attended clutches (Heenan and Seymour 2011; Moreno et al. 2010).

Cavity nests constitute micro-environments very likely to be colonized by ectoparasites that drain resources from avian hosts (Collias and Collias 1984). Given the negative impact of ectoparasites on host fitness (Heeb et al. 1998; Richner et al. 1993), cavity-nesting passerines may have evolved behavioural, physiological and immunological adaptations to counter these effects (Hart 1997; Heeb et al. 1998; Møller and Erritzoe 1996). One possible adaptation concerns the use of insecticidal materials as nesting materials. Thus, some avian species incorporate fresh plant material in order to control nest-dwelling ectoparasites, because their volatile anti-parasitic compounds can delay the development of mites (Clark and Mason 1988; Malan et al. 2002; Tomás et al. 2012). Avian hosts may also react to infestations through behavioural modifications (Cantarero et al. 2013a; Hart 1992; Keymer and Read 1991; Loye and Zuk 1991; Simon et al. 2005) like allocating more time to nest sanitation and grooming in order to control the load of harmful ectoparasites in the nest materials and on the nestlings (Cantarero et al. 2013a).

The Nuthatch is a small cavity-nesting woodland bird that prefers to build their nests in natural cavities in trees but can also use nest-boxes for breeding. Nuthatches *Sitta* spp. use nests made of loose heaps of bark flakes without any structure or nest cup to contain eggs and nestlings (Matthysen 1998). These nesting habits may have evolved in relation to the use of large cavities (Wesolowski and Rowiński 2004). Eggs and nestlings are found buried in these heaps. Several consequences may be derived from the use of loose bark nests. Unstructured nests like these may offer fewer opportunities for hiding to ectoparasites, and nest composition may affect ectoparasite development through the effects of microclimatic conditions associated with different nest materials (Heeb et al. 2000). The Eurasian Nuthatch *Sitta europaea* may prefer pine bark as nest material because it contains toxic secondary compounds that may have insecticidal properties, in particular the monoterpene limonene (Carroll 1994). Limonene (and other plant compounds such as hydrocyanic acid) repel northern fowl mites (*Ornithonyssus sylviarum*), an ectoparasitic mite (Carroll 1994). Bauchau (1998) proposed that Pied Flycatchers *Ficedula hypoleuca* use limonene-rich material from pine trees in order to reduce the abundance of parasites in nests. Matthyssen (1998) found that Nuthatch nests in Sweden contained fewer fleas than Great Tit *Parus major* nests in similar nest-boxes and habitats, and that more fleas were found in nests built of leaves instead of pine bark. Cantarero et al. (2013b) found that the Eurasian Nuthatch nests in central Spain contained fewer fleas and blowflies than Blue Tit *Cyanistes caeruleus* nests. However, some experimental studies have failed to find a clear link between nest type and ectoparasite loads in cavity-nesters (Bauchau 1998; Moreno et al. 2009; Remeš and Krist 2005).

Nesting cavities are not always waterproof (Wesolowski et al. 2002). The insulation quality of nests is dependent on several factors, such as nest structure (McGowan et al. 2004), thickness, height and volume (Alabrudzińska et al. 2003; Grubbauer and Hoi 1996), nest material quality (Mertens 1977) and moisture content (Deeming 2011; Pinowski et al. 2006). Large amounts of nest material, although of benefit to reduce incubation costs (Moreno et al. 2010), may collect and retain humidity above optimal levels. The risk of the nest getting wet could be reduced by incorporating more hydrophobic material such as bark flakes (Wesolowski and Rowiński 2004). Furthermore, bark flakes could help to stabilize thermal fluctuations in the nesting cavity by conserving heat during the cooler hours of the day. Dense accumulations of bark flakes may produce heat due to microbiological activity as observed in composting (Collias and Collias 1984). Other birds like megapodes also use the heat of accumulated vegetal material to keep their eggs warm. Heat production may be especially noteworthy during the night when nest materials cool down after heating up during the day. Eggs and nestlings remain buried within the nest material (Wesolowski and Rowiński 2004) when females leave the nest instead of being exposed to air in the nest cavity as is typical for other cavity nesters. This may favour the maintenance of a suitable egg temperature by the warm nest material (Davis et al. 1984; Wesolowski and Rowiński 2004). Nestlings buried into loose material that remains warm during the night and early part of the day could thereby reduce incubation and brooding costs for females.

Incubation behaviour may be affected by nest microclimate and structure (Álvarez and Barba 2009). When the Nuthatch female returns to the nest, she lowers herself on the clutch and turns about in half-circles until the eggs are free from nest material (Matthysen 1998). This may reduce hatching success in unstructured nests by losing contact with some eggs within the nest material. The same may occur when nestlings are small. Moreover, the dispersion of the nestlings within the nest resulting from the lack of a structured nest cup may reduce contact among nestlings and thereby heat exchange, an important factor during periods of parental inattention (Webb 1993). Heat loss can be reduced by decreasing the area exposed through postural changes of nestlings or huddling (Webb 1993). Nuthatch nestlings may show no tendency for huddling due to the difficulty in keeping together during female absences in unstructured nests made of loose bark flakes. Moreover, nestlings may experience lower thermoregulatory costs while buried in the warm nest material (see above).

The aim of this study is to explore several possible implications of breeding in unstructured bark nests for Nuthatches by conducting an experiment where natural nests were replaced by structured nests made of materials different from bark. To that end we have used the structured moss nests of Great Tits which coexist naturally and breed simultaneously with Nuthatches in our study area. The use of nests built in the same type of nest-boxes by both species control for cavity dimensions and thermal properties. Following the hypotheses presented above, we have predicted that the experimental nest replacements would affect ectoparasite abundance and nest microclimate and possibly the behaviour of nestlings and parents depending on the magnitude and sign of effects on ectoparasites and cavity microclimate. This in turn could affect nestling growth and reproductive success.

MATERIAL AND METHODS

Study area and species

Our study was conducted during the springs of 2012 and 2013 in a montane forest of Pyrenean oak, *Quercus pyrenaica*, at 1200 m.a.s.l. in Valsaín, central Spain (40° 54’ N, 4° 01’ W) where hole-nesting passerine breeding activities in nest-boxes have been studied since 1991. Every year, nest-boxes (see Appendix in Lambrechts et al. 2010 for further details on dimensions and placement) are cleaned after the breeding season and again shortly before the next breeding season. Natural and experimental nests were constructed at the same time in similarly clean nest-boxes.

Nuthatches in the study area narrow the entrance to cavities by plastering mud around it (Enoksson 1993; Wesolowski and Rowiński 2004) and their nests are mainly composed of loose pine *Pinus sylvestris* bark flakes or loose *Cistus laurifolius* bark strips. As in other Nuthatch populations, nests are totally unstructured and do not present a nest cup. Breeding activities are followed routinely every year and laying and hatching dates and brood sizes at hatching and fledging are determined.

On day 13 (hatching day = day 1), we ringed nestlings and measured their tarsal length with a digital calliper to the nearest 0.01 mm and their wing length with a stopped ruler to the nearest mm. Nestlings were weighed with a Pesola spring balance to the nearest 0.25 g.

Experimental nest replacement

The experiment was carried out with 37 nests (20 nests in 2012 and 17 nests in 2013). Of the 37 Nuthatch nests, 19 were built with pine bark flakes and 18 with *Cistus* bark. In 2012, after the first egg, we assigned the nests randomly to two groups to minimize any possible confounding effects such as variation in microclimate or breeding parameters among nest-boxes. In 2013, most pairs of Nuthatches occupied the same or a neighbouring nest-box to those used in 2012. We have therefore assumed that due to Nuthatch intense year-round territoriality (Matthysen 1990; Matthysen 1998), pairs in 2013 were at least partly the same as in 2012, so we assigned the opposite treatment to each territory in 2013 as in 2012. This avoids the problem of confounding treatment with pair or territory identity.

The first group of nests was left unmanipulated (control group, N=23). In the experimental group (N=14), we replaced the natural nest with a fresh and recently built Great Tit nest collected at the same time. Given the possibility that selection of nest materials is affected by the availability and state of nesting materials which may depend on plant phenology and climatic conditions, we chose to exchange Nuthatch nests with nests built by the only cavity nester that constructs nests at the same time as Nuthatches in our study area, namely the Great Tit. We considered a completed Great Tit nest when it presented a defined bowl lined with feathers and/or hair. No eggs had been laid in these nests, so there was no reproductive activity in these nests when collected. Nest replacement was conducted nine days after Nuthatch clutch completion. We did not manipulate ectoparasite loads before nest exchange as both types of nests had been constructed at the same time and therefore under common environmental conditions, and had experienced the same amount of time for being colonized by ectoparasitic arthropods either passively or actively. The only difference between both sets of nests consisted in the brief period of transport between nest-boxes for the experimental great tit nests.

The experimental manipulation was made once the clutch was completed and incubation was advanced in order to avoid possible repercussions on desertion probability. Separating the effects of structure and materials is impossible with real nests given the materials used by cavity nesters in the study area (bark flakes are impossible to structure and alternative materials rapidly attain structure through the birds' own activity).

Ectoparasites

Nuthatch nests in our study area infested by mites *Dermanyssus gallinoides*, blowfly *Protocalliphora azurea* larvae and adult hen fleas *Ceratophyllus gallinae* (Cantarero et al. 2013b). Some authors have shown that fleas have several effects on Great Tit (Allander 1998; Christe et al. 1996; Richner et al. 1993) and Blue Tit reproduction (Tripet et al. 2002; Tripet and Richner 1997), but no experimental studies have been conducted on Nuthatches. Larvae of fleas are not haematophagous, but adult fleas need blood to produce eggs (Tripet and Richner 1997). Therefore, the number of flea larvae in nests indicates the fecundity of adult fleas (Eeva et al. 1994). Fleas may be present in nest materials already during incubation (Harper et al. 1992). Blowfly larvae start to develop after nestlings hatch and feed intermittently of nestling blood (Bennett and Whitworth 1991; Remeš and Krist 2005). In mites, adult and nymphal stages are haematophagous. Populations build up from very few to some thousands of individuals per nest-box during the breeding period as generation time is short. Mites may be present in nest materials even before nestlings hatch and may feed on incubating females (Pacejka et al. 1996).

Ectoparasite abundance estimation

Soon after nestlings fledged (days 20–26 for Nuthatches), all nests were removed in sealed plastic bags and taken to the laboratory, where they were subjected to arthropod removal in Berlese funnels for 48 h until nests were thoroughly dried and no arthropods were moving in the nest material (for arthropods collection and abundance estimations see Moreno et al. 2009). Blowfly puparia were directly counted in the nest material after the removal of living arthropods.

Nest microclimate

One week after clutch completion, we placed on the nest-box floor and under the nest material a climate data logger (Hygrochron iButtons, iButtonLink LLC, Wisconsin, USA) inside 21 nest-boxes with natural nests (9 in 2012 and 12 in 2013) and 10 nest-boxes with experimental nests (all in 2013). Data loggers were programmed to take measurements of temperature and relative humidity every 4 min for 11 days from day 8 of incubation until three days after chicks hatched. We divided each continuous set of temperature (º Celsius) and relative humidity (%) data into segments of 24 h beginning at noon and calculated the mean, maximum and minimum temperature and relative humidity for each segment as well as the range of temperatures. To evaluate thermal changes in the nest-box throughout a random day, we selected a set of 4 nests from each treatment for which we had measurements for the same date with nestlings. For each of these nests we calculated the mean temperature from 9:00 to 21:00 h, thereby excluding the night when females stay in the nest brooding.

To measure microclimate within the nest material (not the cavity as above) in natural and Great Tit nests, we used 3 nests of each type that had been abandoned before any breeding activity had commenced and introduced the data loggers into the middle of the nest material. Temperature and humidity were registered once every 5 min during one complete day while nest-boxes were kept close together outdoors either near the lab (site 1, 19:00-19:00) or in the study area (site 2, 23:00-19:00). We prevented the entrance of any bird to the nest box during this period. Data were collected in different days and were averaged for periods of 4 hours and nest was used sampling unit.

Video recordings

During incubation, all nests were filmed 7 and 9 days after clutch completion (days 7 and 9 of incubation). To reduce the number of disturbances to a minimum, we filmed on the day of the nest exchange. Having waited a few days for filming would have required a new disturbance while placing the camera. However, we did not detect any evidence that birds presented unnatural behaviour while being filmed immediately after the nest exchange. We filmed inside nest-boxes for periods of 90 min (88.63±SE 15.60 min, n=74) with a video camera (Square SONY 1/3* Super HAD CCD) connected to a 3G H.264 CCTV DVR 1 Tb digital recorder installed on the roof inside the nest-box. Both digital recorders and camcorders were powered by batteries (7.2 Ah 12 V).

Nest-boxes were again filmed 3 days after the day of hatching of the young for periods of 90.23±SE10.98 min (n=33) and 9 days after hatching of the young for periods of 94.88±SE10.65 min (n= 31). Nestlings of 9 days are still brooded by females. In one nest no chicks hatched and in three nests all chicks died before day 3, but we have included records taken during incubation. In two nests all chicks died after day 3 but we have included previous records for these nests. Nest desertions were associated with periods of cold and rainy weather in all cases. All films were randomly recorded between 08:00 and 17:00 h, and no differences between experimental groups with respect to time of filming were found (incubation period: *U*=148.0, *P*=0.684; nestling period day 3: *U*=71.0, *P*=0.085; nestling period day 9: *U*=62.0, *P*=0.070). Time of day was used as continuous predictor in all the models and it did not affect the behavioural variables measured (all *P*>0.40). No obvious evidence of stress or unnatural behaviour was observed after the first visit. Males could be distinguished on films due to the colour of their flanks (Matthysen 1990) as they usually climbed on the inside walls of the nest-box while provisioning nestlings.

Behavioural data analysis

Recordings were displayed in the free VLC Media Player software. From films taken during incubation we estimated the proportion of time spent by the female inside the nest-box or “egg attendance” which includes the time allocated to incubating and turning the eggs, and the mean duration of incubation sessions and recesses. Furthermore, we monitored the time devoted to “grooming” and “nest sanitation”. “Grooming” is the combined time which the female spends preening or scratching herself (Cotgreave and Clayton 1994) while “nest sanitation” includes periods of active search with the head buried, sometimes deeply, into the nest material (Cotgreave and Clayton 1994). We obtained the mean duration of these behaviours and the proportion spent in them over time that the female spent inside the box.

From films made on day 3 we obtained hourly provisioning rates by males and females and the amount of time spent by females on “nestling attendance”, “grooming” and “nest sanitation”. “Nestling attendance” includes the proportion of time spent by the female inside the nest-box. “Nest sanitation”and “grooming” were calculated in the same way as during the incubation stage.

From films made on day 9 we obtained total hourly provisioning rates by males and females as we could not always identify the sex of the provisioning adult. We also recorded the begging time of a random nestling, the posture during begging of all nestlings and we estimated the distance between nestlings. Nestling postures were assigned following a scale of increasing intensity (Cantarero et al. 2013a; Leonard et al. 2003): 0 = head down, no gaping; 1 = head down, gaping, sitting on tarsi; 2 = head up, gaping, sitting on tarsi; 3 = same as 2, plus neck stretched upward; and 4 = same as 3, but body lifted off tarsi. The distance between nestlings were estimated following a scale of spatial distribution: 0 = huddling nestlings; 1 = nestlings in contact; 2 = slightly scattered nestlings; and 3 = widely scattered nestlings. On each visit of an adult to the nest with food, we recorded the begging time of a random nestling, the maximum postural begging intensity of each nestling and the distance between nestlings. We then estimated the average value of these variables at each visit for the whole brood.

Data analyses

Natural nests built of pine and of *Cistus* bark were not different in any variable such as laying date, clutch size or microclimate (all $P>0.11$) and have been pooled in the natural nest treatment. Breeding variables and mite abundance were normally distributed or successfully normalized through logarithmic transformation (Kolmogorov-Smirnov, $P>0.20$) and were therefore analyzed with GLM models (STATISTICA package) assuming a normal error with treatment as explanatory factor. Blowfly and adult flea abundances could not be normalized and were analyzed with non-parametric tests (Mann–Whitney U test**)**. Clutch size and brood size were analyzed with GLM models assuming a Poisson distribution with treatment as explanatory factor. Hatching success was normally distributed. Fledging success in nests where at least one nestling fledged was analyzed with GLM.

Microclimatic data were analyzed with GLM models assuming a normal error with treatment as explanatory factor and year as continuous predictors for the incubation stage and year and brood size as continuous predictors for the nestling phase. We selected the 5 days before hatching and the 3 days after hatching to compare treatments as we had microclimatic data for these days for all nests. The two breeding stages have been analyzed separately. The homogeneity of variances for microclimatic data was analyzed with Bartlett's test.

All parametric behavioural variables for the incubation stage were analyzed with repeated measures ANOVA with treatment as explanatory factor and time as repeated-measures factor (before or after nest replacement). All parametric behavioural variables for the nestling phase were analyzed with treatment as explanatory factor, hatching date, time of filming and year as continuous predictors. Grooming variables could not be normalized and were analyzed with non-parametric tests (Mann–Whitney U test**)**.

The effects of treatment on brood-averaged nestling morphometric measurements and mass near fledging (tarsus length, wing length and body mass at day 13) were analyzed with GLM models with treatment as explanatory factor and year, hatching date and brood size as continuous predictors.

RESULTS

Breeding biology

The two treatment groups of nests did not differ with respect to laying date, hatching date, clutch size or brood size (Table 1). In order to assess the effect of our manipulation, we compared the ectoparasite abundances of the two treatment groups of nests. We found no difference in ectoparasite abundances between treatments (Table 1).

	Natural	Experimental	Statistic	p
Breeding data				
Laying date	27.435 ± 6.258(23)	26.286 ± 3.730(14)	$F_1 = 0.389$	0.539
Hatching date	49.478 ± 6.748(23)	47.615 ± 4.053(13)	$F_1 = 0.817$	0.372
Clutch size	6.652 ± 0.775 (23)	6.286 ± 0.611(14)	*Wald* =0.179	0.672
Brood size day 13	5.333 ± 1.560 (21)	5.200 ± 1.033(10)	*Wald* =0.023	0.880
Ectoparasites				
Blowflies	4.762±10.324(21)	0.700±1.636(10)	$U_1 = 74.5$	0.197
Mites	2107.24±3294.80(21	576.300±728.554(10	$F_1 = 2.381$	0.134
Adult fleas	0.762±2.30(21)	1.50±2.718(10)	$U_1 = 89.0$	0.499

Table 1. Differences in breeding variables (April 1=day 1) and ectoparasite abundances (means ± SE, n in parenthesis) between natural Nuthatches nests (Natural) and exchanged Great Tit nests (Experimental) and results of GLM analyses and Mann-Whitney U-tests.

Microclimate

During incubation, nest-boxes with experimental nests had similar temperatures and thermal ranges for the period comprising the 5 days before hatching and the 3 days after hatching (all $P>0.20$). During the nestling phase, cavities with experimental nests experienced higher thermal maximum, averages and ranges than natural nests (Fig. 1A, maximum temperature, $F_{1,30}=2.52$, $F_{1,30}=0.029$; mean temperature, $F_{1,30}=2.31$, $P=0.041$; minimum temperature, $F_{1,30}=1.54$, $P=0.152$; range $F_{1,30}=2.307$, $P=0.02$). During both periods, there were no differences between treatments in variances in temperature (Fig. 1A; all $P>0.07$).

The nests of both treatments showed similar values of mean, maximum, minimum and range in relative humidity (RH) during the incubation (all $P>0.10$) and nestling stages (Fig. 1B, all $P>0.10$). However, during the incubation period, experimental nests showed lower variances in humidity than natural nests (Fig. 1B; relative humidity: $\chi^2 = 9.839$, $P=0.002$; minimum relative humidity: $\chi^2 = 13.356$, $P<0.001$). During the nestling period there were no differences in variances in humidity between treatments (all $P>0.10$). Nest microclimate was not related to year for the incubation stage nor to year or brood size for the nestling phase (all $P>0.20$).

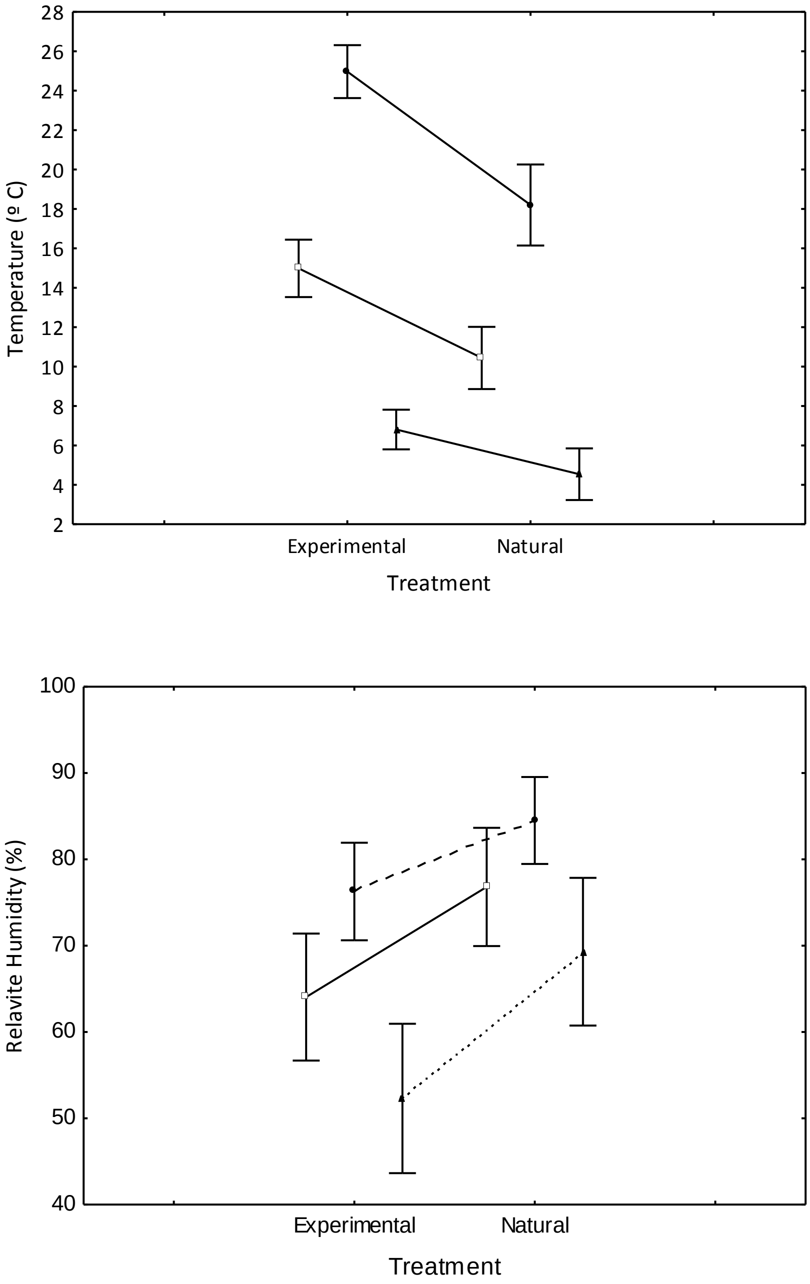

Figure 1. Differences in maximum, mean and minimum temperature (1A) and relative humidity (1B) of nests between the two treatments at nestling phase (±SE) (●Maximum, □ Mean, ▲ Minimum).

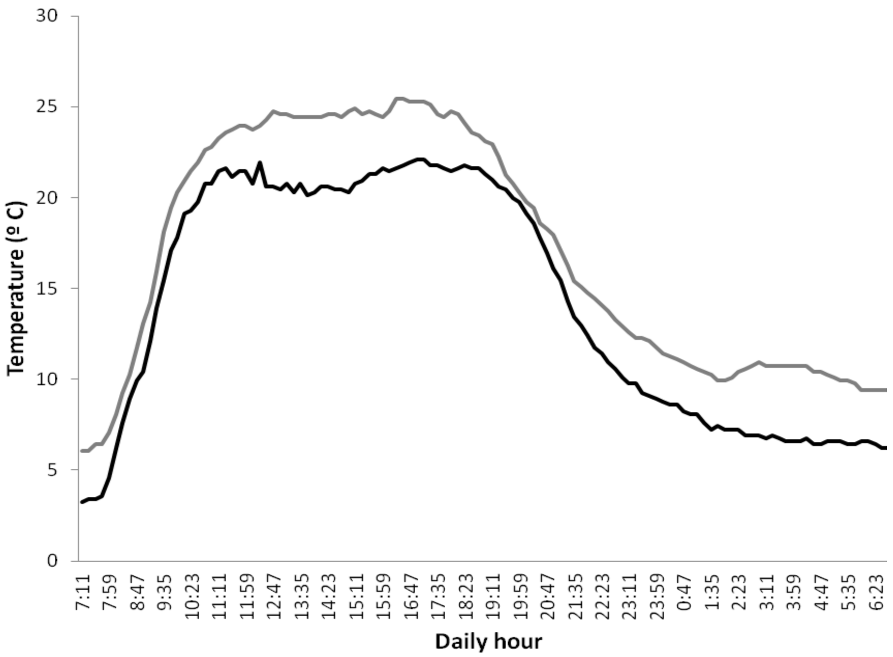

Figure 2. Changes on daily mean temperature inside the nest material in a representative experimental nest (grey line) and in a representative natural nest (black line) during the same day.

When considering the daily thermal fluctuations, cavities with experimental nests showed higher mean temperatures than natural nests throughout the daylight hours of a random day (Fig. 2; 22.9±1.33 ºC versus 20.23±1.07 ºC; $F_{1,7}$=3.11, P=0.021).

Data-loggers inside the material of abandoned nests before any breeding activity registered higher temperatures for natural nests during the night and morning hours at both sites and lower humidity during the morning hours at site 1 (Table 2, Fig. 3). The difference in temperature was of several degrees at site 1 (Table 2, Fig. 3).

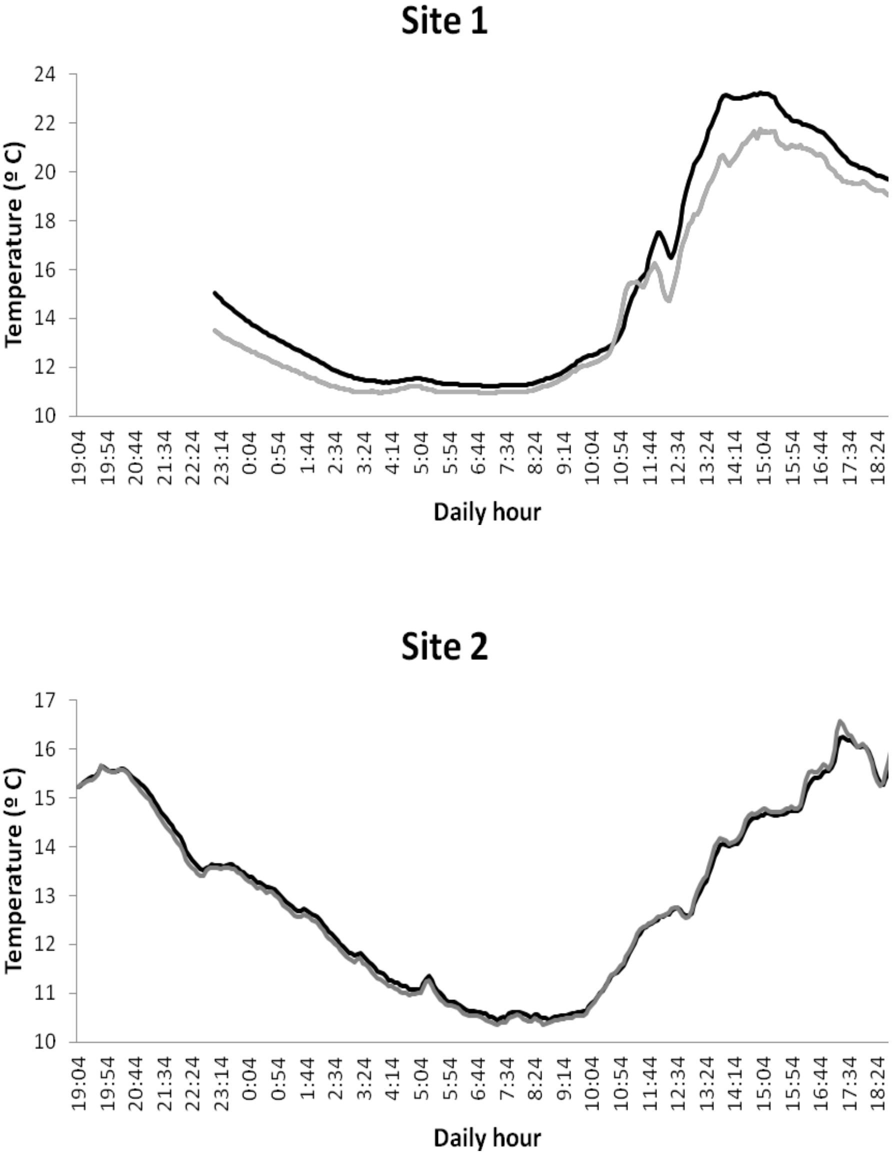

Figure 3. Changes of the temperature inside the nest material between experimental nests (grey line) and natural nests (black line) in nest-boxes near the lab (Site 1, Fig. 3A) and in the study area (Site 2, Fig. 3B).

		Natural	Experimenta	Statistic	p
Temperature					
19:00 – 23:00 h	Site 1	-	-	-	-
	Site 2	14.84±0.03	14.75±0.01	*t*=4.593	**0.010**
23:00 – 3:00 h	Site 1	13.12±0.26	12.13±0.27	*t*=4.534	**0.011**
	Site 2	12.92±0.02	12.82±0.02	*t*=5.182	**0.007**
3:00 – 7:00 h	Site 1	11.43±0.21	11.01±0.22	*t*=2.369	0.077
	Site 2	11.14±0.02	11.03±0.13	*t*=6.764	**0.002**
7:00 – 11:00 h	Site 1	11.94±0.04	11.66±0.14	*t*=3.427	**0.027**
	Site 2	15.40±0.12	15.10±0.08	*t*=3.497	**0.025**
11:00 – 15:00 h	Site 1	20.21±1.62	17.49±0.30	*t*=2.866	**0.045**
	Site 2	14.37±0.14	14.26±0.04	*t*=1.189	0.301
15:00 – 19:00 h	Site 1	20.87±1.36	20.02±0.58	*t*=1.001	0.373
	Site 2	15.10±0.09	15.09±0.02	*t*=0.224	0.834
Relative Humidity					
19:00 – 23:00 h	Site 1	-	-	-	-
	Site 2	49.02±3.01	41.73±11.12	*t*=1.096	0.334
23:00 – 3:00 h	Site 1	47.65±2.47	66.04±3.89	*t*=6.914	**0.002**
	Site 2	49.64±3.03	45.24±7.70	*t*=0.922	0.409
3:00 – 7:00 h	Site 1	55.18±1.95	68.38±2.67	*t*=6.916	**0.002**
	Site 2	51.25±3.23	48.16±5.96	*t*=0.789	0.474
7:00 - 11:00 h	Site 1	61.87±1.57	73.52±1.40	*t*=9.582	**0.001**
	Site 2	46.25±2.10	48.86±2.29	*t*=1.456	0.219
11:00 – 15:00 h	Site 1	61.13±4.14	60.74±11.02	*t*=0.057	0.958
	Site 2	47.77±3.92	50.07±1.61	*t*=0.939	0.401
15:00 – 19:00 h	Site 1	53.08±5.15	49.82±12.17	*t*=0.427	0.691
	Site 2	46.75±3.20	47.06±1.42	*t*=0.149	0.889

Table 2. Differences (means ± SE) in temperature and relative humidity inside nest material in abandoned nests (without presence of incubating females) between 3 nests of each type of treatment in nest-boxes near the lab (site 1) and in the study area (site 2) and results of t-tests (the data from each site were collected in different days).

Parental behaviour and nestling begging

Treatment did not significantly affect incubation attendance or incubation session duration (Table 3). However, the duration of incubation recesses decreased between sequential observations and were significantly shorter in experimental than in natural nests (Table 3). Female grooming behaviour was more intense in the second incubation observation (Table 3), while nest sanitation behaviour was slightly but not significantly more intense in experimental nests (Table 3, P=0.070). We have found no evidence in filmed material that females actively bury their eggs in the nest material when leaving the nest cavity. The eggs remain buried in the material due to its loose structure.

On day 3, nestling attendance (% proportion of time spent by the female inside the nest-box) did not differ between treatments (Table 4). The frequency and mean duration of female grooming and nest sanitation behaviour was not affected by the experiment (Table 4). Provisioning rates of males and females were similar in both treatments (Table 4). On day 9, provisioning rates, begging intensity posture and mean begging time of nestlings were not related to treatment (Table 4) when controlling for year and hatching date. The distance score between nestlings was significantly smaller in experimental nests (Table 4).

Reproductive success

Natural and experimental nests did not differ in hatching success (85.21%±SE20.21 versus 78.84%±SE25.77; $F_{1,31}$=0.109, P>0.70) when controlling for year, laying date and clutch size (all P>0.20). Of 30 unhatched eggs (12.4 % of 241 eggs), 23 contained no visible embryo. Treatment did not significantly affect the number of lost eggs and/ or unhatched eggs ($F_{1,32}$=0.761, P=0.390). Natural nests (0.57±0.51) and experimental nests (0.45±0.52) did not differ in fledging success (χ^2=.39, P=0.239). The low fledging success was due to relatively frequent nest desertions in the two years (2 natural nests, 3 experimental nests) when tending small chicks. In nests where some chicks fledged, fledging success did not differ between control and experimental nests (85.28%±SE4.10 versus 90.48%±SE5.94; $F_{1,29}$=0.518, P>0.40).

Nestlings in experimental nests were significantly smaller with respect to wing length and tarsus length (Table 5), while there were no differences between treatments in body mass (Table 5). Body mass was negatively related to brood size (Table 5, adjusted R^2=0.66).

	Treatment	Before manipulation	After manipulation	Statistic		
				Treatment	**Before/**	**Interaction**
Egg attendance (%)	Natural	75.31±12.41	77.24±8.63	*F*=2.052	*F*=0.746	*F*=0.006
	Experimental	78.46±7.21	80.76±9.87			
Mean session (min)	Natural	26.61±7.50	28.18±13.88	*F*=1.284	*F*=0.023	*F*=1.389
	Experimental	25.26±8.31	25.17±13.52			
Mean recess (min)	Natural	10.78±9.78	9.22±2.55	*F*=8.533**	*F*=9.643**	*F*=0.343
	Experimental	9.02±3.12	5.92±2.55			
Recess (%)	Natural	24.69±12.41	22.76±8.63	*F*=2.052	*F*=0.746	*F*=0.006
	Experimental	21.54±7.21	19.23±9.87			
Grooming (%)	Natural	0.63±0.990	1.20±1.16	*F*=0.185	*F*=4.353*	*F*=0.236
	Experimental	0.61±0.496	0.96±1.94			
Nest Sanitation (%)	Natural	8.25±4.81	6.54±3.95	*F*=3.495	*F*=1.895	*F*=0.036
	Experimental	10.54±6.634	9.25±5.46			

Table 3. Differences (means ± SE) in behavioural variables between treatments before and after manipulation (23 natural Nuthatches nests and 14 exchanged Great Tits experimental nests) and results of repeated-measures ANOVA in the incubation stage including the interaction of treatment with the repeated-measures factor before/after nest exchange (***P*<0.01, * *P*<0.05).

	Natural	**Experimental**	**Treatmen**	**Year**	**Hatching Date**
Nestling day 3					
Nestling attendance (%)	64.172±17.717(23	67.328±13.817(10	*F*=0.350	*F*=10.555**	*F*=0.356
Grooming (%)	0.736±1.163(23)	0.842±1.275(10)	*U*=111.0		
Nest sanitation (NS) (%)	7.794±5.434(23)	8.344±5.256(10)	*F*=0.180	*F*=0.607	*F*=1.337
Male provisioning (h^{-1})	8.840±3.614(23)	7.942±2.799(10)	*F*=1.420	*F*=1.840	*F*=0.007
Female provisioning (h^{-1})	2.542±1.842(23)	2.058±1.688(10)	*F*=0.753	*F*=3.019	*F*=1.195
Nestling day 9					
Adults provisioning (h^{-1})	17.345±7.160(21)	19.148±8.480(10)	*F*=0.011	*F*=5.055*	*F*=6.113*
Begging intensity posture score	1.767±0.509(21)	1.675±0.685(10)	*F*=0.165	*F*=0.016	*F*=0.001
Mean begging time (s)	5.511±2.644(21)	4.437±2.018(10)	*F*=0.889	*F*=0.317	*F*=0.991
Distance score between	0.842±1.085(21)	0.091±0.302(10)	*F*=5.553*	*F*=11.105**	*F*=3.852

Table 4. Differences (means ± SE, n in parenthesis) in behavioural variables between the two treatments (natural Nuthatches nests or exchanged Great Tits experimental nests) and results of GLM analyses and Mann-Whitney U-tests (***P*<0.01, * *P*<0.05).

	Natural	Experimental	Treatmen F	Year F	Hatching Date F	Brood F
Nestling day 13						
Tarsus length	18.81±0.86(21	18.18±1.27(10	5.208*	52.191**	0.001	2.261
Body mass (g)	17.80±1.69(21	17.43±2.41(10	0.645	51.289**	2.144	12.387**
Wing length (mm)	36.83±4.40(21	33.40±5.40(10	4.486*	29.580**	1.516	0.286

Table 5. Differences in brood-averaged nestling morphology and mass (means ± SE, number of broods in parenthesis) and results of GLM models on nestling condition parameters with treatment (natural Nuthatches nests or exchanged Great Tits experimental nests) as explanatory factor and year, hatching date and brood size as continuous predictors (***P*<0.01, * *P*<0.05).

DISCUSSION

We have found some implications of breeding in unstructured nests for Nuthatches. The replacement of unstructured bark nests by structured moss nests did not result in changes in the abundance of blowflies larvae, mites or adult fleas. Nest-boxes containing experimental nests tended to show higher mean and minimum temperatures and larger thermal ranges, as well as lower variances in nest humidity. On the other hand, temperatures inside the nest material were higher in natural nests. Incubation recesses were shorter in experimental nests. Nest replacement did not affect incubation or nestling attendance and there were no effects on parental provisioning rates to the chicks at any stage. Natural nests resulted in structurally larger nestlings shortly before fledging.

Until now, the amount of information about ectoparasite faunas living in Nuthatch nests has been rather limited. Matthyssen (1998) found that Nuthatch nests contained fewer fleas than Great Tit nests in similar nest-boxes and same habitat in Sweden. Cantarero et al. (2013b) in a descriptive study found that Nuthatch nests contained fewer blowflies and fleas than sympatric Blue Tit nests and similar abundances than sympatric Pied Flycatcher nests in similar nest-boxes in Spain. Ectoparasites may be affected by volatile compounds generated by the nest material or through the microclimatic conditions derived from nest properties. The evidence that pine bark in nests may have insecticidal properties is reviewed in Bauchau (1998) and Matthyssen (1998). The bark of these trees contain many compounds with insecticidal properties like limonene that may act as protection against pathogens and herbivores (Pearce 1996). Some studies have found toxic and repellent effect of these natural extracts on northern fowl mites (Carroll 1994) and cat fleas *Ctenocephalides felis* (Hink and Fee 1986). Our results do not confirm predicted trends as we found that Nuthatch nests that were built of bark had no fewer mites or fleas than experimental moss nests. This suggests that the preferences for nest materials in Nuthatches may be unrelated to ectoparasitism. It also explains why there were no differences in grooming or nest sanitation behaviour between treatments as these behaviours have been shown to be related to ectoparasite infestations (Cantarero et al. 2013a; 2013b). Moreno et al (2009) showed that ectoparasite prevalences in Pied Flycatcher nests were independent of nest type (constructed by themselves or Blue Tits) and suggested that interspecific differences in ectoparasite prevalences on hosts are probably related to factors other than nesting material. Remeš & Krist (2005) arrived at similar results for parasitic *Protocalliphora* blowflies in an experimental study with nests of Collared Flycatchers *Ficedula albicollis* and Great Tits.

Birds build nests to provide appropriate protection and microclimate for the development of eggs and chicks (Collias and Collias 1984). Environmental conditions and trade-offs experienced during one stage of development can have important carry-over effects on later life-history stages (Ardia et al. 2010). Nesting material and nest structure may affect cavity microclimate (Hoi et al. 2010; Mertens 1977; Pinowski et al. 2006). Nests with a defined nest bowl like Tit nests may achieve a higher insulation than unstructured nests like those of Nuthatches (Heenan 2013; Heenan and Seymour 2011). Wesolowski et al. (2002) found that the proportion of natural tree nesting holes with wet interior walls was much higher for Nuthatches than for other species. Here we show that nesting material and nest structure affect the variance in humidity in the nest-box although not mean humidity conditions. Moreover, the mean and minimum temperatures were higher in nest-boxes containing experimental moss nests. Reduced fluctuations in humidity and higher mean and minimum temperatures in experimental nest-boxes could reduce incubation and brooding costs to females by reducing heat loss of eggs and chicks to the surrounding air. Microclimatic conditions could thus affect hatching and fledging success. However, we found no difference in these parameters between treatments. Previous arguments are based on eggs and nestlings residing in a nest-cup and thereby being in contact with air within the nest cavity, which is not the case for Nuthatch nestlings in natural nests. Eggs and nestlings in natural Nuthatch nests get buried passively due to the looseness of bark flakes, although we have not observed that females actively bury their eggs in the nest material (Wesolowski and Rowiński 2004).

On the other hand, in our pilot study natural nests retained higher temperatures within the material during the night and morning hours than moss nests. This suggests that bark flakes may contain metabolically active microbes as shown for composting bark flakes (Davis 1992, Ryckeboer et al. 2003), which may contribute to the higher temperatures found within natural nests. Moreover, the lower humidities detected within the nest material at one of the sites may also contribute to reduce heat loss by nestlings. As Nuthatch eggs and nestlings reside within the warmer material itself and are not exposed to cooler surrounding air, they may in fact receive thermal inputs from the nest material itself over and above the thermal savings through not being exposed to air directly. Thus, Nuthatch females would gain a thermal advantage if eggs and nestlings were covered with nesting material during recesses (Hilton et al. 2004). This may explain why incubation recesses were longer at natural nests as slower heat loss rates may allow females to stay away from the nest foraging for longer. This advantage is especially strong as it operates during the morning hours when female energetic needs are higher. The differences detected were moderate to small depending on the site of measurement but covered a large part of the day which includes the coolest hours. Although the results of our pilot study are only suggestive, more detailed studies of the thermal properties of the nest materials used by Nuthatches and of the thermal loss of buried eggs and nestlings are necessary before a conclusion can be reached about the thermal advantages of loose bark flake nests.

Structured nest bowls in experimental nests kept nestlings closer together than at natural nests. The efficacy of huddling in reducing heat loss by nestlings has been shown through reduction in the oxygen consumption of members in a group versus an isolated individual (Glaser and Lustick 1975). However, nestlings in natural nests could have huddled together especially when increasingly mobile at 9 days of age but seemingly preferred to remain apart. The energy savings due to huddling may be compensated by those induced by the heating up of the nest material within which nestlings get buried. Thus, Nuthatch nestlings may show no innate propensity for huddling in the nest.

We found significant effects of nesting material on nestling growth. Tarsi and wing length of nestlings were smaller in experimental nests although we did not find an effect on body mass. These effects could not be explained by either ectoparasite loads, begging intensity or parental provisioning which were not affected by the experiment. The thermal properties of bark flakes and the warming they afford to buried nestlings during female absences may improve their growth.

To conclude, we have experimentally shown clear effects of nesting material type and nest structure on microclimate and breeding behaviour in a cavity-nesting species building nests of loose aggregations of bark flakes. The impaired nestling growth in structured experimental nests could be related to the higher thermal loss for nestlings in open-cup nests compared to being buried into loose and heat-producing bark flakes. Nuthatches appear well adapted to breeding in unstructured bark nests but the physiological basis of their loss of huddling behaviour and the thermal savings afforded by remaining buried into the nest material remain to be further clarified.

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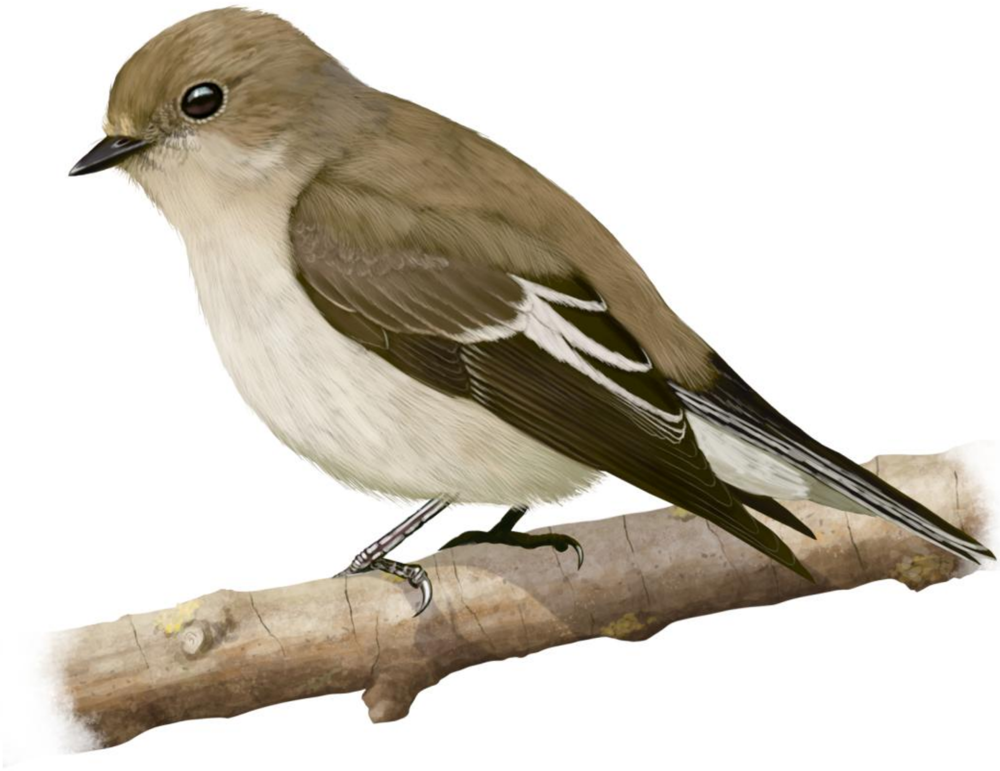
CHAPTER IV



This chapter reproduces entirely the manuscript:

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CHAPTER IV

Behavioural responses to ectoparasites in Pied Flycatchers *Ficedula hypoleuca*: an experimental study

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ABSTRACT. Nests of cavity-nesting birds usually harbor some species of haematophagous ectoparasites that feed on the incubating adults and nestlings. Given the negative impact of ectoparasites on nestlings there will be selection on hosts to reduce parasite infestations through behavioural means. We have experimentally reduced the abundance of all ectoparasites in nests of pied flycatchers *Ficedula hypoleuca* to explore both whether there are changes in the frequency and duration of putative anti-parasite behaviours by tending adults, as well as whether such anti-parasite behaviours are able to compensate for the deleterious effects that parasites may have on nestlings. Heat treatment of nests substantially decreased the density of ectoparasites, and thereby positively affected nestling growth. The frequency and intensity of female grooming and nest sanitation behaviours during the incubation and nestling periods decreased as a consequence of the experimental reduction of ectoparasite infestation. Although nestlings begged more intensely in infested nests, the experiment had no significant effect on parental provisioning effort. Reduction of parasites resulted in larger nestlings shortly before fledging and increased fledging success. This study shows a clear effect of a complete natural nest ectoparasite fauna on parental behaviour at the nest and nestling growth in a cavity-nesting bird. Although ectoparasites induce anti-parasite behaviours in females, these behaviours are not able to fully remove parasite's deleterious effects on nestling growth and survival.

INTRODUCTION

Parasites have been proposed as an important ecological and evolutionary force affecting avian life histories and behaviour (Atkinson and van Riper 1991, Møller 1997). Cavity-nesting birds have been traditionally associated with selective pressures arising from the thermal environment and the impact of nest predation (Hansell 2000). However, the microclimatically stable environment of cavity nests and the presence of an abundant food supply may offer excellent breeding conditions for ectoparasites, and it is thought that many nest ectoparasitic arthropods have evolved specifically in such nesting environments (Waage 1979, Marshall 1981). Thus, ectoparasites in the nest may be an additional important evolutionary factor modulating adaptations of cavity-nesting birds (Heeb et al. 1999, Tripet et al. 2002a).

Ectoparasites cause removal of nutritional and energy resources from hosts that could otherwise be used for growth, maintenance, or reproduction (Møller 1993). They may also induce costly immune and inflammatory responses (Møller et al. 2005, Owen et al. 2009). Conversely the immature immune systems of altricial nestlings result in stronger direct impacts from ectoparasitism faced by nestlings with the need to assign sufficient nutritional resources to growth (Saino et al. 1998, Szep and Møller 1999). Given the negative impact of ectoparasites on host fitness there will be selection on hosts to avoid parasite infestations through behavioural, physiological and immunological responses (Møller and Erritzoe 1996, Hart 1997, Heeb et al. 1998, Cantarero et al. 2013). All these responses are complementary and may be induced in adults, nestlings or both (Hart 1992, Keymer and Read 1991, Simon et al. 2005). Nesting adults may avoid nest sites with high ectoparasite loads (Moore 2002), due to the association between old nest material and higher abundance of certain types of ectoparasites (Mazgajski 2007, Tomás et al. 2007, López-Arrabé et al. 2012) and bacteria (González-Braojos et al. 2012). Adults may also take measures to indirectly minimize the effects of nest parasites through incorporation of fresh plant material containing compounds that either directly affect the development of parasites (Malan et al. 2002, Clark and Mason 1988, Lafuma et al. 2001, Tomás et al. 2012) or stimulate elements of the immune system of chicks that help them to cope better with the harmful activities of ectoparasites (Mennerat et al. 2008). Nevertheless, adult cavity-nesting birds are faced with the presence of nest ectoparasites, and likely have a suite of behaviours directed a minimizing the impacts of parasites (Loye and Zuk 1991, Keymer and Read 1991, Hart 1992, Mazgajski 2009).

The main behavioural defenses against ectoparasites are grooming and nest sanitation (Christe et al. 1996). Grooming behaviour may be operationally defined as manipulation of the plumage with the bill (Nelson et al. 1977, Murray 1990). One of its functions may be to dislodge ectoparasites hiding or residing among feathers (Cotgreave and Clayton 1994, Waite et al. 2012). Thus both adults and nestlings may groom themselves in the presence of ectoparasites (O'Connor et al. 2010). Nest sanitation (Welty 1982) refers to behaviours by parents in altricial species tending to remove ectoparasites on nestlings or nest material (Hurtrez-Boussès et al. 2000), removing from the nest both these as well as eggshells (Montevecchi 1974), fecal material (Blair 1941) or dead nestlings (Skutch 1976). Parents are expected to allocate time to nest sanitation in order to control the load of harmful ectoparasites in the nest materials and on the nestlings. Such anti-parasite behaviours may be time-consuming (Cotgreave and Clayton 1994) and therefore may reduce the time that a parent bird can devote to foraging and provisioning offspring.

Nest ectoparasites are a community of species, and the entire community must be considered when examining the influences of these ectoparasites on host behaviour and fitness. For example the nests of Pied Flycatchers (*Ficedula hypoleuca*) in Iberian populations usually harbour three species of haematophagous ectoparasites, namely mites (*Dermanyssus gallinoides*), blowflies larvae (*Protocalliphora azurea*) and hen fleas (*Ceratophyllys gallinae*) (Merino and Potti 1995, 1996). Nests may also contain haematophagous flying insects such as blackflies and biting midges (Martínez-de la Puente et al. 2009). In mites, adult and nymphal stages are haematophagous, while their short generation times allow the build-up of very large populations with detrimental effects on host reproductive success (Merino and Potti 1995, 1996, Moreno et al. 2009). Mites may be present in nest materials even before nestlings hatch and may feed on incubating females (Pacejka et al. 1996). They have been shown to be the most virulent ectoparasite of Iberian pied flycatcher populations (Merino and Potti 1995, Moreno et al. 2009).

Blowfly larvae live in bird nests and feed intermittently on nestling blood (Bennett and Whitworth 1991, Remeš and Krist 2005). Larvae of fleas are not haematophagous, but adult fleas need blood to produce eggs (Tripet and Richner 1997). Therefore, the number of flea larvae in nests indicates the fecundity of adult fleas (Eeva et al. 1994). Fleas may be present in nest materials already during incubation (Harper et al. 1992).

To explore behavioural anti-parasite strategies it is necessary to conduct field experiments where the levels of infestation are strictly controlled in all treatments (Christe et al. 1996, Heeb et al. 1998, Tripet et al. 2002b, Fitze et al. 2004) or experiments in which nests with a reduced ectoparasite loads are compared with natural controls (Allander 1998, Martínez-de la Puente et al. 2010). Each approach has its advantages and disadvantages. Controlled levels of infestation are useful when dealing with a single parasite and reduce environmentally induced variation. On the other hand, natural controls versus experimental reductions allow manipulations of complete ectoparasite faunas while retaining natural levels of infestation as controls, and are especially useful when the effects of whole ectoparasite faunas with their natural interactions are of interest. Moreover, natural controls reflect the effects of whole ectoparasite faunas on nestlings in the wild.

Our goal is to examine the impacts of an entire nest ectoparasite community on reproductive behaviour of their hosts. In our study of Pied Flycatchers in central Spain, we have reduced the abundance of all ectoparasites by a heat treatment of nestboxes. We have assumed that experimental reduction would have a negative impact on ectoparasite abundance and a positive impact on nestling growth and survival. We have then compared control and experimental host behaviour within the nest-box using data from video films (see Hurtrez-Boussès et al. 2000 for a similar approach). Video-recordings inside the nest-box were made during the incubation and at two stages of the nestling period (nestlings of 3 and 9 days of age). Our objectives were to explore changes in the frequency and duration of parental grooming and nest sanitation behaviours as a consequence of the abundance of ectoparasites, and to examine the impacts of these behaviours of adult birds. We have hypothesized that:

(1) Behavioural responses to ectoparasites should be more frequent in control nests than in experimental nests. This pattern should occur during both the incubation and nestling periods;

(2) There should be a trade-off between brooding nestlings and nest sanitation behaviours at the early nestling stage;

(3) Nestlings should beg more intensely in control nests due to the increased food demand induced by ectoparasites;

(4) Parents should respond to higher begging levels in control nests by increasing provisioning rates only if time consumed by anti-parasite behaviours does not compromise that available for foraging.

MATERIAL AND METHODS

General field methods

The study was conducted during the spring of 2012 in a montane forest of Pyrenean oak, *Quercus pyrenaica*, at 1200 m.a.s.l. in Valsaín, central Spain (40° 54' N, 4° 01' W) where pied flycatchers breeding in nest-boxes have been studied since 1991 (see Sanz et al. 2003 for general description). Of 552 nest-boxes, 91 were occupied by pied flycatchers and the rest by other species, mainly great tits, nuthatches and blue tits (see Lambrechts et al. 2010 for dimensions, structure and placement of nest-boxes).

Egg laying in the Pied Flycatcher population under study typically begins in late May, and modal clutch size is six. The female incubates and broods alone and receives part of her food from her mate (Moreno et al. 2011). No brooding is observed after nestlings attain 7 days of age (Sanz and Moreno 1995). Breeding activities are followed routinely every year and laying and hatching dates and brood sizes at hatching and fledging are determined.

On day 3 (hatching day = day 1), we weighed all nestlings in each brood together with a digital scale to the nearest 0.1 g to give an average nestling mass when divided by brood size. On day 13 (hatching day = day 1), we ringed nestlings and measured their tarsus lengths with a digital callipers to the nearest 0.01 mm and their wing lengths with a stopped ruler to the nearest mm. Nestlings were also weighed with a Pesola spring balance to the nearest 0.25 g. Nestlings from one nest flew before being measured on day 13.

Experimental reduction of ectoparasites protocol

Of the 91 nest boxes occupied by pied flycatchers we selected those whose laying date was between dates 45 and 51 (April 1=day 1). We assigned 56 nests randomly to two groups, to minimize any possible confounding effects such as variation in microclimate among nest-boxes. The first group was left unmanipulated (control group, N=37). In the second experimental group (N=19), we reduced the number of ectoparasites by a heat-treatment for 30 s at 750 W using a microwave oven. For the time that the original nests were treated (around 30 minutes), a fresh substitute nest was introduced into the nest-box (these nests had been collected in previous seasons after being abandoned prior to laying and kept frozen at -20 ºC until use). This treatment ensured that experimental nests did not contain live arthropods when placed in the nest-box (Rendell and Verbeek 1996), although some arthropods may colonize the nest material after the treatment. To avoid the loss of water during the heat-treatment, the nests were placed into a hermetic plastic container. To prevent recurrence of ectoparasite colonization a total of three heat-treatments were made in the experimental group: (1) 7 days after clutch completion, (2) when nestlings were 2 days old (hatching day=day 1) and (3) when nestlings were 8 days old. Furthermore, before returning the nest the flame from a butane jet torch lighter (Microtorch GT-3000) was passed across the walls of the nest-box to kill ectoparasites that might remain there. Nests in the control group were visited on the same days and handled in a similar way to experimental ones.

Ectoparasite abundance estimation

One or two days after nestlings fledged (17 days after hatching), all nests were removed in sealed plastic bags and taken to the laboratory, where they were subjected to arthropod removal in Berlese funnels for 48 h until nests were thoroughly dried and no arthropods were moving in the nest material. Ectoparasite identification was made with the aid of a stereoscopic microscope (Olympus SZX7). We assume that all mites are hematophagous given their red color as a consequence of the ingested blood (for arthropods collection and abundance estimations see Moreno et al. 2009).

Video recordings

Seven days after clutch completion (day 7 of incubation), we recorded nest activity inside nest-boxes for about 90 min (91.45±SE 24.63 min, n=58) with a cold white light (LED 5 mm) powered by a 3 V battery and a camera (GoPro HD Hero1) mounted on the roof inside the nest-box. Video recordings were made one day after experimental treatment and nest handling. Nest-boxes were again recorded two days after the day of hatching of the young (88.63±13.01 min, n=57) and 8 days after hatching of the young (85.04±20.01 min, n= 55). In two nests all chicks died after day 3 but we have included records taken during incubation and day 3. All films were recorded between 08:00-17:00 h, and no differences between experimental groups with respect to time of filming were found (incubation period: *U*=297.0, *P*=0.346; nestling period day 3: *U*=314.5, *P*=0.522; nestling period day 9: *U*=272.5, *P*=0.277). We excluded the time until the first nest visit by parents (14.31±11.55 min, n=164). No evidence of stress or unnatural behaviour like extremely long absence periods from the nest or trying to peck at the camera system were observed after the first visit.

Because of technical problems, we failed to record the behaviour at two different nests in the control group, one from young nestlings and another from older nestlings.

Behavioural data analysis

From recordings taken during incubation we estimated the proportion of time spent by the female inside the nest-box or “egg attendance” which includes the time allocated to incubating and turning the eggs, and the mean duration of incubation sessions and recesses. Furthermore, we monitored two specific types of female behaviour: “grooming” and “nest sanitation”. “Grooming” is the combined time in which female spends preening or scratching herself (Cotgreave and Clayton 1994) while “nest sanitation” is a period of active search with the head buried, sometimes deeply, into the nest material (Cotgreave and Clayton 1994). In our study, we define nest sanitation as burying the bill in the nest material or carrying out nest materials. As scratching by females resting on the nest could not be observed accurately, grooming refers mostly to preening with the bill. We assume that these behaviours in our study population have the functions implied by the terms derived from the literature, although our experiment intends precisely to confirm these functional interpretations. We obtained the proportion and the mean duration of these behaviours over the time that the female was inside the box. In addition, we also counted the number of incubation feedings by males.

From recordings during the early nestling phase we obtained hourly provisioning rates by males and females and the amount of time spent by females in “nest attendance”, “brooding”, “grooming” or “nest sanitation”. “Nestlings attendance” includes the proportion of time spent by the female inside the nest-box. “Brooding” activity is defined as the proportion of time spent by the female inside the nest-box covering young nestlings in relation to the total time spent inside the nest-box. “Nest sanitation” and “grooming” were calculated in the same way as during the incubation stage. Pied flycatcher females do not exhibit “sleeping” behaviours during the incubation or nestling periods like in other species (Tripet et al. 2002b).

From recordings during the late nestling phase we obtained hourly provisioning rates by males and females and nest sanitation behaviour. Nest sanitation at this stage only considers removal of nest material from nest-boxes as the chicks do not need brooding and female visits to the nest-box are just for feeding. We also recorded the posture during begging of nestlings. Nestling postures were assigned based on a modification of the scale used by Leonard et al. (2003) following a scale of increasing intensity: 0 = head down, no gaping; 1 = head down, gaping, sitting on tarsi; 2 = head up, gaping, sitting on tarsi; 3 = same as 2, plus neck stretched upward; and 4 = same as 3, but body lifted off tarsi. On each visit of an adult to the nest with food, we scored the maximum postural begging intensity of each nestling. We then estimated the average value of the maximum begging intensity at each visit for the whole brood.

Statistical analyses

Many response variables were normally distributed or successfully normalized through logarithmic transformation (Kolmogorov-Smirnov, *P*>0.20) and were therefore analyzed with GLM models (STATISTICA package) assuming a normal error with treatment as explanatory factor. Hatching success (proportion eggs that hatched) was not normally distributed even when transformed but its residuals were, so it was analyzed with a GLM with treatment as explanatory factor and laying date and clutch size as continuous predictors. Clutch size and brood size were analyzed with GLM models assuming a Poisson distribution with treatment as explanatory factor. Grooming and nest sanitation variables could not be normalized and were analyzed with non-parametric tests (Mann–Whitney U test). Nest sanitation (nestling day 9) and fledging success (proportion hatched chicks that fledged) were analyzed as frequencies (Yes-1/No-0 observation of sanitation in the nest and Yes-1/No-0 cases of all chicks hatched becoming fledglings) with Chi-squared contingency tables.

All parametric behavioural variables were analyzed with treatment as explanatory factor and hatching date, brood size, date and time of filming as continuous predictors. Nonsignificant predictors were sequentially removed until only significant effects remained in the final model. Only the effects of treatment are presented in all cases, even when non-significant.

RESULTS

The two treatments did not differ with respect to laying date, hatching date, clutch size or brood size (Table 1). In order to assess the efficiency of our manipulation, we compared the ectoparasite abundances of the two treatments. The experiment was successful because the experimental nests differed from controls in the abundances of ectoparasites sampled in the predicted direction (Table 1).

	Control	**Experimental**	**Statistic**	**p**
Breeding data				
Laying date	48.162 ± 1.642(37)	47.386 ± 1.012(19)	$F_1 = 3.70$	0.060
Hatching date	66.351 ± 1.230(37)	66.947 ± 1.311(19)	$F_1 = 1.30$	0.260
Clutch size	5.622 ± 0.594(37)	5.84 ± 0.501(19)	*Wald* =0.107	0.743
Brood size 13	4.722 ± 1.446(36)	5.263 ± 0.733(19)	*Wald* =0.741	0.389
Ectoparasites				
Blowflies	6.162±8.748(37)	0.684±1.887(19)	$U_1 = 172.0$	<0.005
Mites	3347.57±4543.55(37	274.053±906.913(19)	$F_1 = 17.76$	<0.001
Fleas	24.946±88.329(37)	0.000±0.000(19)	$U_1 = 247.0$	<0.01

Table 1. Differences in breeding variables and ectoparasite abundances (means ± SE, n in parenthesis) and results of GLM analyses and Mann-Whitney U-tests.

The control (3.36±0.49) and the experimental group (3.76±0.61) differed in the mean nestling mass (g) on day 3 (F=6.87, P=0.011). We then tested for the effects of treatment on brood-averaged nestling morphometric measurements and mass near fledging (tarsus length, wing length and body mass at day 13), controlling for hatching date and brood size. Nestlings in experimental nests were significantly larger with respect to wing length and tarsus length (Table 2), while there were no differences between treatments in body mass (Table 2). Tarsus length was negatively related to hatching date (adjusted R^2=0.25). The control (90.09±14.13) and the experimental (90.30±11.61) groups did not differ in hatching success (F=0.003, P>0.90) when controlling for laying date and clutch size (both P>0.20), while fledging success was marginally lower (χ^2=2.82, P=0.093) in control nests (0.89±0.32) than in experimental nests (1.00±0.00).

	Control	**Experimental**	**Treatment**	**Hatching Date**	**Brood size**
Nestling day 13					
Tarsus length (mm)	17.42±0.47(34)	17.79±0.38(19)	F=6.615*	F=9.027**	F=0.133
Body mass (g)	13.98±0.94(34)	14.17±0.92(19)	F=0.510	F=0.231	F=0.478
Wing length (mm)	46.85±2.40(34)	48.57±2.32(19)	F=7.126*	F=1.050	F=0.072

Table 2. Differences in brood-averaged nestling morphology and mass (means ± SE, number of broods in parenthesis) and results of GLM models on nestling condition parameters with treatment as explanatory factor and hatching date and brood size as continuous predictors (**P<0.01, * P<0.05).

Treatment did not significantly affect incubation attendance (% incubation time) or the mean of recess and incubation session durations of females (Table 3). Female grooming behaviour was less frequent and the mean duration of grooming sessions were significantly shorter in the experimental group than in the control group (Table 3). Nest sanitation behaviour time was also more intensive in the control group (Table 3).

	Control	**Experimental**	**Statistic**	**P**
Incubation				
Grooming (%)	0.400±0.586(37)	0.122±0.240(19)	U= 182	**0.012**
Mean grooming (s)	3.00±3.00(37)	2.00±3.00(19)	U=185	**0.015**
Egg attendance (%)	63.844±11.74(37	60.750±12.645(19	F_1= 0.81	0.372
Mean session (min)	10.516±4.433(37	9.317±6. 167(19)	F_1 = 0.68	0.412
Mean recess (min)	5.950±2.000(37)	4.217±2.217(19)	F_1 = 1.55	0.218
Nest sanitation (%)	1.905±1.596(37)	1.041±1.289(19)	F_1= 5.50	**0.029**
Nestling day 3				
Nestling attendance (%)	53.274±14.59(36	48.358±13.106(19	F_1= 1.34	0.254
Brooding (%)	93.045±3.127(36	95.997±3.981(19)	F_1= 7.60	**0.008**
Mean grooming duration	2.00±2.00(36)	1.00±2.00(19)	U=172	**0.048**
Grooming (%)	0.108±0.143(36)	0.047±0.121(19)	U = 168	**0.039**
Nest sanitation (NS) (%)	5.367±2.562(36)	2.879±3.659(19)	F_1 = 7.80	**0.007**
Mean NS duration (s)	5.00±3.00(36)	3.00±2.00(19)	U = 121	**<0.005**
Male provisioning (h^{-1})	9.298±5.102(36)	11.108±4.566(19)	F_1 = 1.62	0.209
Female provisioning (h^{-1})	5.950±3.076(36)	5.791±2.557(19)	F_1 = 0.03	0.853
Nestling day 9				
Nest sanitation (yes/no)	0.176±0.387(34)	0.000±0.000(19)	χ^2=3.78	0.052
Male provisioning (h^{-1})	11.934±5.685(34	11.290±5.268(19)	F_1 = 0.16	0.688
Female provisioning (h^{-1})	11.384±5.541(34	10.345±5.074(19)	F_1 = 0.45	0.503
Begging intensity score	1.149±0.636(34)	0.741±0.376(19)	F_1 = 6.16	**0.016**

Table 3. Differences (means ± SE, n in parenthesis) in behavioural variables between the two treatments and results of GLM analyses (significant p-values in bold), Mann-Whitney U-tests and Chi-squared contingency tables (sanitation present or absent).

In relation to the second hypothesis, the proportion of brooding time on day 3 was significantly higher in the experimental group than in the control one as predicted (Table 3). Grooming variables showed the same pattern between treatments as during incubation (Table 3). Nest sanitation time was again higher in the control group (Table 3). There was no experimental effect on provisioning rates of males and females (all $P>0.2$). Control nests where nest sanitation behaviours occurred showed higher mite infestations than control nests where these behaviours did not occur (Fig. 1). This relationship was not found for blowfly larvae or fleas. There were marginally more nest sanitation events in control nests than in experimental nests (Table 3).

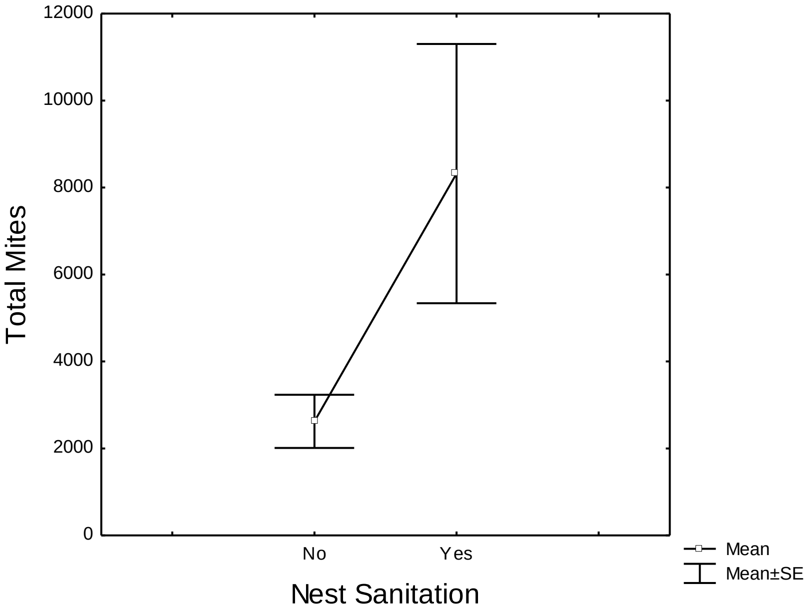

Figure 1. Total mites (±SE) in relation to the presence (Yes) or absence (No) of nest sanitation behaviour in the control group ($P=0.0045$) in the late nestling phase.

In accordance with our third hypothesis, begging intensity of nestlings was higher in control nests (Table 3) and was positively associated with parental provisioning rates (Fig. 2).

Contrary to our fourth hypothesis and despite the association with begging intensity male and female provisioning rates on day 9 were not related to treatment (Table 3).

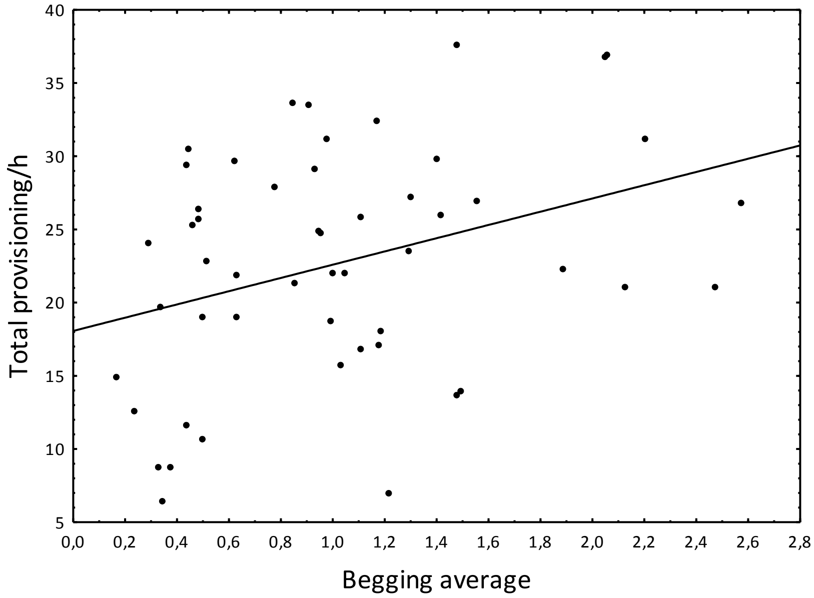

Figure 2. Association between hourly provisioning rates (male and female) and begging average intensity in the late nestling phase (Spearman correlation: r=0.48, *P*<0.005).

DISCUSSION

This study shows that the behaviour of pied flycatcher females inside the nest-box was clearly affected by ectoparasite abundance. The heat treatment of the nests decreased strongly the nest density of blowflies, mites and fleas, and thereby positively affected nestling growth. Experimental nests resulted in larger nestlings shortly before fledging. The frequency and intensity of female grooming and nest sanitation behaviours during the incubation and nestling periods decreased as a consequence of the experimental reduction of ectoparasite infestation. The experimental treatment did not affect incubation attendance and there were no effects on male or female provisioning rates to the chicks at any stage.

There is mixed evidence concerning the impact of ectoparasites on reproductive success in altricial cavity-nesting birds. While some experimental studies have found strong deleterious effects on nestling growth and survival (Heeb et al. 1998, Richner et al. 1993), others have only found weak effects or none at all (Tripet et al. 2002b, O'Brien and Dawson 2008, Bouslama et al. 2002). These differences among host species and populations may depend on the absolute levels of infestation found in different regions or habitats (Hurtrez-Boussès et al. 1997, Eeva et al. 1994, Fitze et al. 2004). Strong effects are thus mostly found in areas where climate is favourable for arthropod survival and dispersal during and between avian breeding seasons (Merino and Potti 1996, Dufva and Allander 1996). Reproductive success in Iberian pied flycatcher populations has been previously shown to suffer the impacts of nest-dwelling ectoparasites (Merino and Potti 1995, 1996, Merino et al. 1998, Merino and Potti 1998, Moreno et al. 2009).

In agreement with several previous experimental studies we found marked effects of ectoparasites on nestling growth (Heeb et al. 2000, Tomás et al. 2008, Brommer et al. 2011). Tarsus and wing length of nestlings were negatively affected by ectoparasite abundance although we did not find an effect on body mass. Tarsus length of pied flycatcher nestlings has been related to their recruitment probability from fledging until breeding (Alatalo and Lundberg 1986), so the effects of ectoparasites may affect the future fitness of nestlings. For the observation that we found no effect of treatment on nestling body mass, there are two non-mutually exclusive alternative interpretations of our results. On the one hand, nestling growth improves under favourable conditions for breeding (Sanz 1995). Conditions during the year of study (2012) must have been especially favourable as nestlings attained their largest masses since the inception of the study (1991), which may explain why we found no effect of treatment on body mass. Additionally, control nestlings could reduce ectoparasite effects on body mass by increasing self preening or standing on top of one another (O'Connor et al. 2010).

Control nestlings showed as expected an increase in begging intensity, which is positively associated with parental provisioning rates, as was found in the Great Tit *Parus major* (Christe et al. 1996). Older nestlings suffering from higher ectoparasite loads begged more intensely as a response to their higher nutritional needs. Parental provisioning frequency depends on begging intensity as found in other studies on begging intensity (Kedar et al. 2000, Kölliker et al. 2000, Wright et al. 2002). Like Fitze et al. (2004) we noticed no effect of ectoparasite reduction on parental provisioning rates at any nestling age despite the strong correlation between begging intensity and provisioning rates. However, we found no effects of the experiment on parental provisioning frequency which could explain the lack of difference in the body mass of nestlings between treatments and it could have been caused by factors for which we did not control such as prey quality, ectoparasite virulence, nestling resistance or environmental constraints (e.g. Møller 1994, Lehmann 1993). Roger et al. (1991) also showed no effect in parental provisioning frequencies in response to ectoparasites (see also Tripet et al. 2002b). There is also evidence that parental effort in pied flycatchers is energetically tightly constrained thereby precluding responses to variation in brood demand (Moreno et al. 1997, Moreno et al. 1999). This lack of parental response may explain why their food provisioning was incapable of compensating for ectoparasite effects leading to smaller size at fledging in control nests. It is also possible that increased dedication to nest sanitation in control nests contributed to reduce the capacity of parents to augment their provisioning rates sufficiently to be detectable. That fledging success was marginally higher in experimental nests supports the existence of ectoparasite effects on nestling survival (Lundberg and Alatalo 1992, Moreno et al. 1999) although other fitness costs such as the probability of recruitment could be expressed after fledging (Thomas et al. 2007).

It is assumed that as a consequence of the negative impact of ectoparasites on nestlings, hosts have evolved behavioural responses (Cantarero et al. 2013). Ectoparasites present during incubation in pied flycatcher nests are mites and fleas. Females groom themselves more in control nests which may imply a direct response to the attachment of these ectoparasites on their skin and plumage. Nevertheless, grooming activity may not occupy sufficient time to constrain incubation attendance in females (Hurtrez-Boussès et al. 2000). However, our results on grooming behaviour indicate that tending females may suffer some costs induced by nest ectoparasites through attachment and possibly blood-sucking even before nestlings hatch (see also Tomás et al. 2008). When the nestlings hatch, the blowflies lay their eggs on their skin, and emerging larvae then begin feeding on nestling blood. The blood-sucking larvae of blowflies feed intermittently on the blood of nestling birds (Rognes 1991), although they may try to attach also to brooding females (Bennett and Whitworth 1991) given that their belly skin is naked at this stage. The combined effect of nest ectoparasites induced a lower body mass of chicks in control nests compared with treated nests already two days after hatching. This indicates that ectoparasites impair the growth of nestlings from hatching, a cost for which parents are apparently not able to compensate. If variation in parasite abundance is obvious to attending parents, we should expect that females in the control group compared to those in the treated group should allocate more time to anti-parasite behaviours and restrict the time spent on brooding chicks, sleeping (Tripet et al. 2002b) or foraging and provisioning nestlings (Christe et al. 1996). We found that control females reduced their proportion of time spent in the nest-box brooding compared to experimental females, but not with respect to total nestling attendance. The fact that females from control nests increased anti-parasite behaviours but maintained similar brooding attendances and provisioning rates as at experimental nests suggests that the time costs of these behaviours are not sufficiently important to reduce time available for foraging at this early stage of nestling development.

The function of nest sanitation behaviour by introducing the bill in the nest material has been debated (Haftorn 1994). One possibility is that birds actually destroy and even consume ectoparasitic arthropods (Rothschild and Clay 1952). This behaviour may also occur in pied flycatchers because we observed females swallowing some collected items on video-recordings of control nests. Nest sanitation could also be used to chase blowfly larvae or adult fleas away from their own body or that of their nestlings, thereby preventing them from biting the incubating female or the nestlings. We also observed one female attacking an adult blowfly that entered the nest-box while she was brooding which could prevent oviposition in the nest. The difference in the time invested in behavioural defences indicates that females may be able to choose to increase the amount of time allocated to control of nest ectoparasites.

Changes in the frequency and duration of grooming or nest sanitation may be interpreted as responses to ectoparasites. Our results are consistent with several previous studies in Great Tits (Richner et al. 1993) and Blue Tits (Christe et al. 1996, Tripet et al. 2002b, Hurtrez-Boussès et al. 2000) that showed that females spent more time on nest sanitation when the nest was infested with fleas. The number and duration of grooming sessions also increased in control nests. This suggests that this behaviour may have evolved in response to ectoparasites and that females could thereby minimize the fitness costs associated with ectoparasite infestations (Richner et al. 1993).

This is the first study showing a clear effect of a complete natural ectoparasite fauna on parental behaviour and nestling growth in a cavity-nesting bird. Our test of effects of ectoparasites is conservative as we were not able to completely remove all ectoparasites and as the study was performed under especially good conditions for breeding. Ectoparasites induce significant changes in female grooming and nest sanitation behaviours which are not able to fully remove their natural deleterious effects on nestling growth and survival.

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CHAPTER V

This chapter reproduces entirely the manuscript:

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CHAPTER V

Factors affecting the presence and abundance of generalist ectoparasites in three sympatric hole-nesting species

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ABSTRACT. Nesting cavities constitute micro-environments very likely to be colonized by ectoparasites which feed on blood of the incubating female and the nestlings. Given the negative impact of ectoparasites on nestlings there will be selection on hosts to minimize ectoparasite loads through behavioural defenses. We have addressed the implications of ectoparasitism in three sympatric avian cavity-nesters, namely Pied Flycatchers *Ficedula hypoleuca*, Blue Tits *Cyanistes caeruleus* and Nuthatches *Sitta europaea*, to explore if differences in prevalence and abundance of generalist ectoparasites (blowflies, fleas and mites) can be related to interspecific differences in their nest size, nest composition and cavity microclimate. Furthermore, we have aimed at detecting if interspecific variation in the incidence and intensity of anti-parasite behaviours is a consequence of the abundance of ectoparasites. Differences in nest composition among host species appear not to be the main factor explaining ectoparasite loads, while nest size, breeding phenology, brood size and nest-cavity micro-climate may affect them in different ways for each host-parasite association. Behavioural defenses against parasites are exhibited by all host species but are more intense in the host species with the highest infestation levels (Blue Tits). This study shows different sources of variation in associations between three sympatric avian cavity-nesters and their generalist ectoparasites.

INTRODUCTION

Hole-nesting has been usually associated with selective pressures arising from the thermal environment and the impact of nest predation (Hansell 2000). Nesting cavities offer conditions of relatively constant temperature and humidity as well as protection from rain, solar radiation and predators. Nesting cavities constitute micro-environments very likely to be colonized by bacteria, decomposers and detritivores due to the presence of faeces and food remains of breeding birds, and by ectoparasites that feed on blood, skin and feathers of avian hosts (Collias and Collias 1984, Mazgajski 2007b). Nest ectoparasites feeding on the blood of nestlings and adults constitute an important selective force affecting avian life history evolution as they remove nutritional and energy resources from hosts that could otherwise be used for growth, maintenance, or reproduction (Møller 1993, 1997). They may also induce costly immune, inflammatory responses (Møller et al. 2005, Owen et al. 2009) and physiological stress (Martínez-de la Puente et al. 2011). Accordingly, ectoparasite presence and abundance in nesting cavities may have constituted an additional important evolutionary factor modulating adaptations of hole-nesting birds (Heeb et al. 2000, Tripet et al. 2002a).

There is mixed evidence concerning the impact of ectoparasites on reproductive success in altricial cavity-nesting birds. While some experimental studies have found deleterious effects on nestling growth and survival (Richner et al. 1993, Heeb et al. 1998, Tomás et al. 2008), other have only found weak or absent effects (Bouslama et al. 2002, Tripet et al. 2002a, O'Brien and Dawson 2008). These differences among host species and populations may depend on the absolute levels of infestation found in different regions or habitats (Eeva et al. 1994, Hurtrez-Boussès et al. 1997, Fitze et al. 2004). Strong effects are thus mostly found in areas where climate is favourable for arthropod survival and dispersal during and between avian breeding seasons (Dufva and Allander 1996, Merino and Potti 1996).

Given the negative impact of ectoparasites on host fitness, there will be selection on hosts to minimize ectoparasite loads through behavioural, physiological and immunological responses (Møller and Erritzoe 1996, Hart 1997, Heeb et al. 1998). One option is the avoidance of nest sites with high ectoparasite loads in order to avoid, or at least reduce, negative effects of parasitism on the survival and condition of offspring (Moore 2002). Certain studies have revealed associations of old nest material with an increased abundance of certain types of ectoparasites (Mazgajski 2007a, Tomás et al. 2007a, López-Arrabé et al. 2012) and bacteria (González-Braojos et al. 2012). Some avian species incorporate fresh plant material in order to control nest-dwelling ectoparasites, because their volatile anti-parasitic compounds can delay the development of mites (Clark and Mason 1988, Malan et al. 2002, Tomás et al. 2012) or stimulate elements of the immune system of chicks that help them to cope better with the harmful activities of ectoparasites (Gwinner et al. 2000). It has been suggested that some species like Nuthatches *Sitta* spp. prefer pine bark as nest material because it contains toxic secondary compounds that may have insecticidal properties (Carroll 1994), in particular the monoterpene limonene. Limonene (and other plant compounds) repel northern fowl mites (*O. sylviarum*), an ectoparasitic mite (Carroll 1994). Bauchau (1998) proposed that Pied Flycatchers *Ficedula hypoleuca* use limonene-rich material from pine trees in order to reduce the abundance of parasites in nests.

Avian hosts may try to compensate for the deleterious effects of ectoparasitism through behavioural modifications (Loye and Zuk 1991, Keymer and Read 1991, Hart 1992, Simon et al. 2005). Hosts can increase their provisioning rates to the offspring (Tripet and Richner 1997b) which may affect their current and future reproduction (Richner and Tripet 1999). Given these fitness costs they can evolve behavioural responses to minimize ectoparasite loads (Christe et al. 1996, Tripet et al. 2002a, Waite et al. 2012). The main behavioural defenses against ectoparasites are grooming and nest sanitation (Christe et al. 1996). Grooming behaviour may be operationally defined as manipulation of the plumage with the bill (Nelson et al. 1977, Murray 1990). One of its functions may be to dislodge ectoparasites hiding or residing among feathers (Cotgreave and Clayton 1994, Waite et al. 2012). Thus both adults and nestlings may groom themselves in the presence of ectoparasites (O'Connor et al. 2010). In addition to combating ectoparasites on their bodies, birds must defend themselves from parasites in their nests (Clayton et al. 2010). Nest sanitation (Welty 1982) refers to parental behaviours tending to remove ectoparasites on nestlings or inside the nest materials (Hurtrez-Boussès et al. 2000), nest cavity eggshells (Montevecchi 1974), faecal material (Blair 1941) or dead nestlings (Skutch 1976). Parents are expected to allocate time to nest sanitation in order to control the load of harmful ectoparasites in the nest material and nestlings (Cantarero et al., submitted). If such anti-parasite behaviours are time-consuming (Cotgreave and Clayton 1994), they may reduce the time that a parent bird can devote to foraging and to provisioning offspring. Behavioural adaptations to control and reduce ectoparasite impacts may mainly be detected in host populations where ectoparasites have important effects on reproductive success. That nest sanitation may be important is suggested by the fact that the condition and health of breeding females can determine the rates of ectoparasite infestation (Tomás et al. 2005, Tomás et al. 2007b, López-Arrabé et al. 2012).

For hole-nesting passerines, fleas, blowflies and mites constitute the most important groups of nest-dwelling ectoparasites (e.g., Merino and Potti 1995, Rendell and Verbeek 1996, Allander 1998, Merino and Potti 1998). Species of these arthropod taxa are usually not host-specific (Tripet and Richner 1997a, Mazgajski 2007b, Moreno et al. 2009, López-Arrabé et al. 2012). Their relative abundance differs according to host species even in conditions of strict sympatry (Bennett and Whitworth 1991, Bauchau 1998, Moreno et al. 2009). Nuthatches *Sitta europaea*, Pied Flycatchers and Tits *Paridae* coexist frequently in European deciduous woodlands and present different prevalences and intensities of infestation by the different ectoparasite taxa (Bauchau 1998, Moreno et al. 2009). Matthyssen (1998) found that Nuthatch nests in Sweden contained fewer fleas than Great Tit *Parus major* nests in similar nest boxes and habitats, and that more fleas were found in nests built of leaves instead of pine bark. Bauchau (1998) found that Great Tit nests showed higher abundances of mites, fleas and blowflies than Pied Flycatcher nests in the Netherlands. One of the factors suggested to explain differences in ectoparasite loads between species is nest design and composition (Bauchau 1998, Remeš and Krist 2005, Moreno et al. 2009). Unstructured nests like those of Nuthatches offer fewer opportunities for hiding to ectoparasites, and nest composition may affect ectoparasite development through the effects of microclimatic conditions associated with different nest materials (Heeb et al. 2000). Large amounts of nest material, although of benefit to reduce incubation costs (Moreno et al. 2010), may collect and retain humidity above optimal levels and attract parasitic arthropods and pathogenic bacteria (Moreno 2012a). Interespecific differences in ectoparasite abundances could be explained by interespecific differences in nest composition.

We have addressed the implications of ectoparasitism in avian cavity-nesters in a montane oak forest in central Spain with coexisting populations of Nuthatches, Pied Flycatchers and Blue Tits *Cyanistes caeruleus* that are parasitized by mites *Dermanyssus gallinoides*, blowfly *Protocalliphora azurea* larvae and hen fleas *Ceratophyllus gallinae* (Moreno et al. 2009). In this area, Nuthatch nests are composed of pine bark and strips of bark of *Cistus* shrubs, Pied Flycatcher nests are composed of dry grass, *Cistus* and pine bark and dry leaves (Moreno et al. 2009) and Blue Tits build nests mainly of moss and hair (Cramp and Perrins 1993). The aim of this study is to explore if differences in prevalence and abundance of ectoparasites between sympatric avian hosts breeding in the same type of nest-boxes can be related to interspecific differences in their nest size, nest composition and cavity microclimate. Furthermore, we aim at detecting if interspecific variation in the incidence and intensity of parental grooming and nest sanitation behaviours is a consequence of the abundance of ectoparasites. We have explored if:

(1) Variation in ectoparasite abundance between host species is associated with interspecific differences in nest size and composition;
(2) Avian hosts using pine bark as nest building material (Nuthatches and some Pied Flycatchers) show lower prevalence and abundances of some ectoparasites;
(3) Cavity microclimate affects ectoparasite abundance;
(4) Behavioural responses to ectoparasites are more frequent in avian hosts with higher infestations. This pattern should occur during both the incubation and nestling periods;
(5) There is a trade-off in time allocation between brooding nestlings and nest sanitation behaviours during the early nestling stage.

MATERIAL AND METHODS

Study area and host species

Our study was carried during the springs of 2011 and 2012 in a Pyrenean oak *Quercus pyrenaica* forest located in Valsaín (Segovia, 40° 54’ N, 4° 01’ W, 1200 m.a.s.l.), where breeding activities in nest-boxes have been studied since 1991 (see Sanz et al. 2003 for general description). For details about nest-box design and placement see Lambrechts et al. (2010). All the nest-boxes are cleaned every year after the breeding season. Scattered pines *Pinus sylvestris* are found among the oaks while the shrub layer consists mainly of *Cistus laurifolius*. Breeding activities are followed routinely every year and laying and hatching dates and brood sizes at hatching and at fledging are determined.

The Pied Flycatcher is a small (12 g) passerine bird, which breeds in many forested areas of the Palaearctic region (Lundberg and Alatalo 1992). It is a summer visitor, which adapts readily to breeding in nest-boxes. Egg laying in the population under study typically begins in late May, and clutch sizes range from 4 to 7 eggs. In our study area Pied Flycatchers incorporate strips of bark of *Cistus laurifolius*, pine *Pinus sylvestris* bark and dry grass as nest material (Moreno et al. 2009). The female incubates alone and receives part of her food from her mate (Moreno et al. 2011). Both sexes feed the young._From the first egg, the mean duration of the breeding cycle is 36 days.

The Blue Tit is a small (10 g) hole-nesting passerine of European woodlands, which breeds mainly in deciduous forests (Cramp and Perrins 1993). It is a resident bird, which adapts readily to breeding in nest-boxes. Egg laying in central Spain typically begins in the second half of April, and clutch sizes range from 4 to 14 eggs (Fargallo 2004). Blue Tits build their nests mainly of moss and hair. Females incubate and brood the chicks alone, receiving part of her food from her mate, and both sexes feed the young (Moreno et al. 1996, Fargallo and Johnston 1997). From laying of the first egg, the mean duration of the breeding cycle is 42 days.

The Nuthatch is a small (23 g) cavity-nesting woodland bird that prefers to build their nests in existing cavities in trees but can also use nest-boxes for breeding. Nuthatches narrow the entrance of cavities by plastering mud around it and their nests are composed in our study area mainly of pine bark flakes and strips of bark of *Cistus laurifolius.* Egg laying in central and western Europe typically occurs during the second half of April, and clutch sizes range from 5 to 9 eggs (Matthysen 1998). Eggs are incubated by the female alone which receives part of her food from her mate (Matthysen 1998). In our population, females always cover the eggs with flakes of bark before leaving the nest during incubation. Both sexes feed the young (Matthysen 1998). From the first egg, the mean duration of the breeding cycle is 48 days.

Ectoparasites

Fleas live mostly in the nest material (Harper et al. 1992). Only the adults are blood-sucking; the larvae feed on organic matter in the nest (Tripet and Richner 1997a). Therefore, the number of flea larvae in nests indicates the fecundity of adult fleas (Eeva et al. 1994). Some authors have demonstrated that fleas have negative effects on Great Tit (Richner et al. 1993, Christe et al. 1996, Allander 1998) and Blue Tit reproduction (Tripet and Richner 1997b, Tripet et al. 2002a).

Blowfly females oviposit in the nests in spring. Blowfly larvae live in bird nests and feed intermittently of nestling blood (Bennett and Whitworth 1991, Remeš and Krist 2005). These larvae start to develop only after nestlings hatch (Bennett and Whitworth 1991). In the Pied Flycatcher, the presence of blowfly larvae in the nest is associated with increased nestling mortality (Merino and Potti 1995) and lower growth rate (Eeva et al. 1994) of the nestlings. In Blue Tit nestlings, infestation by blowflies is associated with higher levels of stress proteins in blood (Arriero et al. 2008).

In mites, adult and nymphal stages are haematophagous. Populations build up from very few up to some thousands of individuals per nest during the breeding period; generation time is short. In Pied Flycatchers some authors have observed detrimental effects of mites on host reproductive success (Merino and Potti 1995, 1996, Merino et al. 1998, Lobato et al. 2005, 2008, Moreno et al. 2008, 2009). Mites may be present in nest materials even before nestlings hatch and may feed on incubating females (Pacejka et al. 1996).

Ectoparasite abundance estimation

We studied 34 Pied Flycatcher nests, 11 Blue Tit nests and 13 Nuthatch nests in 2011 and 35 Pied Flycatcher nests, 20 Blue Tit nests and 13 Nuthatch nests in 2012. Nests were processed for estimating ectoparasite abundances. Soon after nestlings fledged (days 17–18 for Pied Flycatchers, days 19–20 for Blue Tits, days 20–26 for Nuthatches), all nests were removed in sealed plastic bags, were weighed on electronic balances with 0.1 g precision to obtain the fresh nest mass (g) and were taken to the laboratory, where they were subjected to arthropod removal in Berlese funnels for 48 h. The content of the jars from Berlese funnels was examined to estimate of the total number of mites and fleas (adults and larvae) (for abundance estimations see Moreno et al. 2009). Blowfly puparia were directly counted in the nest material (Merino and Potti 1996). Only in 2012 were all nests subsequently separated into different components. Nest composition was expressed in proportions of mass of the following materials: *Cistus* bark, dry grass, pine bark and moss.

Nest microclimatic measurements

Temperature and humidity in nest-boxes were recorded only during the spring of 2012. Seven days after clutch completion, we placed on the nest box base and under the nest material a climate data logger (Hydrochron DS1923 iButtons, Eclo 2008) inside 35 randomly selected nest-boxes (15 occupied by Pied Flycatchers, 10 by Blue Tits and 10 by Nuthatches). Data loggers were programmed to take measurements every 4 minutes for 11 days so we measured the temperature and humidity inside nest-boxes from day 8 of incubation until two days after chicks hatched. We retrieved our data after the loggers were removed from the nest-boxes. We divided each continuous set of temperature and humidity data into segments of 24 h with daytime beginning at noon, and we then calculated the daily average, maximum and minimum for temperature and humidity.

Video recordings

Behavioural data were taken only during the spring of 2012. Seven days after clutch completion (day 7 of incubation), we filmed inside nest-boxes for 90 min (92.67±SE 11.50 min, n=67) with a cold white light (LED 5 mm) powered by a 3 V battery and a camera (GoPro HD Hero1) mounted on the roof inside the nest-box (35 Pied Flycatchers nests and 20 Blue Tits nests). To avoid opening the nest-box and damaging the nest with the possible fall of mud on eggs, Nuthatches (n=12) were recorded by a camera (Square SONY 1/3* Super HAD CCD) connected to a 3G H.264 CCTV DVR 1 Tb digital recorder installed on the roof inside the nest-box. Both digital recorders and camcorders were powered by batteries (7.2 Ah 12 V).

Nest-boxes were again filmed two days after the day of hatching of the young (87.45±13.40 min, n=67) and 8 days after hatching of the young (86.88±17.47 min, n= 67). In two Pied Flycatcher nests all chicks died after day 3 so only basic breeding variables for this nest could be used. All films were recorded at 08:00-17:00 h and the effect of the time of filming was only noted in provisioning rates of large nestlings (effect of time of day in other cases $p > 0.20$). We excluded the time until the first nest visit by parents (14.35±13.20 min, n=201). No evidence of stress or unnatural behaviour was observed after the first visit.

Behavioural data analyses

Recordings were displayed in the free VLC Media Player software. From films taken during incubation we estimated the proportion of observation time spent by females inside the nest-box or “incubation attentiveness”. Furthermore, we estimated the proportion of time spent inside the nest-box allocated to incubating and turning the eggs or “egg attendance”, the mean duration of incubation sessions and recesses and the proportion of time spent on three specific types of female behaviour: “grooming”, “nest sanitation” and “sleeping”. “Grooming” is the combined time which females spend preening their plumage or scratching themselves (Cotgreave and Clayton 1994), while “nest sanitation” is any period of active search with the head buried, sometimes deeply, into the nest material (Cotgreave and Clayton 1994). “Sleeping” is defined as the time when the beak is pointed backwards and tucked under the scapulars (Amlaner and Ball 1983). We obtained the proportion and the mean duration of these behaviours over the time that the female was inside the nest-box. In addition, we also counted the number of incubation feedings by males.

From films during the early nestling phase we obtained hourly provisioning rates by males and females and the amount of time spent by females on “nestling attentiveness”, “brooding”, “grooming”, “nest sanitation” or “sleeping”. “Nestling attentiveness” represents the proportion of time spent by the female inside the nest-box. “Brooding” activity is defined as proportion of the time spent inside the nest-box by the female used to cover young nestlings. “Nest sanitation”, “sleeping” and “grooming” were calculated in the same way as for the incubation stage.

From films during the late nestling phase we obtained the total hourly provisioning rates by males and females and the amount of time spent by females on “nestling attendance”, “brooding”, “grooming” or “nest sanitation”. All these variables were calculated in the same way as before.

Statistical analyses

Breeding variables, ectoparasite abundances, nest composition and some behavioural variables could not be normalized and were analyzed with non-parametric tests (Kruskal-Wallis tests, STATISTICA package) with species as explanatory factor. Hatching success and fledging success were calculated as the proportions of eggs that hatched and the proportion hatched chicks that fledged, respectively. These parameters could not be calculated for Blue Tits and Nuthatches in 2011 as we did not register exactly how many eggs hatched in these species in that year (some nestlings may die and disappear unrecorded during the first days after hatching, see (Moreno 2012b). Hatching date of each species was standardized by subtraction from the annual mean hatching date for this species in the study area. We conducted Spearman correlations between grooming and nest sanitation activities with ectoparasite abundances for each host species. Provisioning rates (h^{-1}) were normally distributed (Kolmogorov-Smirnov test, $p > 0.20$ in all cases) and were analyzed with species as explanatory factor and blowflies, fleas and mites abundance as continuous predictors.

Microclimatic data were analyzed with non-parametric tests (Kruskal-Wallis test) with species as explanatory factor. We conducted Spearman rank correlations between numbers of ectoparasites and maximum, mean and minimum temperature and humidity on the day of hatching. We selected hatching day because it is a biologically important time in the breeding season and is potentially comparable between different species.

Within each host species, the ectoparasite abundances that were normal or could be normalized through logarithmic transformations were analyzed with General Linear Models (GLM) with year as explanatory factor and hatching date, brood size and nest mass (g) as continuous predictors. If ectoparasite abundances could not be normalized, they were analyzed with non-parametric tests (Spearman rank correlation and Mann–Whitney U test).

RESULTS

Breeding biology

Breeding parameters differed between species (Table 1). In 2011, laying date of Nuthatches and Blue Tits was earlier than Pied Flycatchers and, in 2012, Blue Tits showed a temporal delay in their laying date so much larger than the other species (Table 1). Hatching date also differed between species with Nuthatches and Blue Tits hatching earlier than Flycatchers in 2011 and with Nuthatches hatching earlier than Blue Tits and Flycatchers in 2012 (Table 1). Host species differed also in clutch size and brood size (Table 1). We found no differences in hatching or fledging success between species (Table 1, 2012).

2011	**Pied Flycatcher (n=34)**	**Blue Tit (n=11)**	**Nuthatch (n=13)**	**Statistic**	**p**
Laying date	12 May±4	22 Apr±4 ^{x}	19 Apr±3 ^{x}	H_2 = 42.51	**<0.005**
Hatching date	30 May±4	14 May±5 ^{x}	10 May±3 ^{x}	H_2 = 42.28	**<0.005**
Clutch size	5.88±0.77 ^{x}	9.45±1.51	6.50±0.79 ^{x}	H_2 = 42.60	**<0.005**
Brood size 13 days	4.91±1.31 ^{x}	8.00±2.45	5.42±1.73 ^{x}	H_2 = 14.63	**<0.005**
Hatching success	89.13±11.94	-	-	-	-
Fledging success	82.73±19.34	-	-	-	-

2012	**Pied Flycatcher (n=35)**	**Blue Tit (n=20)**	**Nuthatch (n=13)**	**Statistic**	**p**
Laying date	18 May±2	13 May±5	30 Apr±6	$H_2 = 40.61$	**<0.005**
Hatching date	6 Jun±1 ^{x}	5 Jun±4 ^{x}	21 May±6	$H_2 = 30.93$	**<0.005**
Clutch size	5.62±0.59 ^{x}	9.00±0.97	6.50±0.79 ^{x}	$H_2 = 48.40$	**<0.005**
Brood size 13 days	4.72±1.45 ^{x}	8.20±1.47	5.42±1.73 ^{x}	$H_2 = 36.06$	**<0.005**
Hatching success	90.09±14.13	95.50±8.72	84.24±24.0	$H_2 = 2.13$	0.345
Fledging success	96.33±11.48	92.54±14.1	94.87±14.2	$H_2 = 3.53$	0.171

Table 1. Means ± SE for breeding variables for Pied Flycatcher *Ficedula hypoleuca,* Blue Tit *Cyanistes caeruleus* and Nuthatch *Sitta europaea* (n in parenthesis) in the two years of study (values followed by the same symbol (^{x}) do not differ significantly).

Variation in nest mass and composition among species

Nest mass (2011 and 2012) and composition (2012) differed strongly between species (Table 2). Blue Tits nests were heavier in 2011 ($F = 7.204$, $p = 0.012$) while we found no differences in Pied Flycatchers and Nuthatches fresh nests mass between years (Pied Flycatchers: $F = 1.920$, $p = 0.170$; Nuthatches: $F = 0.025$, $p = 0.876$). Pied Flycatchers incorporated strips of *Cistus* and pine bark of and dry grass as nest material while Blue Tits built their nests mainly of moss and hair and Nuthatches nests are composed of pine bark flakes and strips of *Cistus* bark. Nest mass differed between species (Table 2) with Nuthatches building heavier nests than the other species.

2011	**Pied Flycatcher (n=34)**	**Blue Tit (n=11)**	**Nuthatch (n=13)**	**Statistic**	**p**
Total mass (g)	35.453±10.591	33.909±6.737	54.854±9.793	$H_2 = 21.66$	**<0.005**

2012	**Pied Flycatcher (n=35)**	**Blue Tit (n=20)**	**Nuthatch (n=13)**	**Statistic**	**p**
Cistus bark	0.573±0.401 ^{x}	0.005±0.015	0.296±0.333 ^{x}	$H_2 = 28.64$	**<0.005**
Dry grass	0.287±0.323	0.025±0.064 ^{x}	0.000±0.000 ^{x}	$H_2 = 22.83$	**<0.005**
Pine bark	0.100±0.131	0.000±0.000	0.704±0.333	$H_2 = 39.60$	**<0.005**
Moss	0.040±0.123 ^{x}	0.970±0. 064	0.000±0.000 ^{x}	$H_2 = 56.21$	**<0.005**
Total mass (g)	31.426±8.633	28.485±4. 510	56.825±15.382	$H_2 = 26.18$	**<0.005**

Table 2. Proportional (means ± SE) composition (2012) and total fresh mass (2011, 2012) for the 3 host species nests (n in parenthesis) in the study locality (2012) and Kruskal-Wallis test comparisons between species (species followed by the same symbol (^{x}) do not differ significantly).

Variation in ectoparasite abundance among species

We have compared ectoparasite prevalences between avian hosts (Table 3). An outlying value on mite abundance for a Blue Tit nest from 2012 was excluded from the analysis. The proportion of nests infected by fleas (all $p < 0.005$) and blowflies (2011, $p = 0.039$; 2012, $p = 0.046$) was significantly higher for Blue Tits than for the other species in the two years (Table 3). In 2011 Pied Flycatcher and Nuthatch nests showed similar flea prevalences but Pied Flycatcher nests were less infected by blowflies than Nuthatch nests (Table 3). In 2012 blowfly prevalence was similar in both species but Pied Flycatcher nests were less infected by fleas (Table 3). Mites were present in most nests of the three species during the two years (Table 3).

	Pied Flycatcher	**Blue Tit**	**Nuthatch**	**Statistic**	**p**
Blowflies					
2011	0.58 (20)	1 (11)	0.69 (9)	$H_2 = 6.47$	**0.039**
2012	0.66 (23) ^{x}	0.95 (19)	0.67 (8) ^{x}	$H_2 = 6.16$	**0.046**
Fleas					
2011	0.47 (16) ^{x}	1 (11)	0.61 (8) ^{x}	$H_2 = 25.99$	**<0.005**
2012	0.26 (9)	0.95 (19)	0.42 (5)	$H_2 = 22.61$	**<0.005**
Mites					
2011	1 (34)	1 (11)	0.92 (12)	$H_2 = 3.46$	0.177
2012	1 (35)	1 (20)	1 (12)	$H_2 = 0.00$	1

Table 3. Results of Kruskal-Wallis tests for the proportion of infected nests and differences during 2011 and 2012 (number of infected nests in parenthesis) in the study area by each type of ectoparasite and avian host species (species followed by the same symbol (^{x}) do not differ).

We have compared ectoparasitism intensities between avian hosts. Blowflies and fleas were significantly more abundant in Blue Tits nests (Table 4). We found no differences between mite abundances among host species (Table 4).

	Pied Flycatcher	**Blue Tit**	**Nuthatch**	**Statistic**	**p**
Blowflies					
2011	11.1±14.9	22.0±20.6	7.1±8.8	$H_2 = 6.11$	**0.047**
2012	9.9±9.3 ^{x}	23.1±18.2	11.7±14.6 ^{x}	$H_2 = 15.64$	**<0.005**
Fleas					
2011	12.0±22.4 ^{x}	895.3±306.9	330.3±517.2 ^{x}	$H_2 = 25.99$	**<0.005**
2012	24.8±88.1 ^{x}	360.6±519.3	49.4±113.4 ^{x}	$H_2 = 30.82$	**<0.005**
Mites					
2011	2603.3±4713.8	381.3±358.8	1821.9±2252.5	$H_2 = 0.73$	0.695
2012	3347.6±4543.5	1972.2±3737.2	3225.2±3990.3	$H_2 = 2.19$	0.334

Table 4. Results of Kruskal-Wallis tests for differences in ectoparasites abundance (means ± SE) for 2011 and 2012. Species followed by the same symbol (^{x}) do not differ.

Flea abundance increased with hatching date and brood size in Blue Tit nests and was higher in 2011 than in 2012 (Tables 4, 5). Blowfly abundance increased with hatching date in Blue Tit nests (Table 5). Neither year, brood size nor standardized hatching date showed any significant effect on mite abundance for Blue Tits (Table 5).

	Parameter	**SE**	**df**	**F**	**p**	**Adjusted R^2**
Blowflies						
Full model			26			0.215
Hatching date	0.349	0.185	1	3.554	0.071	
Brood size	0.175	0.180	1	0.946	0.340	
Nest mass (g)	0.336	0.209	1	2.570	0.121	
Year	-0.171	0.189	1	0.819	0.374	
Minimal model			29			0.145
Hatching date	0.417	0.169	1	6.099	**0.012**	
Fleas						
Full model			26			0.332
Hatching date	0.342	0.171	1	4.018	0.056	
Brood size	0.368	0.166	1	4.917	**0.036**	
Nest mass (g)	-0.036	0.193	1	0.035	0.852	
Year	0.523	0.174	1	9.013	**0.006**	
Minimal model			27			0.356
Hatching date	0.328	0.150	1	4.763	**0.038**	
Brood size	0.356	0.151	1	5.600	**0.025**	
Year	0.506	0.148	1	11.901	**0.002**	

	Parameter	SE	df	F	p	Adjusted R^2
Mites						
Full model			26			-0.042
Hatching date	-0.107	0.213	1	0.254	0.619	
Brood size	0.075	0.207	1	0.131	0.720	
Nest mass (g)	0.150	0.241	1	0.387	0.540	
Year	-0.314	0.218	1	2.085	0.161	

Table 5. Results of GLM analyses for effects of year, hatching date, brood size and nest mass on abundances of fleas, blowflies and mites in Blue Tit nests (n=30), (minimal models are selected by backward elimination of non-significant terms).

In Nuthatch nests no association was found between ectoparasite abundances and either year, hatching date, brood size or nest size (Table 6). In Pied Flycatcher nests mite abundance showed a negative association with nest size (Table 7).

	Parameter	SE	df	Statistic	p	Adjusted
Blowflies						
Full model			21			0.022
Hatching date	0.025	0.213	1	*F*=0.729	0.403	
Brood size	0.255	0.214	1	*F*=0.014	0.906	
Nest mass (g)	0.327	0.198	1	*F*=1.425	0.246	
Year	0.048	0.199	1	*F*=2.679	0.116	
Fleas						
Hatching date				$r_s = -0.057$	0.784	
Brood size				$r_s = -0.111$	0.589	
Nest mass (g)				$r_s = -0.029$	0.890	
Year				$U = 57$	0.158	
Mites						
Full model			21			0.011
Hatching date	-0.053	0.214	1	*F*=0.060	0.808	
Brood size	0.240	0.215	1	*F*=1.242	0.278	
Nest mass (g)	0.229	0.199	1	*F*=1.324	0.263	
Year	-0.221	0.200	1	*F*=1.226	0.281	

Table 6. Results of GLM analyses for effects of year, hatching date, brood size and nest mass on blowfly and mite abundance in Nuthatch nests (n=26) (minimal models are selected by backward

elimination of non-significant terms) and results of correlations of flea abundance in Nuthatches nests (n=26) with hatching date, brood size and nest mass and Mann-Whitney U-test (year).

	Parameter	SE	df	Statistic	p	Adjusted
Blowflies						
Hatching date				$r_s = 0.080$	0.510	
Brood size				$r_s = 0.194$	0.110	
Nest mass (g)				$r_s = 0.085$	0.490	
Year				$U = 523$	0.387	
Fleas						
Hatching date				$r_s = 0.187$	0.123	
Brood size				$r_s = 0.159$	0.191	
Nest mass (g)				$r_s = -0.107$	0.379	
Year				$U = 528$	0.421	
Mites						
Full model			65			0.105
Hatching date	-0.003	0.118	1	F=0.001	0.977	
Brood size	-0.234	0.119	1	F=3.583	0.054	
Nest mass (g)	-0.233	0.119	1	F=3.818	0.055	
Year	-0.131	0.118	1	F=1.230	0.271	
Minimal model			68			0.080
Nest mass (g)	-0.305	0.116	1	F=6.887	**0.011**	

Table 7. Results of correlations of blowflies and fleas abundance in Pied Flycatchers nests (n=69) with hatching date, brood size and nest mass, Mann-Whitney U-test (year) and GLM analyses for effects hatching date, brood size and nest mass on mites abundance in Pied Flycatchers nests (n=69), controlling for year (minimal models are selected by backward elimination of non-significant terms).

Nest microclimatic data

The nest-boxes of the three species did not differ in thermal variables (Fig. 1; all $p > 0.1$) but Nuthatch nest-boxes nests tended to have higher mean ($p = 0.072$), maximum ($p = 0.085$) and minimum ($p = 0.090$) relative humidity values than Tit and Flycatchers nest-boxes (Fig. 2).

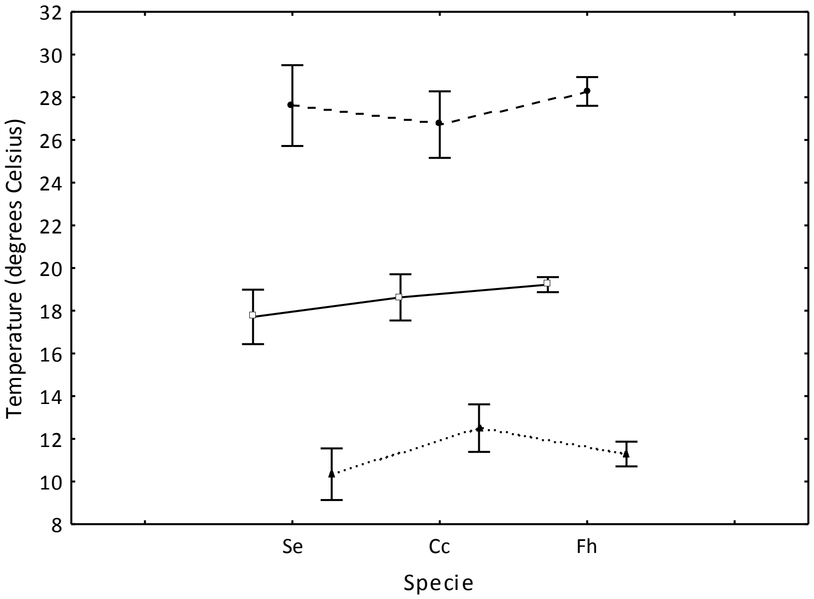

Figure 1. Maximum (*p*=0.524), mean (*p*=0.181) and minimum (*p*=0.315) temperatures at hatching (±SE) in relation to species (*Sitta europaea*, Se, n=10; *Cyanistes caeruleus*, Cc, n=10; *Ficedula hypoleuca*, Fh, n=15) (●Mean maximum temperature, □ Mean temperature, ▲ Mean minimum temperature).

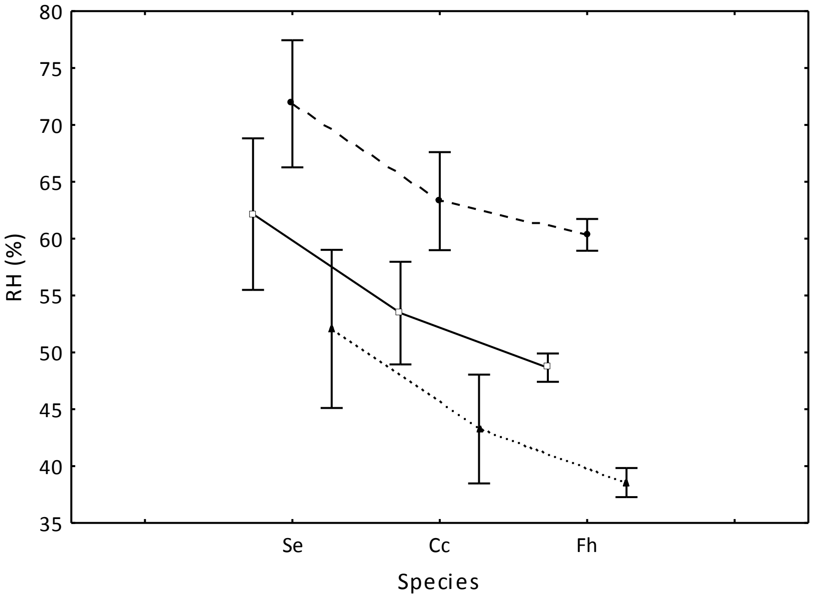

Figure 2. Maximum (*p*=0.085), mean (*p*=0.072) and minimum (*p*=0.090) relative humidity (RH) at hatching (±SE) in relation to the hole nest species (*Sitta europaea*, Se, n=10; *Cyanistes caeruleus*, Cc, n=10; *Ficedula hypoleuca*, Fh, n=15). (●Mean maximum RH, □ Mean RH, ▲ Mean minimum RH).

We have found significant differences in prevalences of infestation by three sympatric ectoparasites, with Blue Tits showing higher values for blowflies and fleas and mites having 100% prevalences in the three host species. We found no correlations between flea and mite abundances and microclimatic conditions inside nest-boxes for any host species (Table 8; all *p* > 0.1). Blowfly abundance was negatively related to minimum temperature in Nuthatch nest-boxes (Table 8) and positively related to mean and minimum relative humidity in Pied Flycatcher nest-boxes (Table 8). Ectoparasite abundances in Blue Tit nest-boxes showed no association with microclimatic conditions (Table 8).

	Pied Flycatcher (15)		**Blue Tit (10)**		**Nuthatch (10)**	
	Statistic	**p**	**Statistic**	**p**	**Statistic**	**p**
Mean temperature	-0,008	0,977	-0,127	0,726	-0,178	0,623
Mean maximum	-0,448	0,094	-0,188	0,603	-0,215	0,551
Mean minimum	0,486	0,066	-0,188	0,602	-0,658	**0,038**
Mean humidity	0,549	**0,034**	0,055	0,881	-0,067	0,853
Mean maximum humidity	0,260	0,349	0,164	0,651	-0,092	0,800
Mean minimum humidity	0,599	**0,018**	0,116	0,751	0,018	0,960

Table 8. Results of correlations of blowfly abundance with nest-box microclimatic variables on the day of hatching (Spearman rank correlation, number of nest-boxes in parenthesis).

Antiparasitic behaviours

During incubation, the frequency and mean duration of female grooming behaviour did not differ between species (Table 9). Incubation attentiveness, egg attendance and the mean of incubation session durations of Pied Flycatchers females were significantly shorter (Table 9). The mean time outside de nest-box was higher in Nuthatches than in the other species (Table 9). Nest sanitation behaviours were more frequent for Blue Tits and Nuthatches (Table 9), while Pied Flycatchers and Nuthatches showed shorter mean durations of these behaviours (Table 9). We found no correlation between grooming or nest sanitation activities with ectoparasite abundances for any host species (Spearman correlation, all *p* > 0.1). We have found no differences between species in male incubation feeding frequency (Table 9). During daytime incubation, only Blue Tits and Nuthatches showed sleeping behaviour (Table 9).

	Pied Flycatcher	Blue Tit	Nuthatch	Statistic	p
Incubation					
Grooming (%)	0.412±0.590	1.104±1.565	0.430±0.418	H_2 = 3.144	0.208
Mean grooming (s)	4.00±2.00	4.00±3.00	4.00±2.00	H_2 = 2.221	0.329
Incubation attentiveness (%)	64.391±11.456	77.982±9.557 ^{x}	69.771±12.592 ^{x}	H_2 = 14.981	**<0.001**
Egg attendance (%)	97.727±1.727	81.471±12.360 ^{x}	79.929±17.080 ^{x}	H_2 = 46.860	**<0.001**
Mean session (min)	10.63±4.45	20.01±9.78 ^{x}	25.97±7.12 ^{x}	H_2 = 33.421	**<0.001**
Mean recess (min)	5.90±2.00 ^{x}	6.22±2.93 ^{x}	13.17±3.53	H_2 = 25.732	**<0.001**
Nest sanitation (NS) (%)	1.860±1.597	12.93±11.020 ^{x}	8.315±5.278 ^{x}	H_2 = 46.452	**<0.001**
Mean NS duration (s)	4.00±2.00 ^{x}	15.00±10.00	3.00±1.00 ^{x}	H_2 = 37.847	**<0.001**
Male provisioning (h^{-1})	0.561±1.471	1.775±4.145	0.102±0.240	H_2 = 1.693	0.428
Sleeping (yes/no)	No	Yes	Yes		
Nestlings day 3					
Nestling attentiveness (%)	55.291±12.868	57.982±16.693	55.025±16.144	H_2 = 0.604	0.739
Brooding (%)	93.108±3.161 ^{x}	75.205±13.348	89.245±7.106 ^{x}	H_2 = 27.858	**<0.001**
Mean grooming (s)	2.00±2.00	5.00±5.00 ^{x}	5.00±6.00 ^{x}	H_2 = 7.539	**0.023**
Grooming (%)	0.106±0.145	1.597±3.778 ^{x}	1.209±1.464 ^{x}	H_2 = 11.130	**<0.001**
Nest sanitation (NS) (%)	5.319±2.592 ^{x}	23.198±11.893	9.545±6.911 ^{x}	H_2 = 33.369	**<0.001**
Mean NS duration (s)	5.00±3.00 ^{x}	15.00±10.00	3.00±2.00 ^{x}	H_2 = 31.358	**<0.001**
Male provisioning (h^{-1})	12.185±8.639 ^{x}	13.702±10.345 ^{x}	7.957±5.703	F = 3.497	**0.037**
Female provisioning (h^{-1})	5.851±3.078 ^{x}	5.614±4.093 ^{x}	3.164±1.438	F = 3.202	**0.048**
Sleeping (yes/no)	No	Yes	Yes		
Nestlings day 9					
Provisioning rates (h^{-1})	22.968±8.765 ^{x}	23.675±6.465 ^{x}	11.885±4.062	F = 10.696	**<0.001**

Table 9. Differences (means ± SE) in frequencies of parental and antiparasitic behaviours between species (35 Pied Flycatchers nests, 20 Blue Tits nests and 12 Nuthatches nests) and result of Kruskal-Wallis tests and GLM analyses.

On day 3, nestling attentiveness did not differ between species (Table 9). The proportion of time allocated to brooding was significantly lower in Blue Tits (Table 9). The frequency and mean duration of female grooming behaviour was higher in Blue Tits (Table 9). Incidence and mean duration of nest sanitation was longer and more frequent in Blue Tits (Table 9). Provisioning rates by males and females were lower for Nuthatches than for the other species (Table 9). At this stage, only Blue Tits and Nuthatches showed sleeping behaviour (Table 9).

On day 9, provisioning rates of Nuthatches were lower than for Pied Flycatchers and Blue Tits (Table 9). We found no effects of ectoparasites on provisioning rates (Table 10).

	Parameter	SE	df	Statistic	p	Adjusted R^2
Provisioning rates						
Full model			65			0.272
Blowflies	0.068	0.069	1	*F*=0.3159	0.576	
Fleas	0.199	0.000	1	*F*=3.6289	0.061	
Mites	0.208	0.003	1	*F*=3.1387	0.081	
Species	0.249	1.571	2	*F*=10.485	<0.001	
Minimal model			68			0.234
Species	0.374	1.316	2	*F*=11.717	<0.001	

Table 10. Results of GLM analyses for effects of blowflies, fleas and mites abundance on Provisioning rates (h^{-1}) controlling for species (minimal models are selected by backward elimination of non-significant terms).

DISCUSSION

We have found in two years significant differences in breeding phenology, nest size and nest composition among three sympatric cavity-nesting passerines breeding in the same type of nest-boxes, with Nuthatches breeding earliest and building the largest nests, Blue Tits laying the largest clutches and the three species differing among themselves in nest composition. We have also found significant differences in prevalences of infestation by three sympatric ectoparasites, with Blue Tits showing higher values for blowflies and fleas and mites having 100% prevalences in the three host species. Intensities of infestation were also higher in Blue Tit nests for blowflies and fleas, with no differences for mites among host species. Nuthatch nest-boxes tended to show higher humidity while there were no differences in nest-box temperature among the three host species. Incubation attendance patterns differed also among host species with Flycatchers incubating proportionally less of nest-box time and spending shorter periods incubating, while Nuthatch females spent longer periods outside the nest-box. Nest sanitation activity during incubation was less intense in Pied Flycatchers. Blue Tit females spent less time inside the nest-box brooding small nestlings and showed higher frequency of nest sanitation behaviours than the other species, while provisioning rates by males and females were lower for Nuthatches than for the other species at both nestling ages.

This is to our knowledge the first study to compare the nest-dwelling ectoparasitic faunas as well as factors affecting it among three sympatric host species. Interspecific studies of ectoparasite faunas have usually concerned Tits and Flycatchers (Harper et al. 1992, Eeva et al. 1994, Kedra et al. 1996, Bauchau 1998, Moreno et al. 2009) and no detailed information about the ectoparasites in Nuthatch nests has been published. The structure and composition of the nests of Blue Tits, Nuthatches and Pied Flycatchers are markedly different, but their effect on ectoparasites is poorly understood. Ectoparasites may be affected by volatile compounds generated by the nest material or through the microclimatic conditions derived from nest properties. The evidence that pine bark in nests may have insecticidal properties is reviewed in Bauchau (1998) and Matthyssen (1998). The bark of these trees contain many compounds with insecticidal properties like limonene that may act as protection against pathogens and herbivores (Pearce 1996). In contrast to the results reported by other authors on northern fowl mites (Carroll 1994) and cat fleas (Hink and Fee 1986), the Nuthatch nests that were built mostly of pine bark had not fewer mites or fleas than other nests. However, the preferences for different materials may be unrelated to ectoparasitism. Moreno et al (2009) showed that ectoparasites prevalences in Pied Flycatcher nests were independent of nest type (constructed by themselves or Blue Tits) and suggested that interspecific differences in ectoparasite prevalences on hosts are probably related to factors other than nest composition. Remes & Krist (2005) arrived at similar results in an experimental study with nests of Collared Flycatchers and Great Tits. Nest size has been shown to be advantageous to reduce incubation costs for Pied Flycatcher females in our study area (Moreno et al. 2010). Here we show that large nests may contain fewer mites as well. It has been shown that mite abundance in Pied Flycatcher nests is unrelated to the presence of old nest material in contrast to fleas and blowflies (López-Arrabé et al. 2012) which again suggests that mites do not benefit from the presence of large amounts of nest materials in nest-boxes.

The effects of ectoparasites seem to vary over time and also among host populations, which implies that they may interact with other environmental factors (Allander 1998). It is known that weather conditions determine patterns of prevalence and abundance of ectoparasites (Merino and Potti 1996). According to some studies an abiotic factor like nest humidity within nests could affect ectoparasite infracommunity structure (Heeb et al. 2000, Remeš and Krist 2005). The brood size could explain the nest humidity due to the higher evapotranspiration from the metabolism of the chicks (Dubiec and Mazgajski 2013). Here we show a lack of association between flea and mite infestations and nest-box microclimate. Only blowflies were apparently affected by temperature and humidity inside the nest-box. This may be due to the active search of optimal conditions for larvae by blowfly females, while mite and flea dispersal is more passive (Harper et al. 1992, Tripet et al. 2002b, Bajerlein et al. 2006). Humidity in the nest-box was positively related to the abundance of blowflies in Pied Flycatcher nests. Bennet & Whitworth (1991) have shown that there was no effect of humidity on the rate of development of blowfly adults but, at the same temperature, the survival of pupae of some species of *Protocalliphora* was lower in extreme humidity conditions. Thus, blowflies could be attracted to more humid Flycatcher nests in order to avoid desiccation.

Intraspecific differences in parasite prevalence within the same region and habitat type have normally been attributed to host traits, including disease resistance ability, age, breeding cycle, and behavioural antiparasite mechanisms (Møller 1997). Antiparasitic behaviours like grooming and nest sanitation may constitute some of the main defenses of breeding birds against ectoparasites which may partly compensate their potentially pathogenic effects on adults and nestlings (Christe et al. 1996, Hart 1997, Tripet et al. 2002a, Mazgajski 2007a). Ectoparasites present in the nest during incubation are mites and fleas. In fleas only the adults are blood-sucking (Harper et al. 1992). Host females groom themselves as a direct response to the attachment of these ectoparasites on their skin and plumage (Cotgreave and Clayton 1994). However, the ectoparasite load during incubation may not be so different between species to establish interspecific differences in grooming behaviour, which were not found. Blowflies may lay their eggs in the nest material when the nestlings hatch with resulting fly larvae intermittently feeding on nestling and brooding adult blood afterwards (Rognes 1991). The emergence of large ectoparasites such as blowfly larvae could induce a significant change in female behaviour.

The function of nest sanitation behaviour by introducing the bill in the nest material has been debated (Haftorn 1994). One possibility is that birds actually destroy and even consume ectoparasitic arthropods (Rothschild and Clay 1952). We have actually filmed two instances in which a Blue Tit female collected a fly larva from the nest material and immediately flew out of the nest-box carrying the larva in her bill (films available on demand). Nest sanitation could also be used to chase blowfly larvae or adult fleas away from their own body or that of their nestlings, thereby preventing them from biting or laying eggs (we have actually filmed one Blue Tit and one Pied Flycatcher female capturing a searching blowfly and flying out of the nest-box with it in the bill). It is known that females of Great and Blue Tits (Christe et al. 1996) and Pied Flycatchers (Cantarero et al., submitted) exhibit nest sanitation, but it has never before been described in Nuthatches. Our behavioural interspecific differences based on higher rates of nest sanitation in the species, Blue Tits, with the highest rate of ectoparasite infestations are consistent with several previous studies (Christe et al. 1996, Hurtrez-Boussès et al. 2000, Tripet et al. 2002a) who showed that Blue Tit females spent more time in nest sanitation when nests were infested. This suggests that this behaviour may have evolved in response to ectoparasites and that females could thereby minimize the fitness costs associated with ectoparasite infestations (Richner et al. 1993). However, we could not detect any intraspecific trends in nest sanitation activities with ectoparasite abundances. Only experimental studies (Hurtrez-Boussès et al. 2000; Cantarero et al., submitted) may be able to tease out such associations.

The difference in the time invested in behavioural defences indicates that females may be able to choose to increase the amount of time allocated to the control of nest ectoparasites. If variation in parasite abundance is obvious to attending parents, we should expect that, compared with the other species, Blue Tits should allocate more time to anti-parasite behaviours and restrict the time spent on brooding chicks or sleeping (Tripet et al. 2002a), or foraging and provisioning nestlings (Christe et al. 1996) due to the higher infestations in their nests. We found that, because of their greater investment of time in behavioural defenses, Tit females reduced the proportion of time spent in the nest-box brooding compared to Pied Flycatcher and Nuthatch females, but not with respect to total nestling attendance.

Species vary widely in their incubation rhythms (Kendeigh 1952), but the underlying causes of this variation remain obscure. Our results show that the mean duration of incubation sessions is lower in Pied Flycatcher females than in the other species and that they do not sleep while on the nest in contrast with the other species. Conway & Martin (2000) have suggested that nest predation could have affected the evolution of passerine incubation behaviour. The more restive incubation behaviour of Pied Flycatchers may be associated with higher levels of risk of predation at the nest for females of this species in the evolutionary past (Martin 2002).

Blue Tits and Pied Flycatchers parents showed higher provisioning rates than Nuthatches throughout the nestling period. The fact that females of these two species have different time allocations inside the nest-box to nest sanitation but maintained similar provisioning rates suggests that the time costs of these behaviours are not sufficiently important to reduce time available for foraging or provisioning nestlings (Rogers et al. 1991, Tripet et al. 2002a, Nilsson 2003).

To conclude, generalist ectoparasites infest nests of avian cavity-nesting passerines as a response to different factors exhibited by host species. However, differences in nest composition among host species may not be the main factor explaining ectoparasite prevalences and abundances, while nest size, breeding phenology, brood size and nest-cavity micro-climate may all affect levels of infestation in different ways for each host-parasite association. Grooming and nest sanitation is exhibited by all host species but is more intense in the host species with highest infestation levels. Further studies are required to experimentally tease out the relative importance of different factors explaining the marked differences among similar host species in infestation levels of different generalist ectoparasites.

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CHAPTER VI

This chapter reproduces entirely the manuscript:

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CHAPTER VI

Males respond to female begging signals of need: a handicapping experiment in the pied flycatcher, *Ficedula hypoleuca*

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ABSTRACT. The ‘female nutrition’ hypothesis proposes that food provided by males during incubation is an important energy source for females in bird species in which females alone incubate. Females should be able to communicate their needs through begging signals to mates and males may compensate for the energetic limitations of females through their feeding visits, owing to their overlapping reproductive interests. To test whether female begging during incubation is an honest signal of energetic need and whether mates respond to it we experimentally handicapped female pied flycatchers at the beginning of incubation by clipping two primary flight feathers on each wing. Experimental manipulation led females to intensify begging displays arising from condition impairment and males accordingly increased their incubation feeding rates. Female begging intensity explained more than half of the variation in male incubation feeding rate, thereby showing that female nutrition is the main factor explaining male incubation feeding. Moreover, handicapped females consumed a higher proportion of male food deliveries during the first few days after hatching and weighed less at the end of the nestling period than control females. Handicapping had no influence on female incubation behaviour, hatching and breeding success, nestling and male condition or female nestling provisioning. The provisioning rates of males in the late nestling stage were higher in experimental nests. This is the first experimental study showing that males adjust incubation feeding rates to behavioural displays of need by their mates. The ability of females to modify their begging displays according to need may be an important adaptation that allows females to maintain a good energetic condition during incubation.

INTRODUCTION

Males of many avian species in which only the female incubates provision their mates during the incubation period (Lifjeld and Slagsvold 1986; Lyon and Montgomerie 1985; Ricklefs 1974). In some species, for example hornbills, females are totally dependent on males for food during the incubation stage (Poonswad et al. 2004). In a variety of other species it is more common for incubating females to receive only some of their food from their mates, although they also leave the nest to forage in order to maintain their energy requirements (Boulton et al. 2010). Mate feeding has evolved as a behavioural strategy to compensate for energetically costly activities for the female during reproduction (Galván and Sanz 2011), which may include the posthatching stage.

Food provided by males during incubation has been proposed to be an important energy source for females, a proposal termed the 'female nutrition hypothesis' (Niebuhr 1981). In fact, several studies have demonstrated that higher rates of male incubation feeding to their mates can improve female body condition (Lifjeld and Slagsvold 1986) and increase nest attentiveness by reducing the amount of time the female spends foraging off the nest (Halupka 1994; Leclaire et al. 2011; Lloyd et al. 2009; Matysioková et al. 2011; but see Lifjled & Slagsvold 1989; Matysioková & Remeš 2010; Boulton et al. 2010; Stein et al. 2010; Moreno and Carlson 1989; Pearse et al. 2004; Smith et al. 1989; Stein et al. 2010) and thereby help to advance hatching (Lyon and Montgomerie 1985; Nilsson and Smith 1988), improve hatching success (Galván and Sanz 2011; Lyon and Montgomerie 1985; Nilsson and Smith 1988) or improve fledgling condition (Lifjeld and Slagsvold 1986; Røskaft 1983). This suggests that incubation feeding has evolved as a behavioural strategy to partly compensate for the energetic limitations of females while incubating (Galván and Sanz 2011). Although there are probable fitness advantages for the breeding pair derived from male incubation feeding, there may also be costs for males induced by intensified foraging activity at an early stage of the season (Leclaire et al. 2011; Lifjeld and Slagsvold 1986; Smith et al. 1989). Thus males may experience a trade-off between provisioning their mate and feeding themselves (Lifjeld and Slagsvold 1986; Lifjeld et al. 1987; Lyon and Montgomerie 1985; Moreno et al. 2011). They may also allocate more or less effort to finding and copulating with extrapair mates (Hill et al. 2011; Wagner 1992). Male incubation feeding intensity could thus be more a product of differences in male age, condition and mating strategy than of female nutritional needs (Lifjeld and Slagsvold 1986; 1989; Lifjeld et al. 1987).

To distinguish between the 'female nutrition' and alternative scenarios it is necessary to experimentally manipulate female condition and study male responses, as males may adjust their feeding activity to the optimal level of attendance at each nest in a nonexperimental situation (Moreno et al. 2011). Only according to the 'female nutrition' hypothesis would we expect a direct male response by either increasing (experimentally reduced female condition) or reducing (experimentally increased female condition) his provisioning rate. Both experimental approaches have provided support for the female nutrition hypothesis (reduced condition: Moreno et al. 2011; improved condition: Smith et al., 1989; Paillisson et al., 2007; Wright and Cuthill 1989; 1990a; 1990b).

To improve their condition during incubation, females should be able to communicate their needs to mates. As both sexes have at least partially overlapping reproductive interests (Moore and Rohwer 2012), communication between incubating females and their mates should be reliable (Searcy and Nowicki 2005). Begging by nestlings has received a fair amount of attention as an honest system of communication (Cotton et al. 1996; Mock et al. 2011; Wright and Leonard 2002), whereas begging between mates has received scant attention. Females beg to their mates in courtship contexts (Clancy 2005; East 1981; Ellis et al. 2009; Otter et al. 2007; Tobias and Seddon 2002), while incubating (Ellis 2008; Moore and Rohwer 2012; Tobias and Seddon 2002) and also during the nestling feeding phase before apportioning food to the nestlings (Clancy 2005). Female begging displays include loud vocalizations, body postures and wing fluttering, which closely resemble the begging displays of older nestlings (Ellis et al. 2009; Godfray 1991; Harper 1986). The striking similarity of female and nestling begging displays suggests the retention into adulthood in females of typically juvenile behaviours (Moore and Rohwer 2012). Otter et al. (2007) manipulated the hunger levels during egg laying of black-capped chickadee, *Poecile atricapillus*, females and showed no effect on male provisioning, even finding a decrease in female food solicitation. Furthermore, Moore and Rohwer (2012) found a correlation between begging displays of incubating yellow warbler, *Setophaga petechia*, females and mate provisioning rate in relation to environmental conditions. However, to our knowledge, it has never been confirmed experimentally that males adjust incubation feeding effort to female begging intensity. To test this link, begging intensity could be manipulated directly although this is difficult. Several behavioural components (posture, vocalizations, wing fluttering) presumably contribute to begging behaviour but the information content expressed in each component is still unknown. Alternatively, begging behaviour may be manipulated through hunger. Hunger depends on energy balance which may be experimentally altered through either food supplementation or handicapping (see above).

In many passerines such as the pied flycatcher, females incubate alone and receive some of their food from their mates (Cantarero et al. 2013b; Moreno et al. 2011). Some experimental studies have shown that pied flycatcher males seem able to adjust incubation feeding to female requirements (Moreno et al. 2011), although the behavioural mechanism behind male responses remains unknown. To test whether female begging during incubation is an honest signal of energetic need and whether mates respond to it in the pied flycatcher we followed previous experiments with this species (Lifjeld and Slagsvold 1986) by experimentally handicapping some females by clipping two primary flight feathers (Moreno et al. 2011). Handicapping is a useful and widely employed method to study the effects of energetically challenging situations on bird behaviour (Harrison et al. 2009). We assumed that clipping should increase the female's flight costs and therefore her energy requirements during incubation (Matysioková and Remeš 2011; Pennycuick 1982). We then compared control and experimental females by videoing their behaviour within the nestbox during incubation (before and after female manipulation) and at two stages of the nestling period (3 and 9 days of age).

We predicted following the 'female nutrition hypothesis' that impaired flight ability caused by handicapping would (1) lead to increase female begging displays during incubation because of the manipulation of female condition and hunger and (2) induce more male incubation feeding in response to female needs. Depending on the effects of the experiment on male incubation feeding rate, we might or might not expect changes in female incubation behaviour and in body mass loss between incubation and the nestling phase.

METHODS

Study area and species

The study was conducted during the spring of 2013 in a montane forest of Pyrenean oak, *Quercus pyrenaica*, at 1200 m above sea level in Valsaín, central Spain (40°54'N, 4°01'W) where pied flycatchers breeding in nestboxes have been studied since 1991 (see Sanz et al. 2003 for a general description). Of 570 nestboxes, 102 were occupied by pied flycatchers (see Lambrechts et al. 2010 for dimensions, structure and placement of nest-boxes).

Egg laying in the pied flycatcher population under study typically begins in late May (Cantarero et al. 2013b), and the modal clutch size is six. The female incubates and broods alone and receives some of her food from her mate (Cantarero et al. 2013b; Moreno et al. 2011). No brooding is observed after nestlings attain 7 days of age (Sanz and Moreno 1995). Breeding activities are followed routinely every year and laying and hatching dates and brood sizes at hatching and fledging are determined (Cantarero et al. 2013a).

On day 3 (hatching day = day 1), we weighed jointly all nestlings with a digital scale to the nearest 0.1 g. On day 13, we ringed nestlings and measured their tarsus length with a digital calliper to the nearest 0.01 mm and their wing length with a stopped ruler to the nearest mm. Nestlings were also weighed with a digital balance to the nearest 0.01 g. The parents were captured in their nestboxes with traps and weighed and measured in the same way as nestlings. Parents were not inside the trap for more than 5 min. All the procedures between capture and release of the bird took less than 10 min.

Female handicapping

Of the 102 nestboxes occupied by pied flycatchers we selected those with laying dates between days 43 and 65 (1 April =day 1). We assigned 71 nests randomly to two groups. We included 39 females in the control and 32 females in the experimental group.

Seven or eight days after clutch completion, incubating females were captured in the nestbox during the day without traps as they are not easily frightened away from the nest at this stage (see Moreno et al. 2011 for a similar protocol). They were banded if necessary, identified and weighed to the nearest 0.01 g with a digital balance. Primaries 7 and 9 on each wing (counting inwards from the distal margin of the wing) were clipped at the base of the rachis with scissors in experimental females while only the tips of these primaries were clipped in control females (Moreno et al. 1999; 2011). The whole procedure took around 5–10 min. The experimental manipulation was admittedly mild to simulate natural situations rather than to enforce drastic effects with possible repercussions on desertion probability (Moreno et al., 2011; but see Matysioková and Remeš 2011 for a more drastic manipulation). The wing manipulation had no observable effect on female behaviour outside the nestbox. No female deserted after manipulation.

Video recordings

Five and 10 days after clutch completion (days 6 and 11 of incubation) we recorded nest activity inside nestboxes for about 100 min (101.68 ± SE 17.42 min, *N*=135) with a cold white light (LED 5 mm) powered by a 3 V battery and a camera (GoPro HD Hero1) mounted on the roof inside the nestbox (Cantarero et al. 2013a). We obtained two incubation records for each nest, before and after treatment.

Nestboxes were again filmed 2 days after the day of hatching of the young for periods of 99.63 ± SE 9.47 min (*N*=69) and 8 days after hatching of the young for periods of 97.08 ± SE 14.93 min (*N*=67). Because of technical problems, we failed to record the behaviour at seven nests during incubation and two nests with young nestlings. In four nests all chicks died after day 3 (one control and three experimental nests) but we have included earlier records for these nests. The death of nestlings was associated in all cases with rainy weather and occurred at least 10 days after female manipulation and 2–3 days after the nests were filmed in the early nestling phase. All the nestboxes in the study area are protected against predators. All films were recorded between 0800 and 1500 hours, and no differences between experimental groups with respect to time of filming were found (first incubation record: *U*=467.5, *P*=0.373; second incubation record: *U*=503.5, *P*=0.548; nestling period day 3: *U*=472.5, *P*=0.150; nestling period day 9: *U*=533.5, *P*=0.969). As in previous studies (Cantarero et al. 2013a; 2013b), no evidence of stress or unnatural behaviour such as extremely long periods of absence from the nest or trying to peck at the camera system were observed after the first visit.

Behavioural data analysis

Recordings were displayed in the free VLC Media Player software. From records taken during incubation we estimated the proportion of time spent by the female inside the nestbox or 'egg attendance' which includes the time allocated to incubating and turning the eggs, and the mean duration of incubation sessions and recesses (Cantarero et al. 2013a). In addition, we counted incubation feedings by males. We also recorded female begging displays by quantifying the call duration, the posture during begging and the prey brought by the male (Fig. 1).

Female begging postures were assigned following a scale of increasing intensity: 0 = no arrival of male; 1 = female does not move upon arrival of male; 2 = takes the prey with low calls; 3 = takes the prey with loud calls; 4 = same as 3, but leaves the nest cup to approach the nest entrance without reaching it; and 5 = same as 3, but leaves the nest cup and puts her head out of the nest entrance in order to collect the prey. We identified prey as caterpillars (value 1) or other prey (value 0). On each visit of the male to the nest with food, we recorded the begging time of the female, the female posture during begging and the prey brought by the male. We then estimated the average value of these variables at each visit for the incubating female.

Figure 1. Example of female begging behaviour when males visit the nestbox with prey during incubation.

From recordings during the early nestling phase we obtained hourly provisioning rates by males and females and the amount of time spent by females on 'nestling attendance'. 'Nestling attendance' includes the proportion of time spent by the female inside the nestbox (Cantarero et al. 2013a). We also counted male feeds aimed at the female and those aimed at the nestlings. We quantified the posture of females and the prey brought by the male while the female was brooding. These variables were estimated in the same way as during the incubation stage. From recordings during the late nestling phase we obtained hourly provisioning rates by males and females.

Data analyses

Breeding variables were normally distributed and were therefore analysed with GLM models (STATISTICA, Statsoft, Tulsa, OK, U.S.A.) assuming a normal error with treatment as explanatory factor. Clutch size and brood size were analysed with GLM models assuming a Poisson distribution with treatment as explanatory factor. The effects of treatment on brood-averaged nestling morphometric measurements and mass near fledging were analysed with GLM models with treatment as explanatory factor and hatching date and brood size as continuous predictors. Hatching success (proportion of eggs that hatched) and fledging success (proportion of hatched chicks that fledged) were analysed as frequencies (Yes-1/No-0 cases of all chicks hatched and Yes-1/No-0 cases of all chicks hatched becoming fledglings) with chi-square contingency tables.

All parametric behavioural variables for the incubation stage were analysed with repeated measures ANOVA with treatment as explanatory factor and time as repeated measures effect (before or after female capture). All parametric behavioural variables for the young nestling phase were analysed with treatment as explanatory factor and hatching date, brood size and mate provisioning rate as continuous predictors. All parametric behavioural variables for the late nestling phase were analysed with treatment as explanatory factor and hatching date and brood size as continuous predictors.

Ethical note

We were authorized to handle pied flycatchers by Consejería de Medio Ambiente de Castilla y León and J. Donés, director of 'Centro Montes de Valsaín' to work in the study area (protocol number EP/SG/193/2013). The experiments comply with current Spanish laws, and grant holder and field researchers were officially licensed for animal manipulation following current EU regulations on animal manipulation (authorization types C and B by regional authorities).

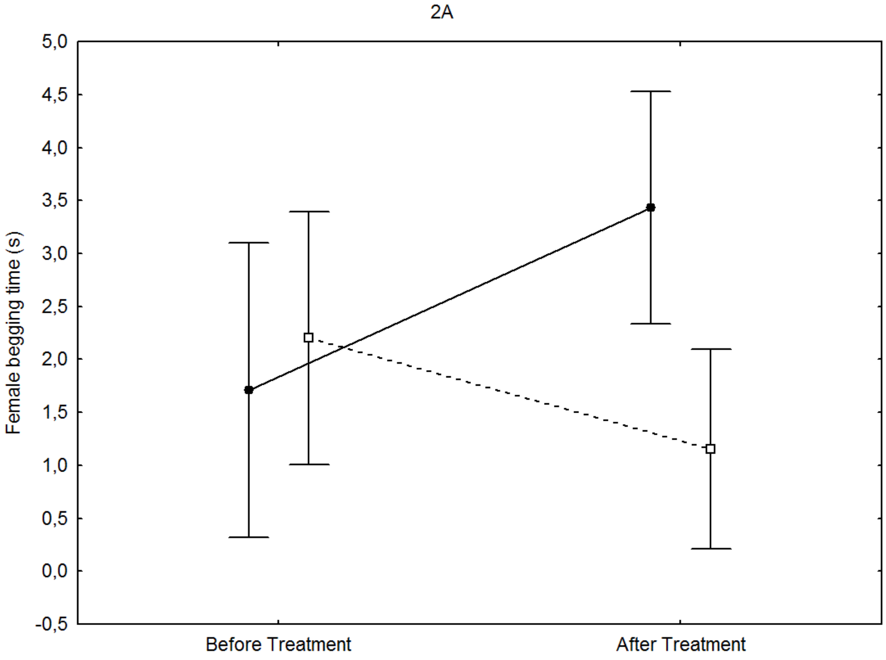
RESULTS

Females in the two treatments did not differ with respect to breeding variables or mass at incubation during capture (Table 1). Female mass during the nestling phase was positively correlated with female incubation mass (r_S=0.56, $F_{1,60}$=17.201, P<0.001), and was affected by treatment (control: 12.57 ± 0.70 g, N=38; experimental: 12.17 ± 0.68 g, N=30; $F_{1,65}$=5.28, P=0.025). Male mass during the nestling phase was similar in the two treatments (control: 12.17 ± 0.51 g, N=34; experimental: 12.20 ± 0.59 g, N=29; $F_{1,61}$=1.35, P=0.820).

	Control	**Experimental**	**Statistic**	***P***
Laying date	57.308 ±	58.406 ±	F_1 = 0.851	0.359
Hatching date	76.231 ±	77.281 ±	F_1 =1.340	0.252
Clutch size	5.256 ± 0.938 (39)	5.344 ± 0.971(32)	Wald =0.025	0.874
Brood size 13 days	4.462 ± 1.274 (39)	4.218 ± 1.660(32)	Wald =0.238	0.626
Female incubation mass	14.184 ±	14.159 ±	F_1 = 0.010	0.917

Table 1. Average ±SE (*N* in parentheses) values for breeding variables of nests included in the two treatments prior to manipulation and results of GLM analyses.

Treatment did not interact significantly with the repeated measures effect for any female incubation variable (all P>0.20), nor for type of prey delivered ($F_{1,63}$=0.007, P=0.935). There was a significant interaction between treatment and the repeated measures effect during incubation for female begging displays and male feeding. While females in the control group showed a similar duration of calling during the two sequential observation periods, females in the experimental group showed a marked increase in begging time after being handicapped (Fig. 2a; interaction of treatment with repeated measures: $F_{1,57}$=7.133, P=0.009). The same pattern was observed in female begging posture (Fig. 2b; interaction of treatment with repeated measures: $F_{1,57}$=7.031, P=0.010).



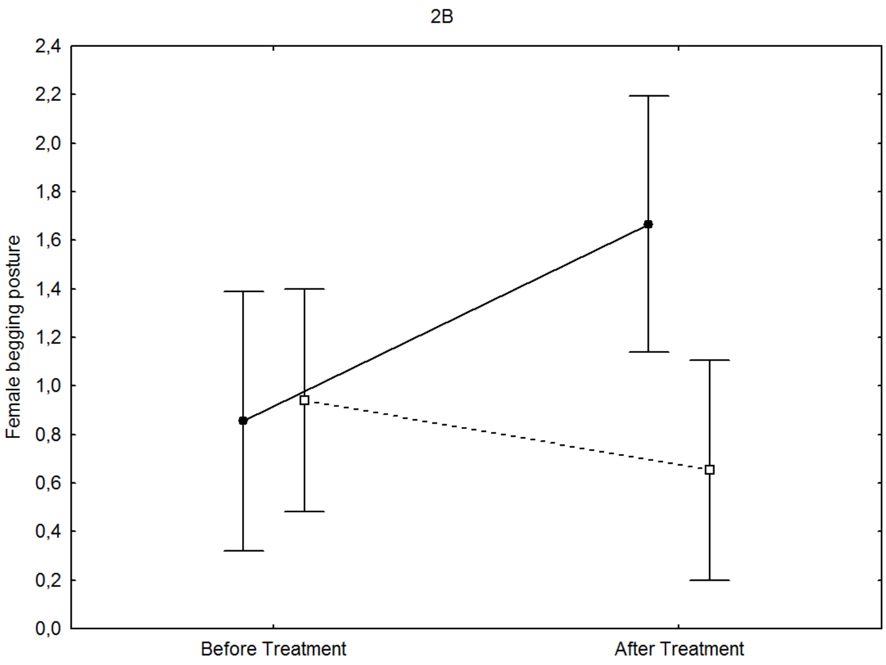


Figure 2. Variation in begging displays of pied flycatcher females during incubation, comparing control (□) and experimental females (●). Means ± SE of (a) female begging time and (b) female begging posture before and after manipulation are presented.

Males increased their incubation feeding rates to females between observations in the experimental group but not in the control group (Fig. 3; interaction of treatment with repeated measures: $F_{1,62}$=7.382, P=0.008). Controlling for treatment, postmanipulation male incubation feeding rate was strongly positively associated with female begging time ($F_{1,60}$=71.231, P=0.008). Female begging time explained 57% of the variation in male incubation feeding. Female mass loss between the two captures was not related to postmanipulation male incubation feeding rate when controlling for treatment ($F_{1,56}$=0.018, P=0.894). However, this mass loss covers the whole posthatching period of adaptive mass loss (Sanz and Moreno 1995) and not just the period for which male behaviour was studied.

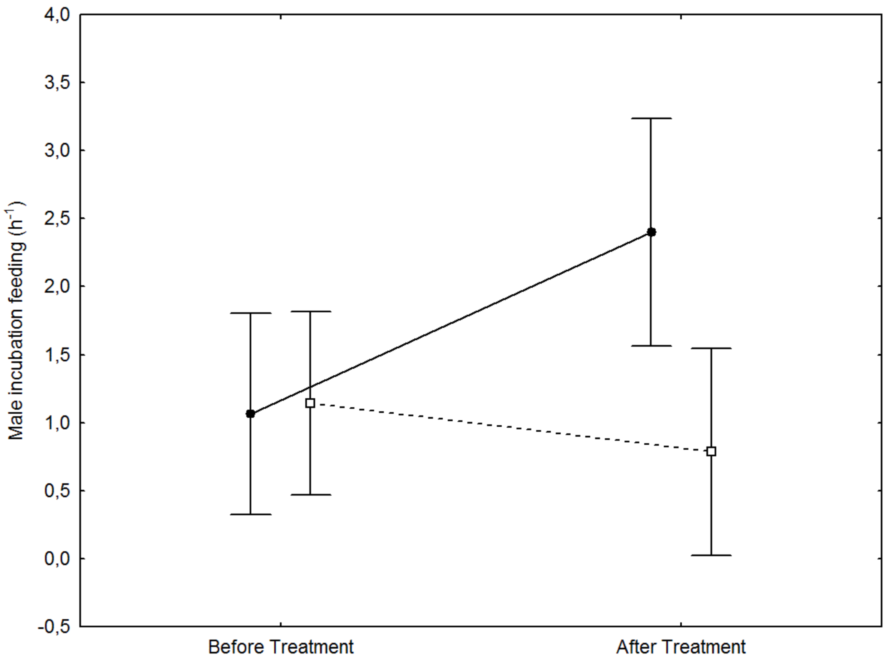


Figure 3. Variation in provisioning rates of pied flycatcher male during incubation, comparing control (□) and experimental nests (●). Means ± SE of male hourly provisioning rate before and after manipulation are presented.

On day 3, nestling attendance (proportion of time spent by the female inside the nestbox) did not differ between treatments and was positively related to male provisioning (Table 2).

Provisioning rates of males were similar in both treatments (Table 2). Female provisioning rates were lower in experimental nests, negatively related to mate provisioning and positively related to brood size (Table 2). There was no difference in direct male feeding rates to females (Table 2), but the proportion of prey consumed by the female with respect to total prey delivered by the male was higher in the experimental group (Fig. 4; $F_{1,41}$=6.027, P=0.018).

	Control	**Experimental**	**Treatment statistic**	**Brood size statistic**	**Hatching statistic**	**Mate statistic**
Nestling day 3						
Nestling attendance (%)	55.75 ± 22.65	59.27 ± 19.69	*F*=1.403	*F*=0.598	*F*=0.868	*F*=4.431*
Male total provisioning (per h)	10.61 ± 4.30	11.18 ± 4.76	*F*=0.011	*F*=0.352	*F*=0.065	*F*=2.746
Male provisioning to female (per	5.62 ± 4.41	5.31 ± 5.31	*F*=0.393	*F*=4.496*	*F*=0.886	*F*=15.227**
Female provisioning (per h)	5.11 ± 3.86	4.80 ± 4.21	*F*=4.841*	*F*=11.309**	*F*=1.273	*F*=6.863*
Female begging posture	1.37±0.39	2.00 ± 0.60	*F*=14.818**	*F*=0.005	*F*=0.192	*F*=1.311
Nestling day 9						
Male provisioning (per h)	11.08 ± 4.24	13.77 ± 4.90	*F*=4.457*	*F*=4.053*	*F*=0.235	-
Female provisioning (per h)	12.57 ± 6.70	10.41 ± 5.95	*F*=1.843	*F*=1.396	*F*=1.187	-
Total provisioning by pair (per h)	19.52 ± 7.28	20.96 ± 9.40	*F*=0.328	*F*=5.978*	*F*=0.658	-

P<0.05; ***P*<0.01.

Table 2. Differences (means ± SE, *N* in parentheses) in behavioural variables between the two treatments and results of GLM analyses.

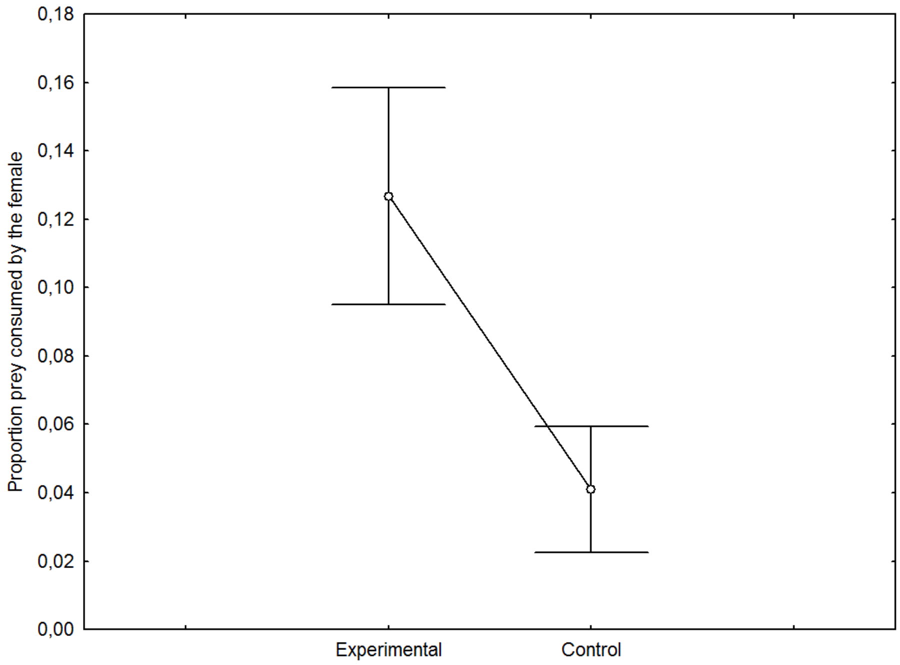


Figure 4. Differences in the proportion of food deliveries consumed by the female with respect to total prey deliveries by the male comparing control and experimental nests on day 3 of nestlings. Means ± SE are shown for each treatment.

Females in the experimental treatment begged more intensely also at this stage (Table 2). On day 9, female provisioning rates were not related to treatment (Table 2) when controlling for brood size and hatching date. Males in the experimental treatment provisioned more at this stage while there was a positive effect of brood size on male provisioning (Table 2). The total provisioning rate by the pair was similar in both treatments and was positively affected by brood size (Table 2).

The control (3.02 ± 0.36 g, N=39) and experimental (2.99 ± 0.68 g, N=32) groups did not differ in mean nestling mass (g) on day 3 ($F_{1,69}$=0.067, P=0.797). There were no differences between treatments in nestling morphometric measurements on day 13 when controlling for hatching date and brood size (all P>0.20). Nestling body mass was negatively related to brood size ($F_{1,62}$=6.428, P=0.014). The nests from the two treatments did not differ in hatching success (χ^2_2=0.52, P=0.473; control nests: 0.56 ± 0.50, N=39; experimental nests: 0.69 ± 0.47, N=32) or fledging success (χ^2_2=1.03, P=0.311; control nests: 0.87 ± 0.34, N=39; experimental nests: 0.78 ± 0.42, N=32).

DISCUSSION

This study shows that experimentally handicapping female pied flycatchers during the incubation stage led to intensified begging displays arising from condition impairment and that males were able to respond by increasing their feeding rates to females. Experimental females produced longer vocalizations and modified their begging posture after being manipulated. Female begging largely explained male feeding behaviour. Handicapping had no influence on female incubation behaviour or hatching success. Moreover, handicapped females fed their chicks at the same intensity as control females, but they showed a greater decrease in body mass. They also took a higher proportion of male food deliveries to the nest for themselves than control females during the first few days after hatching. The provisioning rates of males in the late nestling stage were higher in experimental nests. Male and nestling condition were unaffected by the treatment.

Female begging displays are honest and adaptive if males can assess the nutritional state of their mates (Tobias and Seddon 2002) and males may gain an advantage by responding to female needs through food provisioning (Otter et al. 2007). Communication between females and their mates should be an honest system because of their common interests (Moore and Rohwer 2012; Searcy and Nowicki 2005). Females probably benefit from begging by increasing male incubation feeding rates (Moore and Rohwer 2012) and, by supplying food, males may directly enhance their own fitness. Our results are consistent with this scenario, as we found that incubating females communicate energetic needs to their mate and adjust their begging intensity when their nutritional state is manipulated through handicapping. Most studies involving flight feather removal assume that a reduced wing area affects wing loading and thereby increases flight costs (Slagsvold and Lifjeld 1988; Wright and Cuthill 1990a; Wright and Cuthill 1990b). The higher flight cost imposed by handicapping in experimental females could negatively affect foraging efficiency and thereby reduce energy input. Moreover, the short flights involved in foraging are energetically very costly which may increase energy output in small passerines (Carlson and Moreno 1992; Tatner and Bryant 1986). The combination of the two effects may therefore negatively affect energy balance.

During intense begging displays, females flutter their wings and are constantly calling to their mate during male visits. Different components of begging displays vary in the same direction to enrich the information content of the female signal (Gottlander 1987; Moore and Rohwer 2012) and our results show that this signal is honest over time. Begging behaviour may be a finer predictor of nutritional need as evidenced by several previous studies based on food deprivation experiments in nestlings (Budden and Wright 2008; Marques et al. 2009; Villasenor and Drummond 2007), food supply experiments in females (Otter et al. 2007) or by correlating environmental conditions with female condition (Moore and Rohwer 2012). In our study, female begging behaviour continued during at least the first 3 days after hatching. When males visited the nest during female brooding bouts, they were met by female begging which was more intense in handicapped females. As females can then allocate food to themselves or the nestlings, they can adjust self-feeding to their needs. This has only been observed previously in hornbills (Ng et al. 2011) and raptors (Durant et al. 2004; Sonerud et al. 2013). Contrary to our second prediction but in agreement with the results obtained by Matysioková & Remes (2010; Matysioková and Remeš 2011) in great tits, *Parus major*, we found that male incubation feeding did not predict female nest attentiveness. Since handicapping increases wing loading and thus the costs of flight (Pennycuick 1982), male incubation feeding may compensate for changes in female energy demand, thereby removing effects on attendance or reproductive success (Moreno et al. 2011). Smith et al. (1989) found that males decreased their rate of incubation feeding when females increased incubation attentiveness as a result of a supplementary food experiment, a result that could be obtained by reduced female begging intensity in the experimental situation and not through direct observation by males of female nest attendance.

Handicapped females did not reduce parental care intensity as shown by nestling provisioning rates on day 9. In most manipulative studies, the main effect of handicaps is a decrease in the experimental birds' nestling provisioning rate (Sanz et al. 2000; Slagsvold and Lifjeld 1988; Slagsvold and Lifjeld 1990) or an increased mass loss (Ardia and Clotfelter 2007; present study; Sanz et al. 2000). To maintain the same provisioning effort and attendance as in control females, handicapped females may forage just enough to adequately feed their chicks but not enough to sustain their own body condition (Leclaire et al. 2011), in this way bearing the costs of the handicap themselves (Matysioková and Remeš 2011 , but see Moreno et al., 1999). The higher declines in mass observed in experimental females may be interpreted as a physiological consequence of reduced foraging efficiency (Winkler and Allen 1995) or as an increase in reproductive costs by reducing long-term physiological condition (Alonso-Álvarez and Tella 2001). Another possible explanation for body mass reduction may be an adaptation to compensate for the higher flight cost imposed by increased wing loading (Moreno 1989; Pennycuick 1982).

Although there were opposing trends in provisioning rates for males and females with respect to treatment, the difference was only significant for males. However, the total provisioning rate by the pair did not differ with respect to treatment which agrees with the lack of differences in nestling size and condition between treatments. The absence of effects of provisioning rates on male condition suggests that our measure of condition may be insufficiently precise to detect them.

To conclude, our study is the first to confirm experimentally a basic assumption of the 'female nutrition' hypothesis, namely that males adjust feeding rates to behavioural displays of need by their mates. Female begging behaviour should be considered a communication system enabling successful reproduction in birds with female uniparental incubation (Galván and Sanz 2011). The ability of females to modify their begging displays may be an important adaptation that allows females to maintain an adequate energetic condition during incubation. Further studies should explore the full information content of this intersexual communication channel.

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GENERAL DISCUSSION

The results of the present thesis answer several interesting questions about the ecology and behaviour of cavity-nesting birds. The thesis contains six chapters that deal with behavioural strategies arising during different stages of the reproductive cycle. On the one hand, **chapters I, II and III** explore the behavioural mechanisms that small cavity nesters have evolved to select a nest-box, how protect it from competitors, and to construct a nest with adequate nest material and structure. On the other hand, **chapters IV, V and VI** deal with the different behaviours that these birds have evolved to reduce the impact arising from ectoparasites or an impaired body condition in order to maximize their fitness.

For obligate cavity-nesting birds, nest-holes and/or nest-boxes erected by humans constitute a scarce resource that may limit the availability of breeding opportunities, leading to a strong competition over them (Ingold 1994; Leffelaar and Robertson 1985; Li and Martin 1991; Nilsson 1984). This competition may constitute an important selective force for the evolution of aggressive female behaviours which may be mediated by testosterone (T) levels. **Chapter I** shows that these levels differ between populations of the same species, being higher in populations where the likelihood of nest-site usurpation by intruders is greater. In contrast to some studies which have found strong positive associations between female aggressive behaviour and endogenous T (Cain and Ketterson 2012; Elekonich and Wingfield 2000; Gill et al. 2007), we found that the level of female aggressiveness against intruders decrease with higher T levels in high density areas. Females with higher T may experience a lower threat imposed by intruders and consequently ignore rather than attack intruders, a result which should be considered in future studies of female territorial aggression.

After obtaining a nest-hole or nest-box, nest building begins. Nest construction may be influenced by factors such as the availability of nest materials (Moreno et al. 2009) and may involve a large expenditure of time and energy (Moreno et al. 2008) due to the costs of transporting material to the nest site (Putnam 1949). **Chapter II** shows some clear patterns in the preference of certain nesting material and nest-box for breeding in Nuthatches. The selection of nesting material and the amount of mud in Nuthatch nest-boxes can be explained as a trade-off between requirements for nest construction and availability as a function of transport distance. This trade-off may be stronger in species building nests composed of scarce or specialized materials and may have led to marked territorial habits as in nuthatches in relation not only with food availability but with the requirements for nest construction as well.

Nesting holes constitute micro-environments very likely to be colonized by ectoparasites that feed on blood, skin and feathers of avian hosts (Collias and Collias 1984; Mazgajski 2007). It has been suggested that Nuthatches *Sitta* spp. use pine bark as nesting material because it contains toxic secondary compounds that may have insecticidal properties (Carroll 1994). Furthermore, nests made of loose heaps of bark flakes without any structure may offer fewer opportunities for hiding to ectoparasites and nest composition may affect ectoparasite development through the effects of microclimatic conditions associated with different nest materials (Heeb et al. 2000). In order to explore several possible implications of breeding in unstructured bark nests for Nuthatches in **Chapter III** we conducted an experiment where natural nests were replaced by structured nests made of materials different from bark. The replacement of unstructured bark nests by structured moss nests did not result in changes in ectoparasite loads, which suggests that the preferences for nest materials in Nuthatches and possibly other cavity nesters may be unrelated to ectoparasitism (this does not include the addition of specific insecticidal materials on top of nests). These results were also confirmed in **Chapter V**. We suggest that Nuthatches build nests of loose aggregations of bark flakes to reduce the thermal loss of nestlings experienced in open-cup nests compared to being buried into loose and heat-producing bark flakes. Thus, nuthatches have evolved to fill relatively large and well-isolated cavities due to mud plastering, a resource for which there might have been less interspecific competition, with loose composting nest materials. Nestlings may thereby have lost huddling instincts and depended on composting nest materials for thermal savings during their heterothermic stage. The joint or successive evolution of mud plastering, use of composting materials and loss of huddling in nuthatches remains to be resolved through comparative analyses.

Generalist ectoparasites infest nests of avian cavity-nesting passerines as a response to different factors exhibited by host species. In **Chapter V** we show that differences in nest composition among host species may not be the main factor explaining ectoparasite prevalences and abundances, while nest size, breeding phenology, brood size and nest-cavity micro-climate may all affect levels of infestation in different ways for each host-parasite association. These results confirm some previously obtained experimentally in the same populations. Future studies on the implications of nest structure and composition should avoid concentrating on implications for ectoparasites and focus on implications of availability of nest materials, phenology and possibly antibacterial properties. Given the negative impact of ectoparasites on nestlings there will be selection on hosts to reduce parasite infestations through behavioural means. In **Chapter IV** we experimentally reduced the abundance of all ectoparasites in nests of pied flycatchers to explore changes in the frequency and duration of anti-parasite behaviours by tending adults. The frequency and intensity of female grooming and nest sanitation behaviours during the incubation and nestling periods decreased as a consequence of the experimental reduction of ectoparasite infestation (**Chapter IV**), and these behaviours were more intense in the host species with highest infestation levels (**Chapter V**). The difference in the time invested in behavioural defences both intra and interspecifically indicates that females of cavity nesters may experience a trade-off between increasing the time allocated to the control of nest ectoparasites and other uses of time spent in the nest like resting or tending eggs or nestlings. Moreover, avoidance tactics by the parasites may reduce the efficacy of nest sanitation. Thus, females are not able to remove completely their natural deleterious effects on nestling growth and survival (**Chapter V**). The importance of alternative functions of sitting on the nest and the efficiency of parasite avoidance tactics in different types of nests remains to be resolved.

In **Chapter IV** we also showed that nestlings begged more intensely as a response to their higher nutritional needs arising from higher ectoparasite loads. While begging by nestlings has received a fair deal of attention as an honest system of communication (Cotton et al. 1996; Mock et al. 2011; Wright and Leonard 2002), begging between mates has received scant attention. It is known that females beg to their mates in courtship contexts (Clancy 2005; East 1981; Ellis et al. 2009;

Otter et al. 2007; Tobias and Seddon 2002) and while incubating (Ellis 2008; Moore and Rohwer 2012; Tobias and Seddon 2002). In **Chapter VI** we tested if female begging during incubation is an honest signal of energetic need by experimentally handicapping some females through clipping of two primary flight feathers. We found that experimentally handicapping female pied flycatchers during the incubation stage intensified begging displays arising from condition impairment and that males were able to respond by increasing their feeding rates to females. Females are able to modify their begging displays and it may be an important adaptation to maintain a good energetic condition during incubation. The postural and acoustic communication channel between mates may be important in other contexts such as conflicts about investment in nest construction or nestling care. The evolutionary implications of this type of communication of need may be crucial for understanding the evolution of biparental care and the reduction of sexual conflict.

CONCLUSIONS

- The relationship between testosterone and competitive behaviour in females can be complex and differ between populations of the same species. The population differences in testosterone levels of females reflect the need to defend nesting cavities and this need is stronger where the likelihood of usurpation by intruders is greater.
- The selection of nest sites and nest materials in Nuthatches may be constrained by costs of transport of nest material. Nuthatches use pine bark as nesting material only when nest-sites are situated close to pines, and use more mud when breeding close to streams.
- Nest structure and nesting material have important consequences on microclimate and breeding behaviour in the Nuthatch.
- Adaptations of Nuthatches nestlings for remaining buried in the nest material beside the heat-conserving properties of loose bark flakes nests reduce energy costs for nestlings during female absences.
- Ectoparasites have deleterious effects on nestling growth and survival and induce significant changes in female anti-parasite behaviours.
- Nest composition is not the main factor explaining ectoparasite prevalences and abundances, while nest size, breeding phenology, brood size and nest-cavity micro-climate may all affect levels of infestation in different ways for each host-parasite association.
- Anti-parasite behaviours are more intense in species with higher infestation levels.
- Female pied flycatchers are able to modify begging displays arising from their condition and males adjust incubation feeding rates to behavioural displays of need by their mates.

SUMMARY OF RESEARCH AND SCHOLARLY ACTIVITIES (PHD)

- **GRANTS**

2011-2015 Pre-doctoral Fellowship

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✓ CANTARERO, A., LÓPEZ-ARRABÉ, J. y MORENO, J. 2015. Selection of nest-site and nesting material in the Eurasian Nuthatch *Sitta europaea*. Ardea (in press).

✓ GONZÁLEZ-BRAOJOS, S., VELA, A.I., RUIZ-DE CASTAÑEDA, R., BRIONES, V., CANTARERO, A. y MORENO, J. 2015. Bacteria on nestling skin in relation to growth in pied flycatchers. Journal of Ornithology 156: 327–330.

✓ CANTARERO, A., LÓPEZ-ARRABÉ, J., SAAVEDRA-GARCÉS, I., RODRÍGUEZ-GARCÍA, V., PALMA, A. y MORENO, J. 2014. The significance of nest structure and nesting material for hole-nesting passerines: an experimental study with Nuthatches *Sitta europaea*. Acta Ornithologica 49: 143-155.

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✓ CANTARERO, A., López-Arrabé, J., RODRÍGUEZ-GARCÍA, v., GONZÁLEZ-BRAOJOS, S., RUIZ-DE-CASTAÑEDA, R., REDONDO, A. J. y MORENO, J. 2013. Factors affecting the presence and abundance of generalist ectoparasites in nests of three sympatric hole-nesting bird species. Acta Ornithologica 48: 39-54.

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