Efectos comportamentales, fisiológicos y reproductivos del riesgo de depredación y del parasitismo de cría en la reproducción de las aves: ¿importa el miedo?

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A mis padres, Carmen y Martín.

A mi hermana, Marta.

Resumen/Abstract

RESUMEN

La depredación y el parasitismo de cría interespecífico son dos fuerzas selectivas fundamentales que han contribuido a modular el comportamiento reproductivo y las estrategias vitales de las aves. Recientemente se ha sugerido que, además de los efectos directos de la depredación, el riesgo de sufrir depredación (miedo) podría tener efectos sub-letales afectando rasgos comportamentales y reproductivos en tiempo ecológico y evolutivo. No se ha estudiado aún si el miedo a sufrir parasitismo de cría podría igualmente afectar a comportamientos distintos de los implicados en la defensa frente a los parásitos o a rasgos reproductivos de los hospedadores.

La presente Tesis tiene por objetivo fundamental identificar los efectos indirectos o sub-letales del miedo a la depredación y al parasitismo de cría interespecífico en varias especies de aves. Para abordar este objetivo general, se estudiaron las respuestas comportamentales, modulación de rasgos de historia vital y respuestas fisiológicas a una serie de manipulaciones experimentales del riesgo de depredación y de parasitismo de cría antes y durante la reproducción y a distintas escalas espaciales, en una comunidad de aves no excavadoras que anidan en cajas nido y en una población de Urracas *Pica pica*, que sufre parasitismo de cría por el Críalo europeo *Clamator glandarius*. Además, evaluamos la respuesta al riesgo percibida a través de distintas pistas que pueden ser percibidas por canales de comunicación distintos (i.e. pistas olfativas, acústicas y visuales).

En resumen, esta tesis aporta nuevas evidencias de los efectos sub-letales de la depredación y parasitismo de cría sobre las decisiones previas a la reproducción de las aves en ambientes naturales. Estos efectos se han mostrado a través de cambios en la elección de los territorios de reproducción y tienen efectos indirectos sobre la estructura, composición y nivel de interacción entre gremios dentro de la comunidad. Encontramos también efectos del riesgo de depredación percibido durante la reproducción mediante

1

Resumen/Abstract

cambios en el cuidado parental. Sin embargo, no hemos encontrado evidencias claras de los efectos del riesgo de parasitismo sobre rasgos reproductivos y fisiológicos en un hospedador de un parásito obligado de cría una vez comenzada la reproducción. Finalmente, los resultados sugieren que la actividad humana también puede ser percibida como un factor de riesgo modulando el estado fisiológico de especies en declive poblacional como consecuencia de la alteración de sus hábitats. Por tanto, los resultados conjuntos de esta tesis ofrecen nuevos avances en el estudio del riesgo de depredación y parasitismo de cría en tiempo ecológico de gran interés tanto en el campo de la Ecología Evolutiva y del Comportamiento como por su aplicación en la Conservación y Gestión Animal. Además, los resultados de la tesis en su conjunto sugieren que el estudio de múltiples pistas es fundamental para llegar a una compresión profunda de los efectos del riesgo puesto que las aves tienen la capacidad de evaluarlo en base a pistas que perciben por distintos canales de comunicación.

ABSTRACT

Predation and inter-specific avian brood parasitism are two fundamental selective forces that have contributed to modulate the reproductive behavior and life-history strategies of birds. Recently it has been suggested that, in addition to the direct effects of predation, the risk of suffering predation (fear) could have sub-lethal effects affecting behavioral and reproductive traits in ecological and evolutionary time. It has not yet been studied whether the fear of suffering brood parasitism could also affect behaviors other than those involved in the defense against parasites or reproductive traits of the hosts.

The main objective of this thesis is to identify the indirect or sublethal effects of fear of predation and inter-specific brood in several bird species. To address this general objective, we studied possible changes in behaviors, life-history traits and physiology in response to a series of experimental manipulations of predation and inter-specific brood parasitism risk before and during reproduction. Experimental procedures were designed at different spatial scales in a community of non-excavating birds, which reproduce in nest-boxes, and in a population of Magpie *Pica pica*, which suffers cuckoo parasitism by the Great Spotted Cuckoo *Clamator glandarius*. In addition, we evaluated the response to perceived risk through different cues that can be perceived by different communication channels (i.e. olfactory, acoustic and visual cues).

In summary, this thesis provides new evidence of the sub-lethal effects of predation and inter-specific brood parasitism on decisions prior to the reproduction of birds in natural environments. These effects have been shown through changes in nest-site choice and have indirect effects on the structure, composition and level of interaction between guilds within the community. We also found effects of the risk of nest predation perceived during reproduction through changes in parental care. However, we have not

Resumen/Abstract

found clear evidence of the effects of the risk of parasitism on reproductive and physiological traits in a host of an obligate brood parasite, once reproduction has begun. Finally, the results suggest that human activity can also be perceived as a risk factor modulating the physiological state of species under population decline, as a consequence of the alteration and disturbances in their habitats. Therefore, the results of this thesis offer new advances in the study of the risk of predation and the risk of cuckoo parasitism in ecological time of great interest both in the field of Evolutionary Ecology and Behavior and its application in Animal Conservation and Management. In addition, the results of the thesis suggest that the study of multiple cues is necessary to deeply understand the effects of risk, given that birds may evaluate threats perceived by cues through different communication channels.

Introducción General

La depredación y el parasitismo de cría como fuerzas selectivas

Los individuos de cualquier especie animal han de enfrentarse durante su vida a una gran variedad de amenazas que se presentan en su entorno y que afectan a su supervivencia y éxito reproductor (Caro 2005). Entre estas amenazas se incluyen fuerzas selectivas como la depredación y el parasitismo de cría, que son las fuerzas que aquí trataremos, pero también otras como el parasitismo en general o la competencia.

La depredación es un tipo de interacción biológica en la que un individuo de una especie animal (el depredador) caza a otro individuo (la presa) para subsistir. Este tipo de interacción constituye una de las mayores fuerzas selectivas de la naturaleza al implicar la muerte directa de una de las partes de la interacción (Lima y Dill 1990; Lima 1998) y ha selecionado en las presas defensas anti-depredatorias para evitar los efectos directos de la depredación (Caro 2005). La reproducción es una fase de elevada vulnerabilidad a la depredación para gran parte de las aves ya que su actividad es más perceptible al ocurrir en torno a un lugar fijo (el nido). Un gran número de estudios seminales han mostrado que la depredación de nidos es la causa principal de fallo reproductivo para la mayor parte de las especies de aves (Ricklefs 1969; Martin 1993; 1995; Thompson 2007).

El estudio de la incidencia de la depredación se ha abordado fundamentalmente mediante aproximaciones comparativas que han estudiado la variación entre especies o grupos taxonómicos mayores. Así, se ha registrado variación interespecífica en aspectos como la biología de reproducción, tamaño de puesta, grado de sociabilidad o comportamiento de alimentación que se relacionan con la variación en la depredación sufrida en los nidos de esas especies (Ricklefs 1969; Martin 1995; Hansel 2000; Roff *et al.* 2005; Martin *et al.* 2007; Hipfner *et al.* 2010).

Por otro lado, el parasitismo de cría en aves es una estrategia reproductiva que afecta aproximadamente al uno por ciento de las aves y en la cual una de las partes (la especie parásita) pone sus huevos en los nidos de otra especie (el hospedador) de la que obtiene recursos en forma de cuidados parentales para su descendencia (Rothstein 1990). A pesar de su baja incidencia, en las especies parasitadas, el parasitismo de cría produce una disminución drástica del éxito reproductor del hospedador puesto que en muchos casos éste no puede sacar adelante sus propios pollos y cría a los del parásito, por lo que, en tiempo evolutivo, el parasitismo actúa como un potente agente selectivo sobre los comportamientos y rasgos de historia vital de sus hospedadores (e.g. Davies y Brooke 1988; Soler y Møller 1990). Así, por ejemplo, se ha descrito la evolución del comportamiento de defensa de nidos y reconocimiento de huevos parásitos o cambios en la apariencia de los huevos dentro de la puesta en respuesta a los parásitos (revisado en Davies 2000; Avilés y Møller 2003; Payne 2005).

De todo lo anterior se desprende que los efectos letales de la depredación y los costes del parasitismo de cría interespecífico, son fuerzas selectivas mayores en la evolución de la expresión de los rasgos (i.e. comportamentales y reproductivos) que intervienen en la biología de reproducción dentro de la clase aves.

Ecología del miedo: efectos indirectos de la depredación y parasitismo de cría

Recientemente se ha sugerido que la respuesta que se produce para minimizar el riesgo (miedo) de depredación podría tener efectos sub-letales que podrían influir sobre numerosos aspectos del comportamiento y la toma de decisiones en tiempo ecológico, esto es, durante la duración de la vida de un individuo (revisado en Cresswell 2008; Lima 2009; Martin y Briskie 2009). El miedo a sufrir depredación propia o de los hijos, podría alterar el uso del hábitat, la inversión en reproducción, los comportamientos de aprovisionamiento de alimento a la descendencia, la fisiología y tener consecuencias en términos de éxito reproductor y en la dinámica a largo plazo de las poblaciones en la naturaleza (Lima 2009). El estudio de los efectos indirectos del miedo a sufrir depredación se denomina Ecología del Miedo (Brown *et al.* 1999).

Un ejemplo clásico que ilustra la importancia del miedo a la depredación y sus efectos es el que describe la evolución poblacional de lobos y ciervos en el parque nacional de Yellowstone (ver Creel *et al.* 2005, 2007; Creel y Christianson 2008). La eliminación de la población de lobos Canis lupus de Yellowstone durante los años 30 del pasado siglo propició un incremento de la población de ciervos Cervus elaphus. Cuando 60 años después los lobos fueron reintroducidos, la población de ciervos descendió. El descenso poblacional de los ciervos no fue consecuencia directa de la depredación por parte de los lobos, sino debido a una bajada en la tasa de reproducción de las ciervas como consecuencia de cambios comportamentales y en la elección de hábitats que afectaron a su fisiología reproductiva. Es decir, el descenso poblacional de los ciervos fue consecuencia directa del miedo a la depredación por los lobos. Existen también numerosas evidencias del papel que juega el riesgo de depredación en las aves (revisado en Lima 2009; Martin y Briskie 2009). La alta prevalencia de la depredación de nidos sugiere que la selección natural favorecería mecanismos que permitieran a las aves evaluar el riesgo de depredación de sus nidos y responder a ello de manera preventiva. De hecho, estudios experimentales han mostrado que el riesgo de depredación es un componente fundamental de la calidad del hábitat que podría ser evaluado por las aves antes de asentarse en un territorio (Fontaine y Martin 2006a; Parejo y Avilés 2011; Parejo et al. 2012b), y que incluso puede tener consecuencias demográficas (Zanette et al. 2011;

Introducción General

Lamanna y Martin 2016; Dudeck *et al.* 2018). Una vez iniciada la reproducción, se espera también que las aves respondan al riesgo de depredación mediante cambios en los rasgos reproductivos y en su comportamiento parental. De hecho, hay evidencias que muestran que rasgos de historia vital como fecha y tamaño de puesta, tamaño de huevos y comportamiento parental son modificados por las aves en respuesta al riesgo de depredación sufrido una vez comenzada la reproducción (e.g. Eggers *et al.* 2006; 2008; Fontaine y Martin 2006b; Dunn *et al.* 2010; Zanette *et al.* 2011; Ghalambor *et al.* 2013; Hua *et al.* 2014; Basso y Richner 2015).

Por otro lado, numerosos estudios han evaluado el papel del riesgo de parasitismo sobre comportamientos implicados directamente en la defensa frente a los parásitos (expulsión de huevos, e.g. Moksness et al. 1993; Brooke et al. 1998) encontrando que un incremento del riesgo de parasitismo en general favorece la expresión del comportamiento defensivo (Fenney y Langmore 2015). Sin embargo, los efectos del riesgo de parasitismo en tiempo ecológico sobre los rasgos reproductivos e inversión parental de hospedadores han sido estudiados sólo preliminarmente. La evidencia de que los hospedadores de los parásitos de cría podrían evaluar el riesgo de parasitismo antes de elegir sus lugares de reproducción es fundamentalmente correlativa (revisado en Krüger 2007), y se restringe a unos pocos trabajos mostrando que los hospedadores localizan sus nidos lejos de posaderos (e.g. Øien et al. 1996; Moskat y Honza 2000; Patten et al. 2011; Antonov et al. 2007; Welbergen y Davies 2012) o en lugares no accesibles para los parásitos (Krüger 2004, Avilés et al. 2005; Grim et al. 2011). Los estudios correlativos, sin embargo, no excluyen la posibilidad que factores ambientales no controlados (e.g. recursos alimenticios, depredadores, o competidores) pudieran estar detrás de los patrones de elección del lugar de nidificación. Tampoco existen evidencias empíricas que muestren cambios en los rasgos reproductivos ni en el comportamiento parental de los hospedadores en respuesta a un incremento del riesgo de parasitismo (Avilés 2017) pese a que esa posibilidad se haya sugerido en estudios teóricos recientes (Medina y Langmore 2016). En esta Tesis Doctoral pretendo aportar nuevas evidencias sobre los efectos del miedo a la depredación en las aves dentro del marco de la Ecología del Miedo y extendiendo su estudio para abarcar los efectos del miedo al parasitismo de cría. De manera análoga a lo que sucede con el riesgo de depredación, cabe predecir que individuos que perciban un riesgo elevado de ser parasitados por parásitos de cría en sus territorios muestren algún grado de plasticidad en sus rasgos reproductivos y sus decisiones de cuidado parental que pueden afectar a la supervivencia de su descendencia.

Evaluación del riesgo: dónde, cuándo y qué

En tiempo ecológico, los riesgos de depredación y de parasitismo de cría interespecífico varían espacial y temporalmente. Por ello, los individuos deben ser capaces de percibir, evaluar y responder de manera flexible a este "paisaje de miedo" (Brown et al. 1999; Laundré et al. 2001). Así, los animales deberían ajustar su distribución y comportamientos como respuesta a los distintos niveles de riesgo existentes en su hábitat (e.g. Thomson et al. 2006). Además, la actividad de depredadores y parásitos puede aumentar a una determinada hora del día o variar estacionalmente (e.g. Mukheriee et al. 2009, Kotler et al. 2010, Molina-Morales et al. 2013; Welbergen y Davies 2012). Ante las variaciones temporales de riesgo de depredación y parasitismo de cría, los animales deben hacer balance entre el tiempo que dedican a comportamientos anti-depredatorios/ defensivos y otras actividades básicas como alimentarse o cuidar de sus crías (hipótesis de reparto óptimo del riesgo, sensu Lima y Bednekoff 1999). Esta hipótesis predice que en periodos cortos de alto riesgo, los individuos mostrarán comportamientos anti-depredatorios elevados pero, si las situaciones de alto riesgo persisten, la

Introducción General

inversión en defensas anti-depredatorias será menor y por tanto, los individuos tomarán más riesgos para seguir con sus actividades (e.g. Eggers *et al.* 2005; Thomson *et al.* 2011). Adicionalmente, la vulnerabilidad a la depredación y parasitismo también varía temporalmente, de modo que la presión que sufren los individuos por parte de depredadores y parásitos de cría es diferente en función de si la reproducción no ha comenzado y entonces la selección de hábitat puede constituir una decisión importante para disminuir el riesgo (Fontaine y Martin 2006a; Feeney *et al.* 2012; Feeney *et al.* 2014), o bien si la reproducción ya se ha iniciado y deben ser otros mecanismos los que ayuden a disminuir este riesgo (Martin y Briskie 2009; Zanette *et al.* 2011; Dudeck *et al.* 2018; Avilés 2017, ver Figura 1).

Los animales también han de ser capaces de percibir y evaluar los diferentes tipos de señales que indican riesgo (Lima y Steury 2005). La información sobre el riesgo se podría adquirir a través señales visuales como la propia presencia del depredador (e.g. Amo et al. 2017) o parásito (e.g. Čapek et al. 2010; Welbergen y Davies 2012), o indirectamente a partir de indicios de su actividad (e.g. restos de heces y orina (Forsman et al. 2013)). Las señales acústicas, vocalizaciones de depredadores (e.g. Zanette et al. 2011; Schmidt y Belinsky 2013) y parásitos de cría (e.g. Kleindorfer et al. 2013; Tolvanen et al. 2017), o cantos de alarma de conespecíficos (Hoover 2003; Suzuki 2015) y heterospecíficos (Forsman et al. 2008; Parejo et al. 2012b) pueden también proporcionar información fiable sobre niveles de riesgo que las aves podrían usar en su beneficio. Así la literatura indica que las aves pueden informarse del riesgo de depredación y parasitismo a través de señales acústicas De esta manera, las aves pueden incorporar la información pública sobre el riesgo disponible en el ambiente (Seppänen et al. 2007; Parejo y Avilés 2016). Por último, las aves podrían obtener información sobre los niveles de amenaza en el ambiente mediante su capacidad olfativa, hasta hace poco desconocida (Roper 1999; Avilés y Amo 2018). Algunos estudios han mostrado que las aves pueden evaluar el riesgo de depredación a través de pistas olfativas proporcionadas por sus depredadores (e.g. orina) (Amo *et al.* 2008; Roth *et al.* 2008; Amo *et al.* 2015; Amo *et al.* 2017) o incluso sus pollos sometidos a riesgo de depredación (Parejo *et al.* 2012a).

La fisiología del miedo

El riesgo de depredación y parasitismo de cría podrían constituir fuentes de estrés que generan repuestas fisiológicas en los individuos. Actualmente existe un interés creciente por conocer los efectos fisiológicos del miedo a la depredación en diferentes taxones (e.g. Clinchy *et al.* 2013; Sheriff y Thaler 2014; Harris y Carr 2016). De hecho, un siglo atrás ya se le otorgaba un importante papel fisiológico al riesgo de depredación (Canon 1915). Sin embargo, como indican algunas revisiones en este campo, todavía son escasos los estudios en aves (Zanette *et al.* 2014; Ibáñez-Álamo *et al.* 2015; Harris y Carr 2016) y hasta la fecha no conocemos ninguno con clara referencia al riesgo de parasitismo de cría interespecífico.

La respuesta fisiológica al estrés generado por el riesgo puede manifestarse a través de variaciones en los niveles normales de los múltiples componentes que conforman el estado hormonal e inmunológico de los individuos. Ante alteraciones ambientales a corto o largo plazo, los individuos inician una respuesta de estrés que resulta en una activación del eje hipotalámico-pituitario-adrenal (HPA) y liberación, entre otras hormonas, de glucocorticoides en plasma (Buchanan 2000; Sapolsky *et al.* 2000; Romero 2004). Concretamente, la corticosterona es una de las principales hormonas liberadas en la respuesta al estrés para favorecer la adaptación, el mantenimiento de la homeostasis y la supervivencia (Wingfield *et al.* 1998) a través de cambios fisiológicos y comportamentales en situaciones estresantes a corto plazo (e.g. Buchanan 2000; Sapolsky *et al.* 2000). Sin embargo, cuando las perturbaciones persisten en el tiempo (estrés crónico), el mantenimiento de altos niveles de corticosterona puede perjudicar el estado inmunológico (e.g. Dhabhar 1998; Martin 2009), las funciones reproductivas (Sapolsky *et al.* 2000; Salvante y Williams 2003) y, en última instancia, afectar a la eficacia biológica (Breuner *et al.* 2008; Boonstra 2013). Además, los efectos de los eventos estresantes pueden proyectarse en la siguiente generación, de modo que los hijos de padres estresados puedan mostrar niveles elevados de glucocorticoides transferidos a través de los efectos maternos (Sheriff y Love 2013) o cambios en los cuidados parentales (Kidawa *et al.* 2017).



Figura 1. Esquema general de los efectos del riesgo de depredación y parasitismo de cría sobre las aves.

OBJETIVO GENERAL

En este marco, el objetivo fundamental de esta tesis es profundizar en el conocimiento y las consecuencias de los efectos subletales de la depredación (tanto la procedente de depredadores como la originada por la actividad humana) y el parasitismo de cría interespecífico para las aves en ambientes naturales. El estudio de los efectos subletales de esos factores sobre la selección de hábitat, los rasgos de historia vital, el comportamiento reproductivo y la fisiología de las aves nos permitirá una mejor comprensión de las consecuencias que dos fuerzas selectivas fundamentales como la depredación y el parasitismo de cría tienen sobre la evolución de rasgos reproductivos. Los capítulos de esta tesis se han ordenado siguiendo una estructura temporal a lo largo de la reproducción de las aves. De esta manera, se podrían dividir en dos partes según si las especies evalúan el riesgo antes (elección de lugares de nidificación) o durante la reproducción.

Objetivos específicos

Antes de la reproducción:

1. Evaluar la capacidad de una comunidad de aves de percibir el riesgo de depredación a través de señales olfativas y su respuesta durante la elección de territorios de reproducción (Capítulo 1). Estudios recientes han demostrado que las aves tienen la capacidad de percibir e integrar señales olfativas que indican riesgo de depredación y responder mediante cambios en sus estrategias de vida (Amo *et al.* 2017). Se desconoce, sin embargo, el papel que las pistas olfativas antes del inicio de la reproducción podrían tener sobre la elección de territorios. En este capítulo se manipula experimentalmente pistas olfativas sobre el riesgo de depredación antes de la reproducción y se analizan las respuestas sobre la estructuración espacio-temporal de una comunidad troglodita compuestas por aves no excavadoras y roedores.

- 2. Conocer el papel de las señales de alarma en la evaluación del riesgo por parte de una comunidad de aves (Capítulo 2). Dentro de una comunidad los individuos pueden obtener información sobre el riesgo de depredación a través de llamadas de alarma de individuos de su misma y otras especies (Parejo *et al.* 2012b; Magrath *et al.* 2015). Por ello, la comunidad puede constituir una red de información social (Seppänen *et al.* 2007) en la que el nivel trófico del receptor puede influir en el valor informativo de la señal, puesto que algunas especies pueden tener entre sí relaciones tróficas y de competencia (Parejo y Avilés 2016). Estudiaremos por primera vez el efecto del riesgo de depredación percibido mediante llamadas de alarma de mochuelo *Athene noctua* sobre la elección de lugares de nidificación en una comunidad de aves trogloditas que incluye varios niveles tróficos, desde competidores hasta presas de la especie informante y todo esto ante situaciones diferentes de competencia por los sitios de reproducción.
- 3. Estudiar la capacidad de percibir el riesgo de parasitismo de cría interespecífico durante la elección de territorios de reproducción de una especie de hospedador (Capítulo 3). Los hospedadores han desarrollado adaptaciones para evitar los costos del parasitismo de cría en diferentes etapas del evento reproductivo, habiendo sido éstas estudiadas sobre todo durante la puesta e incubación de huevos o durante el cuidado de los pollos (e.g. Soler 2014). En este capítulo, se evalúa por primera vez si los hospedadores (la urraca *Pica pica*) de un parásito de cría especialista (el críalo *Clamator glandarius*), evalúan el riesgo de parasitismo de cría percibido a través de señales visuales y acústicas para tomar decisiones en

Introducción General

su elección de territorios de reproducción, es decir, antes del comienzo de la reproducción.

Durante la reproducción:

- **4.** <u>Identificar los efectos del riesgo de depredación sobre los</u> <u>comportamientos reproductivos y cuidado parental</u> (Capítulo 4). El miedo a sufrir depredación puede afectar el cuidado parental (e.g. Martin y Briskie 2009) y llevar a una disminución de la actividad (e.g. Ghalambor *et al.* 2013) para reducir la detección por los depredadores (Briskie *et al.* 1999). Alternativamente, los padres podrían mantener sus niveles de cuidado al nido en respuesta al miedo, pero modificando su estrategia de reparto de alimento dentro de las nidadas en función del valor reproductivo de cada pollo (Tilgar *et al.* 2011). En este capítulo, evaluamos el efecto del riesgo de depredación de nidos sobre los cuidados parentales y el reparto del alimento en la Carraca Europea *Coracias garrulus*, una especie con asincronía de eclosión y una patente jerarquía de tamaño entre sus pollos.
- 5. Evaluar los efectos del riesgo de parasitismo de cría inter-específico sobre los comportamientos reproductivos y rasgos de historia vital del hospedador (Capítulo 5). Se ha sugerido recientemente que los hospedadores de algunos parásitos de cría podrían minimizar los costes del parasitismo a través de cambios en sus rasgos reproductivos (e.g. Medina y Langmore 2016; Avilés 2017; Soler y Soler 2017). En este capítulo, evaluamos si el riesgo percibido a través de señales acústicas afecta a la expulsión de huevos parásitos y/o a la expresión de rasgos de historia vital en urracas.

6. Identificar los costos fisiológicos del riesgo de parasitismo (Capítulo 6). El miedo a sufrir parasitismo podría tener efectos indirectos mediante cambios fisiológicos en los hospedadores y en su descendencia, análogos a los mostrados para el riesgo de depredación (Zanette *et al.* 2014). En este capítulo se evalúa la posibilidad de que el riesgo de parasitismo pueda acarrear costes fisiológicos para los hospedadores (i.e. en términos de

respuesta al estrés e inmunocompetencia) o para su descendencia.

7. <u>Identificar los efectos de la actividad humana en las respuestas</u> <u>fisiológicas de las aves dentro de un gradiente antrópico (Capítulo 7).</u> La actividad humana puede suponer una fuente de amenazas y riesgo para las aves en los medios que habitan (Vitousek *et al.* 1997). Nuestro objetivo en este último capítulo es identificar patrones espaciales de estrés en relación a los gradientes de actividad humana que se dan en medios agrícolas mediante el estudio de indicadores de estrés y función inmune en dos especies insectívoras en declive, la carraca *Coracias garrulus* y el autillo europeo *Otus scops* (Bird International 2018).

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Métodos Generales

Sistema de estudio

Para cumplimentar los objetivos de esta Tesis se utilizaron dos zonas de estudio, ambas situadas en la Hoya de Guadix-Baza, provincia de Granada, en el sureste de España.

En la primera de las zonas de estudio, localizada entre la comarca de Guadix y Baza (37°18'N, 3°11'W), realizamos los experimentos para evaluar los efectos del riesgo de depredación (**capítulos 1, 2, 4 y 7**) sobre una comunidad de aves cavernícolas que se reproduce en cajas nidos. Esta zona tiene una vegetación variable, que incluye áreas de cultivo de cereal, encinares abiertos, plantaciones de almendros y olivos, así como zonas de ramblas y choperas (Fig. 1a). En la actualidad, en el área hay instaladas alrededor de 400 cajas nido, aunque este número varió según el año de estudio (ver Mapa 1). Las cajas nido están hechas de corcho, con unas dimensiones internas de 24 x 24 x 40 cm y una abertura de 6 cm de diámetro (Fig. 1b). Estas cajas se encuentran mayoritariamente colocadas en árboles, en su mayoría encinas *Quercus ilex*, y de forma anecdótica en soportes artificiales como postes eléctricos o paredes (Rodríguez *et al.* 2011).



Figura 1. Fotografía de un encinar de la zona de estudio (a) y de una caja nido colocada en una encina (b).

Métodos Generales

La zona alberga una comunidad de aves cavernícolas que habitan y se reproducen en las cajas nido y se compone mayoritariamente de aves de mediano tamaño y especies más pequeñas que pueden ser presa de las anteriores. Entre ellas, las principales especies de mediano tamaño son el mochuelo *Athene noctua*, el autillo Europeo *Otus scops* y la carraca Europea *Coracias garrulus* (Fig. 2), siendo la densidad en la zona de estudio de 0.024, 0.02 y 0.032 parejas/ha, respectivamente (Parejo y Avilés, 2011).



Figura 2. Especies meso-depredadoras, carracas (a), autillos (b) y mochuelos (c), ocupando las cajas nido del área de estudio durante la reproducción.

Entre las especies de aves de pequeño tamaño que se incluyen en esta comunidad y que también compiten por las cajas nido para reproducirse, están las abubillas *Upupa epops*, grajillas *Corvus monedula*, gorriones chillones *Petronia petronia*, estorninos *Sturnus unicolor* y carboneros *Parus major* (Fig. 3). Estas tres últimas especies pueden ser presas de mochuelos y autillos (e.g. Obuch y Kristin 2004; Marchesi y Sergio 2005; Tome *et al.* 2008; datos propios no publicados).



Figura 3. Algunas de las especies que ocupan cajas nido: abubillas (a), carboneros (b) y estorninos (c).

Todas estas especies cavernícolas, tanto las de tamaño mediano como pequeño, comparten depredadores. Así, en la zona de estudio la culebra de escalera, *Zanechis scalaris* es el depredador más frecuentes de huevos (Fig. 4a) y pollos (Fig. 4d) de las especies que habitan las cajas nido (Parejo *et al.* 2012) e incluso sobre adultos que sorprenden durante la incubación (Fig. 4b). Las cajas nido son también ocupadas por pequeños mamíferos que compiten con las aves por este recurso y que a la vez pueden depredar sobre las puestas, entre ellos, el lirón careto *Eliomys quercinus* (Fig. 4c) y el ratón de campo *Apodemus sylvatucus* y en menor medida ardillas *Sciurus vulgaris* y ratas *Rattus rattus* (datos propios no publicados).



Figura 4. Especies que pueden depredar sobre diferentes especies de aves que habitan las cajas nido: (a) culebra escalera sorprendida depredando huevos de carraca, (b) culebra escalera sorprendida tras depredar una abubilla durante la incubación, (c) lirones careto ocupando una caja nido, (d) culebra escalera depredando pollos de autillo.

Por otro lado, el trabajo para cumplimentar los objetivos relativos a los efectos del riesgo de parasitismo de cría (**capítulos 3, 5 y 6**) fue desarrollado en La Calahorra (37°10´N, 3°03´W). El área de estudio tiene una superficie de unos 12 km², y es un hábitat antropizado donde predomina el cultivo de cereal y almendro, árbol en el que principalmente nidifican las urracas, aunque también pueden usar moreras, álamos y olivos, e incluso arbustos como el rosal silvestre y la retama (Molina-Morales *et al.* 2012; Molina-Morales *et al.* 2013) (Fig. 5).



Figura 5. Fotografía hecha en La Calahorra de una morera con un nido de urraca en la copa.

En este área hay establecida una población de urracas *Pica pica* en la que se viene estudiando a nivel individual su relación con un parásito de cría obligado, el críalo Europeo *Clamator glandarius*, desde 2007 (Molina-Morales 2014). La urraca es un ave territorial, sedentaria y socialmente monógama (Birkhead 1991). En esta población, su reproducción comienza entre los meses de Abril y Mayo y el tamaño de puesta es de entre 6 y 8 huevos (Molina-Morales 2014). Las urracas presentan eclosión asincrónica que genera una evidente jerarquía de tamaño en la pollada (Birkhead 1991; Soler *et al.* 1997). En el periodo de estudio, el número de parejas reproductoras osciló entre 60 y 90 (Mapa 2). La densidad de nidos de urraca en nuestra población es en promedio de 6.25 parejas/Km², distribuidas de forma no uniforme por el territorio (ver Mapa 2).

El críalo es un ave migratoria y parásito de cría obligado que tiene en la urraca, su principal hospedador en la zona de estudio (Soler 1990). En la mayoría de los nidos parasitados naturalmente por el críalo no vuela ningún pollo de urraca (Soler *et al.* 1996) debido a la eclosión más temprana de los pollos de críalo que acaban acaparando todo el alimento y matando de hambre a los pollos de urraca (Soler y Møller 1996; Soler *et al.* 1996). El

parasitismo aumenta a lo largo de la temporada (Molina-Morales *et al.* 2013) y el porcentaje de nidos parasitados en la población de estudio varía según el año (36.2% en 2012 y 18.7% en 2013 y 23.8% en 2014) (ver Mapa 2).

Tareas generales de campo

En cada temporada reproductora de los años que abordamos los objetivos relacionados con el riesgo de depredación (2011-2015), las cajas nido fueron visitadas semanalmente desde principios de Abril para registrar la ocupación de las distintas especies. En las revisiones se registraba la especie que ocupaba la caja, la fecha y tamaño de puesta y el éxito de vuelo. Los nidos de carracas, autillos o mochuelos tuvieron un seguimiento más exhaustivo durante todo el periodo reproductivo aumentando la frecuencia de visitas a estas cajas una vez que se detectaban los huevos en los mismos. Los adultos de estas tres especies eran capturados con trampas colocadas en la entrada de la caja o sorprendidos en ellas al final de la incubación o estancia de los pollos en el nido. Una vez capturados, los adultos eran anillados y se tomaban medidas de su peso, longitud de tarso y ala. También hacíamos un seguimiento de los pollos desde la eclosión hasta el vuelo, siendo estos anillados aproximadamente a los 21 días. Se tomaban sus medidas de peso, longitud de tarso y ala un total de 5 veces a lo largo de toda su etapa de desarrollo. En las dos últimas visitas también extraíamos sangre para evaluar el estado fisiológico de los pollos en relación al riesgo.

Por otro lado, en el periodo 2012-2014, cuando hicimos el trabajo para analizar los efectos del riesgo de parasitismos de cría interespecífico, controlamos los nidos de urraca desde principios de Marzo hasta principios de Julio cada año. Los nidos eran detectados tras búsquedas exhaustivas y su posición registrada con un GPS. Cada nido era observado durante la fase de construcción del nido con telescopio o prismáticos desde un escondite o el coche desde unos 100 m de distancia para detectar si los individuos de la pareja reproductora estaban marcados. Se visitaba el nido antes de la puesta cada 5 días y durante la puesta y la eclosión de los huevos, reducíamos el tiempo de visita a cada 2 o 3 días para determinar si el nido estaba parasitado y determinar la fecha de eclosión. Un nido se consideraba parasitado si había al menos un huevo de críalo en él. Para cada nido registrábamos la fecha de puesta, el número de huevos de urraca y críalo, la fecha de eclosión y el éxito de vuelo. Desde el año 2007 los individuos reproductores han sido capturados con trampas en las que introducíamos una urraca viva en el interior como señuelo durante el periodo de construcción de los nidos o con redes japonesas durante la estancia de pollos en el nido. Los individuos capturados se marcaron con una combinación individual de anillas de colores. Durante la captura también se les extrajo una muestra de sangre para sexaje y sus medidas biométricas (peso, longitud de tarso, ala y cola). Los pollos fueron marcados en el nido a los 15-18 días de la eclosión con una combinación de anillas de colores y les tomamos muestras de sangre en dos visitas diferentes para poder medir parámetros fisiológicos.

Diseños experimentales

Para poder establecer relaciones causa-efecto en relación a de los objetivos planteados usamos una aproximación experimental. Así, llevamos a cabo experimentos diferentes para conocer los efectos del miedo antes (**capítulos 1-3**) o durante la reproducción (**capítulos 4-6**). La manipulación se realizó a nivel de parche (**capítulos 1, 2 y 3**) o a nivel de nido (**capítulos 4-6**). Nuestro objetivo era, además, comprobar la capacidad de las aves de percibir distintos tipos de pistas que indican riesgo. Para ello utilizamos pistas olfativas (**capítulos 1**), visuales (**capítulos 3 y 4**) y acústicas (**capítulos 2, 3, 5 y 6**) simulando el riesgo de depredación (**capítulos 1, 2, 4**) o de parasitismo de cría (**capítulos 3, 5, 6**). A continuación, se describen brevemente los diferentes diseños experimentales utilizados para abordar los objetivos de

esta tesis (Tabla 1), los aspectos metodológicos más específicos se describen de forma detallada en cada capítulo. Los objetivos del **capítulo 7** fueron abordados mediante una aproximación correlativa.

Tabla 1. Breve descripción del procedimiento experimental de manipulación del riesgo

Manipulación de riesgo de :	Momento de manipulación en el evento reproductivo	Pista de riesgo manipulada	Tipo de manipulación	Capítulo correspondiente de la tesis
Depredación	Elección de nidos	Olfativa	Parcheada	Capítulo 1
Depredación	Elección de nidos	Acústica	Parcheada	Capítulo 2
Parasitismo	Elección de nidos	Acústica y visual	Parcheada	Capítulo 3
Depredación	Cuidado de los pollos	Visual	Localizada	Capítulo 4
Parasitismo	Puesta de huevos	Acústica	Localizada	Capítulo 5 y 6

Medidas fisiológicas

Para conocer los posibles efectos fisiológicos del miedo, en el año 2013 (pollos de carraca y autillo (**capítulo 7**)) y en 2014 (pollos de urraca (**capítulo 6**)) obtuvimos diferentes medidas fisiológicas que podemos relacionar con la respuesta al estrés, como son los niveles de hormona de estrés (corticosterona en plasma y plumas), estimadores del estado inmunológico (niveles de aglutinación (NAbs), inmunoglobulinas (Ig Y) y respuesta de células T).

Para obtener el total de medidas fisiológicas se extrajo sangre de cada pollo de las tres especies en dos días diferentes, pero en etapas similares de desarrollo de los pollos. En el primer día de muestreo (12 días de edad del primer pollo en urracas, 15 días de edad del primer pollo en carracas y 12 días de edad del último pollo nacido en autillos) se extrajo una muestra de 225µL de sangre por pollo para obtener las mediciones relativas a inmunidad (niveles de aglutinación. NAbs) v adquirida (niveles innata de inmunoglobulinas, Ig Y). En el segundo día de muestreo (15 días del primer pollo en urracas, 20 días de edad del primer pollo en carracas y 15 días de edad del último pollo nacido en autillos), se obtuvo otra muestra de sangre de 225 µL de sangre por pollo para poder medir los niveles de corticosterona inducida por estrés. El muestreo siempre se realizó por la mañana (8:30 a 11:30 a.m.) para evitar cambios circadianos en los niveles de corticosterona (Breuner et al. 1999) y tras pasar 30 minutos desde la captura (inducción de estrés). Además, este segundo día de muestreo también recolectamos una pluma (8ª cobertera primaria en urracas y 3ª cobertera primaria en carracas y autillos) de cada pollo, que fue almacenada en una caja oscura a temperatura ambiente hasta el análisis en laboratorio de los niveles de corticosterona en plumas.

Las muestras de sangre se extraían de la vena braquial usando un aguja de 0.5 x 16 mm y capilares heparinizados para transferir la sangre cuidadosamente a un Eppendorf de 1.5 mL (Fig. 6b). La sangre se refrigeró hasta su centrifugación dentro de las siguientes 12 horas a 13300 rpm durante 5 minutos. Todas las muestras se almacenaron en un congelador a -20°C hasta el final de la temporada de campo y después se guardaron en un congelador a -80 ° C hasta que se analizaron en el laboratorio. Los polluelos se pesaron (a la edad de 12 días en urracas y 21 días en carracas y autillos) con una precisión de 1 g utilizando Pesolas de 300 g.

Corticosterona

La corticosterona es el glucocorticoide principal en las aves liberado en respuesta a amenazas ambientales estresantes a través de la activación del eje hipotalámico-pituitario-adrenal (HPA) (Romero 2004) y que conlleva la movilización de energía y recursos para la supervivencia (Wingfield et al. 1998; Sapolsky *et al.* 2000). La extracción hormonal y los análisis de

corticosterona en plasma y plumas se realizaron en el Centre d'Études Biologiques de Chizé (CEBC-CNRS, Villiers en Bois, Francia) (Fig. 6a). Las concentraciones de corticosterona se determinaron mediante una técnica de radio-inmunoensayo (RIA) descrita en el protocolo de Lormée *et al.* (2003).

Inmunocompetencia

Las dos ramas principales del sistema inmune de vertebrados son la inmunidad humoral y la mediada por células (Klein 1990; Roitt *et al.* 1996; Wakelin 1996). Además, la respuesta inmune puede ser específica o inespecífica, adquirida o innata (Roitt *et al.* 1996). Nosotros utilizamos tres estimadores diferentes de la respuesta inmune en pollos descritos brevemente a continuación. Las técnicas que precisaron de laboratorio se realizaron en la Estación Experimental de Zonas Áridas (EEZA-CSIC, Almería, España).

1. Estimación de la respuesta inmune innata: Niveles de aglutinación

La inmunidad innata constitutiva representa la primera línea de defensa contra el ataque de parásitos, y está formada por anticuerpos naturales (NAbs) y complemento. La función de estos dos componentes es reconocer e iniciar la cascada de la enzima del complemento (NAbs) que finalmente termina en la lisis celular (Carroll y Prodeus 1998). NAbs y complemento están conectados a la inmunidad adaptativa, proporcionando un vínculo entre las defensas innata y adquirida (Carroll y Prodeus 1998, Ochsenbein y Zinkernagel 2000). Para estimar la respuesta inmune innata utilizamos el protocolo de Matson *et al.* (2005) que determina el estado humoral innato en aves mediante obtención de los niveles de aglutinación y lisis mediante un código de pocillos (Fig. 6d). La aglutinación estima la interacción entre los anticuerpos naturales y el antígeno, en este caso, sangre de conejo (Matson *et al.* 2005). La capacidad de aglutinación se obtiene a partir del último número de pocillo en que se presenta esta actividad (Fig. 6d). La lisis estima la acción del complemento por la cantidad de hemoglobina que libera de los eritrocitos

dela sangre de conejo, sin embargo en esta tesis estimamos y usamos únicamente los niveles de aglutinación ya que no encontramos actividad lítica en los pollos de las especies de estudio.

2. Estimación de la respuesta inmune adquirida: niveles de inmunoglobulinas (Ig Y)

Las inmunoglobulinas son componentes cruciales de la respuesta humoral adquirida. De entre sus tipos, la inmunoglobulina Y (Ig Y) es un anticuerpo en aves, con función similar a la inmunoglobulina G de mamíferos, que puede neutralizar patógenos (Demas *et al.* 2011) y se acumula en la yema de huevo (Warr *et al.* 1995). Para evaluar la capacidad de respuesta inmune humoral adquirida se realizó una técnica de inmunoensayo (ELISA) que permite medir los niveles de Ig Y en el plasma de los pollos siguiendo el protocolo de descrito en Martínez *et al.* (2003).

3. Estimación de la respuesta inmune adquirida: respuesta mediada por células T

Para conocer la respuesta inmune celular adquirida (mediada por células T) usamos un método basado en la intensidad de hinchazón de la piel del patagio del ala en respuesta a la inyección con una lectina, la fitohematoglutinina (PHA) (Cheng y Lamont, 1988) que estimula la proliferación inespecífica de linfocitos T (Hernandez y Leavitt 1984; Bühring *et al.* 1999). Se ha demostrado que el aumento en el grosor del patagio del ala se correlaciona con una serie de componentes de la aptitud en aves (e.g. Saino *et al.* 1997).

Cuando los pollos de carraca y autillo tenían aproximadamente 21 días, realizamos el test de la PHA (Fig. 6c). Previamente marcábamos con marcador indeleble la zona del patagio a inyectar. Los patagios de ambas alas se medían con un espesímetro (Balxo 3000). Después se inyectaba 0.2 mg de PHA (Sigma, L-8754) disuelto en 0.04 mL de PBS en el patagio derecho y

Métodos Generales

solo 0.04 mL de PBS en el patagio izquierdo. Al día siguiente, aproximadamente pasadas 24 horas, se medía el engrosamiento del patagio.



Figura 6. Diferentes metodologías de obtención de estimadores fisiológicos en pollos. (a) Extracción de corticosterona en plumas mediante metanol, (b) extracción de sangre en pollo de autillo (c) medición de la respuesta de PHA en un pollo de autillo, (d) técnica de medición de los niveles de aglutinación en sangre.



Mapa 1. Área de estudio para los capítulos de riesgo de depredación y distribución de las cajas nido. (a) Distribución de las cajas nidos usadas hasta 2013. (b) Distribución de las cajas utilizada a partir de 2014.





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Capítulo 1

Avian and rodent responses to the olfactory landscape of fear in a Mediterranean cavity community



Avian and rodent responses to the olfactory landscape of fear in a Mediterranean cavity community

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Abstract

Avoiding predation is critical for animals, and cues informing on predation risk can trigger stereotyped fear responses in prey which may have cascading effects on community dynamics. Olfactory information may play a fundamental role in the assessment of a predation threat because predators produce characteristic body odors that may act as modulators of memory and emotion in prey, but its role in habitat selection and community structure of birds has been neglected. Here, we demonstrate for the first time that fear to predation induced by odor cues may have ecological consequences altering composition and phenology in a Mediterranean cavity community composed by rodents and non-excavator hole-nesting birds. We experimentally increased the perception of predation risk at a patch scale by applying odors of a carnivore predator and found that Risky-odor plots were less occupied than plots with a Non-risky-odor treatment and than Control plots. Also, there was a trend in birds, but not in rodents to settle down first in Control and Non-risky plots than in Risky ones. Finally, the odor treatment influenced the relationship between avian and rodent abundance: avian and rodent abundances were inversely related in Control and Non-risky odor plots, but avian abundance did not increase with low rodent abundance in Risky-odor plots, suggesting that birds avoided them.

Manuscrito enviado a revista.

Introduction

All animals are exposed to predation at some time through their life (Elton 1927). Risk of suffering a predation event is not random but more frequent in some habitats where predators concentrate or in some periods of life where individuals are more vulnerable. Reproduction is a particularly sensitive period to the effect of predation because offspring mortality accounts for a high proportion of variance in total mortality and because during that period prey became highly conspicuous to predators (Martin 1995). No surprisingly, predation constitutes a major selective force driving the evolution of reproductive life history traits (Reznick and Endler 1982; Martin 1995) and behavior (Farr 1975; Lima and Dill 1990; Conway and Martin 2000), including the election of safe places to reproduce (Fontaine and Martin 2006; Blaustein et al. 2004).

Habitat selection based on cues informing on risk of predation has proved to be a widespread mechanism to reduce predation risk and, hence diminish or avoid predation, influencing survival and fitness of individuals (Lima and Dill 1990; Caro 2005). In birds nest predation is the main cause of reproductive failure (Nice 1957; Martin 1993), and a large body of empirical evidence has shown that birds can perceive and react to a wide array of visual and vocal stimulus informing on predation risk when taking habitat settlement decisions (Eggers et al. 2006; Fontaine and Martin 2006; Monkkonen et al. 2009; Parejo and Avilés 2011; Parejo et al. 2012b; Parejo et al. 2018; Emmering and Schmidt 2011; Peluc et al. 2008).

Olfactory information may play a fundamental role in the assessment of predation risk as predators produce characteristic body odors which may act as modulators of memory and emotion in prey (Apfelbach et al. 2005; Parsons et al. 2018). Indeed, HPLC analysis indicates enriched 2-phenylethylamine urine production by numerous carnivores, and that this volatile chemical detected in the environment can trigger stereotyped fear and

avoidance responses in rodents (Ferrero et al. 2011), which may potentially have cascading effects on community dynamics (Brinkerhoff et al. 2005). Regarding birds, anatomical studies have shown that they possess an olfactory apparatus similar in function and structure to that of other vertebrate species with known olfactory capabilities (Bang 1971; Wenzel and Sieck 1972; Zelenitsky et al. 2011). A growing body of behavioral work demonstrates that birds are capable to recognize and respond to chemical cues in several relevant biological context including prey detection (e.g. Nevitt et al. 1995; Amo et al. 2013), orientation (e.g. Nevitt and Bonadonna 2005; Gagliardo 2013), and social interactions (e.g. Bonadonna and Nevitt 2004; Hagelin and Jones 2007; Caro and Balthazart 2010; Amo et al. 2012; Caspers et al. 2017; Rossi et al. 2017). Moreover, experimental studies have shown that birds can respond modulating their parental investment to the scent of mammals' predator urine placed in their nests (Whittaker et al. 2009; Amo et al. 2008; Stanbury and Briskie 2015), or even to odorous cues informing on recent predation attempts on their offspring (Parejo et al. 2012a), suggesting that avian olfaction may play a key role in the assessment of nest predation risk. Surprisingly, although the study of chemical ecology in birds has considerably expanded in the last two decades, the role of predators' chemical cues in habitat selection and community structure of birds has been almost neglected, with only two studies dealing with this issue. On the one hand, Eichholz et al. (2012) found that ducks were less likely to settle down their nests in plots were red fox Vulpex vupex urine was applied than in control plots. Similarly, Forsman et al. (2013) found that the number of migratory passerine species and their total density were lower in patches where mammal's urine and feces were sprayed compared to patches were water was sprayed as a control. However, mammalian urine is highly reflective in the UV part of the light spectrum that birds can detect (e.g. Viitala et al. 1995; Cuthill et al. 2000) and, hence, as noted by the authors themselves, it cannot be discarded that ducks and passerines were cueing on

Capítulo 1

visual rather than on chemical information when selecting breeding territories.

Here, we demonstrate for the first time that fear to predation induced by odor cues may have ecological consequences altering composition and phenology in a Mediterranean cavity community composed by rodents and non-excavator hole-nesting birds. In this community, rodents and birds are involved in two types of competition. First scramble competition as rodents and birds exploit cavities on trees as roosting or breeding sites, thus one group of species reduces the availability of holes for the other group of species. Scramble competition is likely to be strong in Mediterranean holm oak Quercus ilex forests where long-term pruning activities have promoted a shortage of suitable holes for cavity dependent species (Avilés and Parejo 2018). This is likely to occur in our study site as well, as most of nest-boxes are occupied (see results). In the other hand, there is evidence of interference competition, as garden dormouse *Eliomys quercinus* are common predators of eggs and nestlings of hole-nesting birds (Adamik and Kral 2008) and has been reported to predate on bird eggs in our study area (Avilés and Parejo, unpublished data). Competition between rodents and birds results in inverse changes in the abundance of rodents and birds across plots (see Results). In this community, before the settlement of birds and rodents in cavities, we experimentally increased the perception of predation risk at a patch scale by applying odors of a carnivore predator. We expected i) that settling avian and rodent individuals avoided areas (i.e. reduced their abundance and delayed settlement) with odors of predators because this is likely to indicate dangerous areas, and that these changes have ii) cascading effects on competence between rodents and birds.

Materials and methods

Study system

The study was conducted during the 2015 breeding seasons (April-July) in south-eastern Spain (37°18'N, 3°11'W). In the study area, the cavity-nesting bird community includes little Athene noctua and scops owls Otus scops, Eurasian rollers Coracias garrulus, common hoopoes Upupa epops, great tits Parus major, spotless starlings Sturnus unicolor, rock sparrows Petronia petronia and jackdaws Corvus monedula, which have been using as breeding sites 259 cork-oak nest-boxes held in trees for the last ten years (Rodriguez et al. 2011; Parejo and Avilés 2011; Parejo et al. 2012b; Parejo et al. 2018). In addition, every spring a proportion of next-boxes are regularly occupied by a mammal community composed by garden dormouse Eliomys quercinus and wood mouse Apodemus sylvaticus (Table 1 Supplementary Material). No nest-box was added during the study year, and hence both birds and small mammals are likely to have previous knowledge of these nest-boxes. All used nest-boxes had a base and roof surface of 24 x 24 cm, a height of 40 cm and an opening 6 cm in diameter, which is wide enough to allow easy entrance of all the species in the community. The area is a homogeneous Holm oak Quercus ilex wooded landscape where natural holes are scarce, and when present they are so small that are unsuitable for most of these cavity user species (Avilés and Parejo 2018).

Experimental design

Nest-boxes were assigned by proximity to plots (the mean number of nestboxes per plot was 5.07 and ranged from 3 to 8 nest boxes, N = 259 nestboxes in 51 plots, Table 1 Supplementary Material). Plots were separated by at least 300 m and nest-boxes within each plot were separated by 50-100 m of each other. Aiming to avoid possible spatial influence on our experiment, plots were spatially grouped in triads. Within each triad, plots were randomly assigned to one of the following three treatments: 1) Risky odor, in which we artificially increased perceived predation risk by applying the scent of a predator to all the nest-boxes (N = 17 plots); 2) Non-risky odor, in which we did not modify perception of predation risk but applied lemon essence as a control scent to all the nest-boxes (N = 17 plots); and, 3) Control, in which we did not apply scent but visited as frequently as Risky and Non-risky odor plots (N=17 plots). The number of nest-boxes per plot did not significantly differ between treatments (One-way Anova, $F_{2,48} = 1.90$, P = 0.16; average (±SD) number of next-boxes: 5.47 (±0.87) nest-boxes in Risky odor plots; 5.05 (±1.08) nest-boxes in Non-risky odor plots; and 4.70 (±1.40) nest-boxes in control plots).

The scent was applied by placing a scented paper hidden under a 10 x 3 cm piece of cork oak, attached with pushpins to the inner part of the nest-box near the entrance. In control plots we also attached a piece of cork oak but did not apply scent, so that the internal appearance of the nest-box was not differently affected by treatments and that any differential response to the treatments was due to the applied scent. Predator scent was obtained by placing clean absorbent papers under the cages of two male ferrets (Mustela putorius furo L.) by at least 3 days (see Amo et al. 2008; Amo et al. 2011). Although ferrets are not natural predators of cavity birds they predate ground birds and small mammals (Bodey et al. 2011), and their scent is very similar to that of other common cavity avian predator mustelids inhabiting the study area, such as Mustela erminea or Martes foina (Brinck et al. 1983). Moreover, previous studies have demonstrated that ferret scent is recognized and avoided by other species which are not natural prey as small mammals (Zhang et al. 2007), and birds (e.g. Amo et al. 2008; Amo et al. 2011). As a control scent we used lemon essence obtained diluting 0.5 g of scratch lemon in 1 ml of distilled water. The mixture was maintained 24 hours in the fridge and then the liquid fraction was collected and used to drench absorbent papers to be used in the experiment. Lemon essence has satisfactorily been used as a control harmless and unusual odor in studies of scent recognition in breeding birds (Parejo et al. 2012a).

The experiment started on 15 April, when most bird species in the community are evaluating breeding territories but have not started reproduction yet. Treatments were applied every second day during 20 days, i.e. from 15 April to 5 may, in alternative days in Risky and Non-risky plots and half of the Control plots. We are certain that our odor manipulation was perceived by birds and mammals as we reported that in 31 out of 42 plots occupied by at least one bird, and in 31 out of 40 plots occupied by at least by one mammal, occupation occurred before the end of treatment application (average $(\pm SD, N)$ Julian date of the earliest bird occupation per plot = 113.16 (\pm 16.64, 42 plots with at least one bird) and average (\pm SD, N) Julian date of the earliest mammal occupation per plot = $113.20 (\pm 23.14, 40 \text{ plots})$ with at least one mammal). From 15 April on (i.e. beginning of treatments application) plots were visited weekly to the end of July to record occupation (a nest-box was defined as occupied when at least one egg was laid in it (in the case of birds) or when a mammal occupied a nest-box in two consecutive visits). Before 15 April, territories were visited to be sure that individuals had not occupied nest-boxes.

Response to the odor experiment by the cavity community was evaluated using two estimators of breeding habitat preference: i) The rate of occupation of all nest-boxes in a plot (i.e. the proportion of nest-boxes occupied by cavity community species per plot) on the knowledge that high quality habitats should be more occupied (Fretwell 1972) and ii) the earliest laying date (i.e. for birds) or earliest occupation date (i.e. for mammals) per plot in the knowledge that high quality habitats are occupied first (Alatalo et al. 1986; Parejo et al. 2012b).
Statistical analyses

Analyses were performed using SAS v.9.4 statistical software (SAS 2002-2008 Institute, Cary, NC, USA).

General linear models (GLMs) (GLM SAS procedure) were used to investigate whether the plot odor treatment influenced (i) the plot occupation rate by all species of the cavity community, and, (ii) the earliest laying/occupation date of all species in each plot. In addition, we also run GLMs to test if the relationship between bird and rodent abundance (i.e. plot occupation rate by birds and mammals) on the one hand, and, on the other hand, bird and rodent phenology (i.e. the earliest laving date of a bird and the earliest occupation date of a nest-box by a rodent) in each plot were influenced by odor treatment. In these two last models, bird abundance and phenology were considered dependent variables and mammal abundance and phenology as predictors based on the knowledge that small mammals are competitively superior to birds in our community (see introduction). The number of nest-boxes per plot was introduced as a covariate in all the models to control for the fact that the number of nest-boxes may vary among plots, and based on the knowledge that the level of competition can modify the value of cues informing on predation risk (Parejo and Avilés 2016; Parejo et al. 2018). Pairwise differences in significant models were checked by comparisons of least-squared means of each treatment using Scheffé tests. Standard model validation graphs (Zuur 2009) revealed that model assumptions of homogeneity of variance and normality of residuals were fulfilled.

Results

50 out of the 51 plots were occupied by at least one cavity community species during the experimental year (Table 1 appendix). Mean number of

occupied nest-boxes per plot was 3.49, rendering an average occupation of 69.5%.

Community responses to olfactory cues

Occupation rate of a plot was influenced by odor treatment (Table 1). Riskyodor plots were in average a 23 % less occupied than non-risky-odor plots (Fig. 1A), and a 17 % less occupied than control plots (Fig. 1A). Phenology of plot occupation by the whole cavity community was not influenced by odor treatment (Table 1). However, there was a trend in birds (General linear model: $F_{2,39} = 2.94$, P = 0.06), but not in mammals (General linear model: $F_{2,37} = 0.68$, P = 0.50), to be installed first in Control and Non-risky plots than in Risky ones (Fig. 1B).

Capítulo 1



Fig. 1. Evidence of eavesdropping on olfactory cues informing on predation risk for breeding habitat choice in a cavity community. (A) Average (± standard error) plot occupation rate in relation to odor treatment. (B) Average (± standard error) avian earliest laying dates per plot in relation to odor treatment. Numbers inside bars are number of plots. Pairwise differences are shown, with arrows designating pairs.

Plot occupation rate	Level	Coefficient	SE	Lower 95% CL	Higher 95% CL	DF	F	d
Intercept		75.6	17.1	41.2	110.0	1,47	19.55	0.00
Treatment	Non-risky odor	9.6	5.2	-0.7	20.0	2,47	3.33	0.04
	Risky odor [*]	-13.1	5.3	-23.8	-2.4			
Number of nest-boxes		-1.2	3.3	-7.8	5.4	1,47	0.14	0.71
Earliest laying date	Level	Coefficient	SE	Lower 95% CL	Higher 95% CL	DF	F	р
Intercept		128.3	8.7	111.8	145.8	1,45	218.16	0.00
Treatment	Non-risky odor	2.3	2.7	-3.3	7.5	1,45	1.31	0.28
MbRR	Risky odor*	2.3	2.8	-3.2	7.8	74.0		10.0
Number of nest-boxes		-4.0	I./	-1.9	-1.2	2,40	70.1	10.0

* Reference level in the treatment contrasts parametrization of the model matrix.

Table 1. Results of general linear models of plot occupation probability and earliest laying date in relation to odor treatment. Significant

Avian versus mammal responses to olfactory cues

The odor treatment applied to plots influenced the relationship between avian and mammal abundance (Table 2). Avian and mammal abundances were inversely related in control and non-risky odor plots (Fig. 2). However, avian abundance did not increase in plots treated with odor of a predator and with low mammal abundance (Fig. 2). The relationship between avian and mammal occupation phenology, however, was not affected by odor treatment (Table 2).



Fig. 2. Avian abundance in relation to mammal abundance and odor treatment (N=51 plots, 17 plots per treatment).

Table 2. Results of general linear models testing for bird and mammal relationships in abundance and phenology in relation to odor treatment. Significant terms are highlighted in bold. N=51 plots.

Bird occupation rate	Level	Coefficient	SE	Lower 95% CL	Higher 95% CL	DF	F	d
Intercept		67.4	12.6	42	92.8	1,44	28.51	0.000003
Treatment	Non-risky odor	10.2	6.9	-3.6	24.1	2,44	6.18	0.004
	Risky odor	-21.8	6.3	-34.6	6-			
Mammal occupation rate		-0.4	0.1	-0.6	-0.2	1,44	14.6	0.0004
Mammal occupation rate*treatment	1	-0.2	0.1	-0.5	0.1	2,44	4.89	0.012
	2	0.5	0.2	0.2	0.8			
Number of nest-boxes		-4.1	2.5	-9.1	0.9	1,44	2.74	0.105
Bird earliest laying date	Level	Coefficient	SE	Lower 95% CL	Higher 95% CL	DF	F	р
Intercept		123.7	26.8	68.4	179	1,25	21.25	0.0001
Treatment	Non-risky odor	-10.1	21.6	-54.5	34.3	2,25	0.34	0.71
	Risky odor	17.4	22.4	-28.8	63.6			
Mammal earliest laying date		-0.1	0.2	-0.4	0.2	1,25	0.19	0.67
Mammal earliest laying date*treatment	1	0.1	0.2	-0.3	0.5	2,25	0.14	0.87
	2	-0.1	0.2	-0.5	0.3			
Number of nest-boxes		-0.4	3.1	-6.8	6.1	1,25	0.01	0.91

Discussion

Chemical cues play a fundamental role in the assessment of predation risk in mammals as they can trigger stereotyped fear and avoidance responses in prey (Kavaliers et al. 2005; Ferrero et al. 2011; Apfelbach et al. 2005; Sharp et al. 2015), which may result in cascading ecological effects on communities (Brinkerhoff et al. 2005; Sunyer et al. 2013). This possibility remained not considered in birds due to the lack of direct experimental evidence that the perception of odor cues alone was powerful enough to affect avian settlement decisions. Here we test for the first time whether fear to predation may have ecological consequences in a Mediterranean cavity community composed by rodents and non-excavator hole-nesting birds by experimentally manipulating odor cues at the time of assessing territory quality and testing their influence on settlement patterns and rodent-bird competence. As predicted, community organization was influenced by the olfactory landscape of fear at the time of settlement: we found a lower abundance of cavity-users in plots treated with predator scent. Birds, but not rodents, showed sign of aversion (i.e. delayed occupation compared to Control and Non-risky plots) toward territories treated with odor of a predator. Finally, aviar abundance did not increase with low rodent abundance in plots treated with predator odor, suggesting that birds avoided settlement in risky plots with low rodent abundance because they perceived a high risk of predation based on odor cues. These results cannot be explained by differences in habitat characteristics among plots as these were matched by proximity before the randomization of treatments. Moreover, the number of available nest-boxes per plots did not differ between treatments (see Methods) suggesting that differences in competition could not account for the found patterns. Therefore, our findings provide empirical support for the view that fear to predation induced by odor cues may have ecological consequences altering composition and phenology in a

Mediterranean cavity community composed by rodents and non-excavator hole-nesting birds.

Although we found a net effect on density of the whole cavity community in response to nest predation risk based on odor cues, we detected subtle differences in the effect of these cues on avian and rodent species. Birds, but not rodents, selected plots to settle in the order of low to high predation risk as indicated by odor cues on predation risk. Several factors may explain this pattern. First, birds use nest-boxes to reproduce whereas rodents do it mainly as roosting or food stores. Therefore, selection for the use of cues informing on risk of predation might be larger in birds than in rodents as the former should reduce offspring mortality that accounts for a high proportion of variance in total mortality (Martin 1995). A mutually nonexclusive possibility is that garden dormouse and wood mouse perceived ferrets as not natural predators in cavities. Experimental work has shown that some rodent species possesses a finely tuned sense of smell and that they can recognize levels of predation in a graded way based on odor cues (Taraborelli et al. 2008). Accordingly, they would disregard odor cues on ferrets when settling because they recognized that ferrets are not a major predation threat in cavities. Also, it cannot be discarded the alternative possibility that rodents in our community were not able to recognize predation risk based on mammal odor cues because they are very rarely exposed to mammalian predation in cavities. Previous studies have found that house mice Mus *domesticus* showed little discrimination between traps bearing faecal odours of the predators and traps bearing conspecific odours or no odour in areas without mammalian predators, whereas in areas with mammalian predators mice avoided traps with smell of predators (Dickman 1992). Discriminating among these possibilities clearly deserves further experimental work.

Our study demonstrates an ecological consequence of fear to predation perceived by odor cues on cavity community structure. Abundance of rodents

in our plots is inversely related to abundance of birds, which, together with previous observations of low number of suitable cavities and events of predation (see Introduction), would suggest that rodent and birds are involved in competition by cavities (Dhondt 2012). Perception of predation risk based on odor cues modified competition patterns between rodents and birds as birds avoided to settle down in plots with low mammal abundance when these were treated with odor of a predator.

Our study has some obvious weakness worth mentioning that may affect the strength of our conclusions. First, we cannot make analyses based on single species due to the low number of individuals of each species (see Table 1 Appendix, Supplementary Material). Therefore, we summed the abundance of all observed birds and rodents in each plot to get abundance estimates and consider a wide range of species of birds and mammals to calculate plot phenology. Different species may differ in their olfactory capabilities and in their assessment of predation risk based on ferret odor cues. Therefore, it is not possible to discriminate whether the detected net effects on our Mediterranean cavity community is solely due to aversion induced by odor cues on early plot occupants or it is partly a consequence that newly arriving individuals or species were using the presence of previous settled birds (Parejo et al. 2005; Seppänen et al. 2007). Late breeders may for instance avoid settlement on plots not because of the odor per se but due to the absence of a cue species informing on habitat quality. Regarding this issue our results are thus conservative.

In conclusion, our study has shown that the risk of nest predation perceived by odor cues can have profound effects on habitat settlement decisions made by species in a Mediterranean cavity-dependent community composed by rodents and non-excavator hole-nesting birds. Offspring predation is a potent selective agent favoring the evolution of mechanisms allowing birds to assess offspring predation risk proactively (Lima 2009). A large body of empirical work has previously demonstrated proactive responses to nest predators based on visual and acoustic cues informing on predator presence or density (Eggers et al. 2006; Fontaine and Martin 2006; Monkkonen et al. 2009; Parejo and Avilés 2011; Parejo et al. 2012b; Parejo et al. 2018; Emmering and Schmidt 2011; Peluc et al. 2008). Our findings reinforce the importance of olfactory cues on predation risk in shaping the structure and function of a cavity dependent rodent-bird community through the process of habitat selection.

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Supplementary material for:

Avian and mammal responses to the olfactory landscape

of fear in a Mediterranean cavity community

Jesús M. Avilés, Deseada Parejo and Mónica Expósito-Granados

Capítulo I

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Wood	0	0	-	-	0	-	1	0	0	0	0	-	-	-	0	1	-	-	-
Garden dormouse	0	0	-	1	1	П	1	1	Т	0	0	0	0	0	0	1	0	-	-
Eurasian jackdaw	0	0	0	0	0	Н	0	0	0	0	0	0	0	0	0	0	0	0	¢
House sparrow	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	¢
Spotless starlings	1	1	0	0	0	0	0	0	0	Т	1	0	0	0	0	0	0	0	¢
Great tit	0	0	-	0	0	0	0	0	0	0	-	0	0	0	0	0	0	0	c
Little owl	0	0	0	-	0	-	0	0	0	0	0	0	0	0	0	-	0	0	c
Eurasian scops owl	0	0	0	0	0	0	1	0	П	0	0	0	-	0	0	0	0	-	¢
European roller	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	c
Common hoopoe	0	0	0	0	0	0	0	0	0	Т	0	1	0	-	0	0	0	0	c
earlier laying date	133	130	101	96	160	100	76	26	140	76	76	98	100	96		76	96	96	Š
occupied boxes	2	ŝ	3	4	1	5	5	ŝ	2	4	4	4	3	6	0	9	-	٢	
number of boxes	5	3	5	4	3	9	6	5	4	8	4	4	9	9	9	ę	6	7	t
treatment	odour- predator	control	odour- lemon	control	odour- lemon	odour- predator	odour- lemon	control	odour- predator	control	control	odour- lemon	odour- predator	odour- predator	odour- predator	odour- lemon	control	odour- predator	odour-
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0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0 0	1 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0	0	0	0 0	0 0	0 0	0 0
0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	-	1	0	0	0	1	1
0	0	0	0	1	0	0	0	0	0	0	0	-	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	0	0	0	0	1	0
0	0	0	1	-	-	1	П	0	0	П	0	-	0	-	0	_	1	0	0	0	0
96	76	112	95	135	103	95	100	108	106	76	86		107	102	96	96	98	86	116	112	117
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odour- predator	odour- lemon	control	control	odour- lemon	odour- predator	odour- lemon	odour- predator	control	odour- predator	odour- lemon	control	odour- lemon	odour- lemon	control	odour- predator	odour- predator	control	control	odour- lemon	odour- predator	odour- lemon
0	51	52	23	24	25	26	22	28	50	20	31	32	33	55	35	36	37	88	39	9	⊒

4	0	0	0	0	-	0	0	0	0	0
28	0	0	-	-	1	1	-	1	0	0
22	0	0	0	0	0	0	0	0	0	
6	0	0	0	0	0	0	0	0	1	1
1	0	0	0	0	0	1	0	0	0	0
8	1	Т	0	0	1	0	0	1	0	0
7	1	0	0	-	0	0	0	-	0	-
œ	0	0	0	0	0	0	0	0	0	0
r	0	0	0	0	0	0	1	0	0	0
w	0	0	0	0	0	П	0	0	0	0
15	0	0	0	0	1	0	1	0	0	0
104.62 (13.41)	98	67	100	101	96	104	96	101	121	121
3.33 (1.61)	3	3	-	4	5	4	4	5	2	3
4.87 (1.34)	4	4	5	5	9	S	5	5	5	5
Average (SD)	control	odour- lemon	odour- predator	odour- lemon	control	odour- lemon	odour- predator	control	control	odour- predator
	51	50	49	48	47	46	45	44	43	42

Receivers matter: the meaning of alarm calls and competition for nest sites in a bird community



Receivers matter: the meaning of alarm calls and competition for nest sites in a bird community

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Abstract

Animal communities may constitute information networks where individuals gain information on predation risk by eavesdropping on alarm calls of other species. However, communities include species in different trophic levels, and it is not yet known how the trophic level of the receiver influences the informative value of a call. Furthermore, no empirical study has yet tested how increased competition may influence the value of alarm calls for distinct receivers. Here, we identify the importance of alarm calls emitted by a small owl, the little owl (Athene noctua), on the structure of a cavity-nesting bird community including mesopredators and primary prey under variable levels of competition for nest holes. Competitors sharing top predators with the callers and prey of the callers interpreted alarm and non-alarm calls differently. Competitors chose preferentially alarm and non-alarm patches over control patches to breed, while prev selected alarm patches. In contrast, competition for nest-sites affected habitat selection of prey species more than that of competitors of the callers. This study provides support for a changing value of alarm calls and competition for nest-sites for distinct receivers related to niche overlapping among callers and eavesdroppers, therefore, calling attention to possible cascading effects by the use of information in natural communities.

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Introduction

Many prey species emit alarm calls when encountering a predator (Caro 2005). Individuals producing alarm calls may obtain benefits recruiting conspecifics for mobbing defence (Curio et al. 1978), warning relatives and/or mates who increase their chances to escape (Weary and Kramer 1995), also warning the predator that it has been detected (Hasson 1991), and attracting predators of the predator (Curio et al. 1978). In addition, alarm calls may inform about predation risk to non-related conspecific and heterospecific prey threatened by the same predator (Caro 2005; Magrath et al. 2015). Hence, alarm calls emitted by one species may warn all potential prey of a given predator within the community (e.g. Templeton and Greene 2007; Vitousek et al. 2007; Parejo et al. 2012).

In food webs, top predators often feed at more than one trophic level. Top predators may feed on primary prey (i.e. herbivore or granivore prey), but also, as part of intraguild predation, they may feed on mesopredators, which are the usual predators of primary prey. Killing of mesopredators by top predators is sometimes important enough to impact demography of mesopredator populations (Ritchie and Johnson 2009). Therefore, mesopredators would mainly benefit by detecting top predators, and, meanwhile, primary prey would get more advantages by detecting mesopredators than top predators because the formers are more of a threat. Evidence suggests that prey can recognize their predators' vocalizations to assess predation risk (Blumstein et al. 2008; Emmering and Schmidt 2011; Zanette et al. 2011; Cure et al. 2013; Schmidt and Belinsky 2013), and that this capability may be an important part of the top-down mechanisms mediating food web dynamics (Schmitz et al. 2000). In the landscape of fear, with peaks and valleys of predation risk (Laundré et al. 2001), mesopredators should avoid sites where top predators emit any vocalizations and sites where individuals from species sharing predators indicate high predation risk by means of alarm calls. This is based on the fact that empirical studies in bird communities have shown that individuals at high risk of predation may change their distribution, shifting to safer places (Suhonen 1993; Cresswell 2008). Primary prey, on the other hand, could avoid sites where their predators occur and, hence, avoid sites where the mesopredators produce any vocalizations. Alternatively, primary prey could distinguish between sites where the mesopredator gives different type of vocalizations (alarm and non-alarm calls). Non-alarm calls are variable sounds made by many social animals, which differ from alarm calls in that they are not designed to communicate specific information about predators. Therefore, primary prey could prefer areas where mesopredators give alarm calls, because this would indicate that their predator is stressed, compared to sites where mesopredators emit non-alarm calls, because this would only indicate the occurrence of predators in the area. This idea is based on the "enemy of my enemy is my friend" rule proposed by Sabellis et al. (2001). The last hypothesis assumes that predators in risky situations would be ineffective, which is supported by evidence showing that animals would be unable to simultaneously allocate attention to important tasks such as the detection of predators and effective foraging (Metcalfe et al. 1987; Dukas and Kamil 2000; Dukas 2002). The two proposed hypotheses predict, hence, that alarm calls are differently decoded depending on the trophic level in which the receiver is included. As far as we are aware, however, nobody has yet investigated whether prey species may eavesdrop specifically on different calls of their predators to evaluate its presence, abundance and/or stress when choosing breeding habitats.

Individuals constrained by the same factors, from the same or different species, may be useful to each other because they are likely to provide profitable information on shared limiting factors (Parejo et al. 2005; Seppänen et al. 2007). However, the value of social information encoded in calls for receivers within food webs may change according to the intensity of

competition between receivers and callers, as happens with other sources of social information (Seppänen et al. 2007; Parejo and Avilés 2016). For instance, resident titmice *Parus spp.* usually provides migratory flycatchers Ficedula spp. with information on breeding habitat quality that residents evaluate throughout the year (Forsman et al. 2002). However, this only happens when tit densities are between low and moderate levels. At higher tit densities, information from tits becomes ineffective because both flycatchers and tits suffer the costs of the increased competition for resources (Forsman et al. 2008). A number of studies have analysed the effects of competition on social information use at the intraspecific level (e.g. Doligez et al. 1999; Szymkowiak et al. 2016). But no empirical study has yet tested how changes in the level of competition may influence the value of alarm calls, as a particular type of social information, for distinct receivers in natural communities. For example, increased community density, likely to increase competition, in the face of predation risk might have positive effects either through a dilution effect or through an increase in the probability of spotting predators but also might have some costs when predators respond functionally to prey distribution (Schmidt and Whelan 1999; Szymkoviak and Kuczynski 2015). Therefore, costs and benefits of clustered breeding, and hence of increased competition, emerging from social information use may affect the value of this information, and change the result of the interaction.

Here, we aim to identify the value of alarm calls emitted by a mesopredator for the organization of receivers from different trophic levels in a community of hole-nesting birds (Fig. 1a) under variable competitive pressure. This community includes within one trophic level the resident little owl (*Athene noctua*), which is the call emitter, and two migratory species, the scops owls (*Otus scops*) and the European roller (*Coracias garrulus*). Also, the community includes three resident passerines preyed upon species of the higher trophic level: great tits (*Parus major*), spotless starlings (*Sturnus*)

unicolor) and rock sparrows (*Petronia petronia*). Little owls emit conspicuous alarms calls used in habitat selection by conspecifics and scops owls (Parejo et al. 2012). Scops owls and European rollers compete for hole-nesting sites with little owls, while all share potential predators for example in larger owls (Parejo et al. 2012). Finally, the three passerine species also compete for breeding cavities with rollers and little and scops owls, and are common prey of these two latter species (e.g. Obuch and Kristin 2004; Marchesi and Sergio 2005; Tome et al. 2008; Parejo et al. unpublished data) (Figure 1a).

In a crossed design (Fig. 1b), we manipulated social information on a threat at the patch scale by broadcasting calls of little owls indicating different levels of danger. In addition, we modified availability of holes at the patch scale to manipulate competition for nest-sites. Populations of non-excavator vertebrates, as secondary-hole nesting birds, are limited by the availability of existing cavities (Newton 1998; Aitken and Martin 2007), hence, by modifying availability of holes during the choice of nest sites, competition for nest-sites is likely to be changed. Then, we analysed the effects of these two factors on the structure of this cavity-nesting bird community in which all the species compete for nest sites but where some species share predators with the little owl and others are their prey. Therefore, response to the experimental manipulation was measured separately for species within the same trophic level of little owl's guild hereafter) and prey species of the little owl. Distinguishing the relative impacts of predation risk and competition for nesting-sites on the spatial settlement of small predators is important to lower trophic levels because the spatial distribution of prey will be strongly determined by the distribution of predators (Morosinotto et al. 2017). We predicted first that species from the little owl's guild deciphered little owls' alarm calls as a measure of predation threat by top predators, whereas prey of the little owl could interpret them either as a measure of predator presence and/or abundance, or as a measure of their predator's stress. Second, we

predicted that the value of information encoded by little owls' calls changed with the level of competition for nest-holes and the competitive ability of each guild.



Figure 1. a) Simplification of the food web in which the studied hole nesting bird community is included. The little owl (*Athene noctua*), that is the species emitting alarm calls, is marked in yellow. European rollers (*Coracias garrulus*), little and scops owls (*Otus scops*) compete for hole-nesting sites and share potential predators

for example in larger owls (Parejo et al. 2012). Great tits (*Parus major*), spotless starlings (*Sturnus unicolor*) and rock sparrows (*Petronia petronia*) also compete for breeding cavities with rollers and little and scops owls, and are common prey of the two latter species (e.g. Obuch and Kristin 2004; Marchesi and Sergio 2005; Tome et al. 2008; Parejo et al. unpublished data). Arrows of different width indicates the importance of each species in the diet of the predators. Illustrations were made by ME. b) Schematization of the experimental crossed design. One of the groups of six plots in which all the treatments were applied is represented.

Materials and methods

Study system

The study was conducted during the 2013-2014 breeding seasons (April-July) in southeastern Spain ($37^{\circ}18$ 'N, $3^{\circ}11$ 'W). In the study area, the cavity-nesting bird community, including little and scops owls, rollers, great tits, spotless starlings and rock sparrows, use as breeding sites 346 cork-oak nest-boxes held in trees that have been used by these species for years (Parejo and Avilés 2011; Parejo et al. 2012). That is, birds from the cavity-nesting community may have previous knowledge of these nest-boxes because no nest-box was added during the study years. All used nestboxes had a base and roof surface of 24 x 24 cm, a height of 40 cm and an opening 6 cm in diameter, which is wide enough to allow easy nesting of all the studied species. The area is a homogeneous holm oak (*Quercus ilex*) wooded landscape where natural holes are scarce, and when present they are so small that are unsuitable for most of these hole-nesting species (Parejo et al. unpublished data).

Little owls are resident birds in the study area and, hence, are likely to have more precise information on breeding habitat quality than the other two medium-size migrant species, scops owls and rollers, as has been shown in other communities (Monkkönen et al. 1990). Indeed, experimental work has shown that little owls' vocalizations encode valuable information affecting settlement decisions for conspecifics, and heterospecific migrants (Parejo et al. 2012). Furthermore, the other three species breeding in the community (great tits, spotless starlings and rock sparrows) are more or less commonly preyed by

the little owl. Therefore, the little owl is a suitable informer of all the species from the different trophic levels in this bird community.

Experimental design

In 2014 nestboxes were assigned by proximity to patches, each one containing on average 5.86 nestboxes (range = 4-11, n = 346 nestboxes in 59 patches). Patches were at least 300 m apart of each other and nestboxes within each patch were separated by 50-100 m of each other. As the experiment produced six different kinds of patches (Fig. 1b), we spatially assembled patches into groups of six to avoid spatial influence, and hence of habitat quality, on treatments. Within each group of six patches, three duplets of two spatially paired patches were established and randomly assigned to one of the three following treatments: a) "Alarm" (N = 20 patches), in which we simulated alarmed little owls by broadcasting their alarm calls; b) "Non-alarm" (N = 20patches), in which we simulated the presence of non-stressed little owls by broadcasting non-alarm calls; and, c) "Control" (N = 19 patches), in which no playback was broadcasted but visits were as frequent as to Alarm and Nonalarm patches. In addition, competition for holes to breed was increased during the experimental time, which is the period in which nest-site selection of every species is more likely to occur. This was done by blocking the first day of the experiment the entrance of the 60% of nestboxes (High-competition treatment) in one of the two patches of each duplet (N = 29 patches), while competition was not manipulated at all in the other patch of each duplet (Low-competition treatment) (N = 30 patches) (Figure 1b). After 15 days, the blocking was removed and, thus, all nestboxes were available to birds. Each group of six patches included two Alarm, two Non-alarm and two Control patches, each of which one patch was assigned to the high competition and the other to the low competition treatment (Figure 1b). Despite the blocking of nestboxes, in all patches some nestboxes remained empty through the season (see occupation rates of patches within each treatment in Table 1), which minimizes the possibility that responses to the manipulation were due to the lack of nestboxes and not to changes in the intensity of competition during nest-site choice. Furthermore, we acknowledge that the High-competition treatment might actually represent low competition for individual birds after settlement, whenever this treatment triggers low occupation. However, this treatment clearly causes high competition during nest-site choice, which is the effect we looked for as we aimed to measure the response of birds to treatments in terms of distribution, that is, when choosing a place to breed.

Due to the limited number of patches we could define within the study area, we chose to establish a Silent control treatment instead of a Noise control treatment (as in Betts et al. 2008, Szymkowiak et al. 2016). As we had two noise treatments, we consider that responses to playbacks, which we expected to be different according to previous results in the same study system (Parejo et al. 2012), were not likely to be due to attraction to a novel sound. Moreover, the use of vocalizations of a presumably neutral bird species in a Noise control treatment could cause unexpected reactions in receivers because these vocalizations might be conceived as non-neutral and, hence, being attractive or aversive for receivers.

Call and competition treatments were applied during 15 days, from 21 April to 5 May (as in Parejo et al. 2012). The duration and dates of the treatments comprised the time in which most breeding birds in the community are evaluating territories, and, thus, performed manipulations are expected to influence breeding patch selection (Parejo and Avilés 2011; Parejo et al. 2012).

Little owl calls were extracted from Llimosa et al. (1990). They were broadcasted during 2h just before dusk, to be sure that diurnal birds as rollers and passerine species were still active, on alternative days at the core of patches using portable amplified speakers connected to digital takeMS audio players (model deseo) (as in Parejo et al. 2012). Three non-alarm and three alarm calls from different individuals were used to generate two distinct 1.5-3

min of uncompressed audio files with the recordings of the various calls. These two files were randomly mixed with periods of silence from 3 to 8 min and then randomly broadcast to reduce pseudoreplication (Parejo et al. 2012). Little owl calls and silent periods were recorded in separate tracks so that the exact sequence of calls and silences was randomly decided by selecting the random playback mode. The randomized presentation of the three calling bouts of each type in combination with the silence tracks during the 2 h of broadcasting produces an unique assortment of calls for each day of treatment and patch (see for similar approaches Eggers et al. 2006; Schmidt 2006; Parejo et al. 2012). We chose to use the highest quality available little owl calling bouts instead of own recordings of local little owls to avoid data biases resulting from discrimination of familiar calls in our experiment (Hardouin et al. 2006). Average broadcasting volume was 89.1 (+1.1) dB measured 1 m away from the speaker, which closely resembles by ear to the natural production of real little owl calls.

Two days before the start of the experiment, all nestboxes were visited to be sure that no bird was already breeding. At that moment, we only found 7 occupied nestboxes which were removed from our analyses, which were finally performed on 339 nestboxes. Patches were visited weekly from 21 April to the end of June to register occupation (assuming a nestbox was occupied when at least one egg was laid in it), breeding species, laying dates and reproductive parameters.

The responses of species from the little owl guild and prey species of the little owl to the experimental manipulation were measured by using three variables estimating breeding habitat preference: 1) the occupation of a patch by each group of species, i.e. whether a patch is occupied or not at least by one breeding pair of the corresponding group. Preferred habitats should be more likely occupied. 2) For occupied patches, the specific rate of occupation of a patch by each group of species, i.e. the proportion of nestboxes occupied by

individuals of a group per patch. Preferred habitats should be more occupied (Fretwell 1972). Finally, as preferred patches should be more prone to be reoccupied, we used data from 2013, as a pre-treatment year, on nest-box occupation by the different species of each group to analyse: 3) the re-occupation of patches already used in 2013 by individuals of a group of species, i.e. whether an occupied patch in 2013 by individuals of a group of species is re-occupied or not in 2014, that is the treatment year. During the 2013 breeding season, the pre-treatment year, nest-boxes were monitored following the same field protocol as in 2014. In 2013, 41 out of 59 studied patches were occupied by at least one of the target species, 32 patches by species of the little owl's guild and 20 by prey species of the little owl. Therefore, these patches are the sample for analysing this last variable.

Statistical analyses

Analyses were performed using SAS v.9.4 statistical software (SAS 2002-2008 Institute, Cary, NC, USA).

Generalized linear models (Genmod procedure in SAS), with binomial error structure and link function logit, were performed to analyse the effect of the treatments of both little owls' calls (Alarm calls, Non-alarm calls and Control) and competition (High- and Low-competition) on: 1) patch occupation by the different groups of species during the experimental year; and, 2) patch re-occupation in 2014 of patches already occupied in 2013 by the different groups of species.

With the aim to test whether the experiment affected occupation rate of patches by the two groups of species, we ran General linear models (GLM procedure in SAS), with Gaussian error structure and link function identity, in which patch occupation rate (arcsin transformed) was the dependent variable and the call treatment and the competition treatment were explanatory factors.

In all statistical models the number of nestboxes per patch was introduced as a covariate to control for the fact that the number of nestboxes varied among patches and, thus, the actual level of competition. For each statistical model, we provide a measure of its goodness-of-fit (Pearson χ^2 for the logistic models and R² for regression models). Pairwise differences in significant models were checked by comparisons of least-squared means of each treatment.

Results

In 2014, in 41 out of the 59 studied patches we reported nesting at least one of the species of interest (23% of nestboxes being occupied). Species of the little owl's guild occupied 29 patches, while prey species of the little owl occupied 19 patches. The rate of occupied patches and occupation rate of occupied patches by species of each group in the experimental treatments are shown in Table 1.

Table 1. Proportion of occupied patches and average occupation rates for each group of species (little owl's guild and prey species of the little owl) in the different treatments. Competition treatment: LC = Low competition, HC = High competition.

Trea	tments			Variables		
Call	Competition	Occupied patches by species of the little owl's guild/No. of patches	Occupied patches by prey species of the little owl/No. of patches	Occupied patches by all species of the community/ No. of patches	Mean occupation rates of patches occupied by species of the little owl's guild	Mean occupation rates of patches occupied by prey species of the little owl's guild
Alarm	LC	6/10	4/10	1/10	0.22	0.40
Non-alarm	LC	5/10	5/10	2/10	0.28	0.26
Control	LC	3/10	4/10	0/10	0.23	0.18
Alarm	HC	7/10	4/10	2/10	0.24	0.26
Non-alarm	HC	6/10	1/10	1/10	0.32	0.25
Control	HC	2/9	1/9	1/9	0.14	0.20

Little owl's guild

Occupation of patches by individuals belonging to the little owl's guild was influenced by the call treatment (Table 2), so that individuals occupied more frequently patches in which calls were broadcast (i.e. alarm and nonalarm calls' patches) than control patches (post-hoc comparisons: Alarm *versus* Control patches: z = -2.39, p = 0.017; Non-alarm *versus* Control patches: z = -1.90, p=0.047; Alarm *versus* Non-alarm patches: z = -0.40, p = 0.69) (Fig. 2a). In occupied patches, the occupation rate by species within the little owl's guild was not affected by the call or competition treatments (Table 2).

On the other hand, the call treatment affected the reoccupation of patches previously occupied by owls and rollers in 2013 but in interaction with the competition treatment (Table 2). Thereby, in 2014, patches where little owl non-alarm or alarm calls were broadcasted (informed patches hereafter) were more likely to be reoccupied than control patches when competition was low (call treatment effect for the low-competition treatment: $\chi^2_2 = 4.95$, p = 0.084) and high (call treatment effect for the high-competition treatment: $\chi^2_2 = 5.75$, p = 0.056) (Fig. 2b). However, the effect of the competition treatment was only shown in alarm patches (competition treatment effect for the alarm treatment: $\chi^2_1 = 3.68$, p = 0.055) so that they tended to be more reoccupied when competition was low (Fig. 2b). In non-alarm and control patches the effect of the competition treatment was farther from significance (competition treatment effect for the non-alarm treatment: $\chi^2_1 = 2.68$, p = 0.102; competition treatment effect for the control treatment: $\chi^2_1 = 0.07$, p = 0.787). No other explanatory factor had an effect on any of the response variables of the little owl guild (Table 2).



Figure 2. Effects of the call and competition treatments on organization of species within the little owl's guild. a) Percentage of patches occupied by at least one breeding pair of the little owl's guild in each call treatment. b) Percentage of plots already used by individuals of the little owl's guild in 2013 which were re-occupied in 2014 (the year of the experiment) in each treatment. The number of patches per treatment is shown in bars.

Table 2. Sources of variation in the response variables of the little owl guild to the Little owls' Calls and Competition treatments. Results of: 1) Generalized Linear Models investigating Patch occupation in 2014 (the year of the experiment) and Patch reoccupation in 2014 of patches already occupied in 2013 in relation to Calls and Competition treatments; and 2) General Linear Model in which Patch occupation rate was the dependent variable and the Call and the Competition treatments, together with its interaction, were explanatory fixed factors. For each whole model, sample size and Pearson χ^2 (for logistic regression models) or R2 (for lineal regression models) values are shown as measures of their relevance. Note that a non-significant Pearson χ^2 is indicative of no evidence of lack of fit of the model.

	Patch oc	cupati	on	Patch of	occupation	n rate	Patch re-	-occup	ation
	(N = 59 Pearson	plots) $\chi^2_{52} =$	58.99,	$(N = 2)$ $R^2 = 0$	9 plots) .37		(N = 32) Pearson	plots) $\chi^2_{25} =$	22.96,
	P > 0.1						P > 0.1		
Parameter	χ2	df	Р	F	df	Р	χ2	df	Р
Call Treatment	7.06	2	0.029	1.62	2, 22	0.220	8.78	2	0.012
Competition Treatment	0.03	1	0.855	0.08	1, 22	0.775	0.00	1	0.989
Call *Competition treatment	0.65	2	0.723	0.79	2, 22	0.467	6.41	2	0.040
Number of nestboxes	0.72	1	0.397	4.13	1, 22	0.054	0.05	1	0.817

Prey species of the little owl

Occupation of patches by prey species of the little owl was not influenced by the call treatment, but was influenced, although only marginally, by competition for nest sites (Table 3). Prey species mainly settled down in patches with relaxed competition (Fig. 3a).

In occupied patches, the occupation rate by prey species was unaffected by call and competition treatments (Table 3).

Reoccupation of occupied patches in 2013 by prey species was determined by the call treatment in 2014 (Table 3), so that patches where alarm calls where broadcasted in 2014 were preferentially reoccupied by individuals
of these species (post-hoc comparison: Alarm *versus* Non-Alarm patches: z = -1.99, p = 0.049; the other two pairwise comparisons: p > 0.10) (Fig. 3b).



Figure 3. Effects of the call and competition treatments on organization of little owl's prey species. a) Percentage of patches occupied by at least one breeding pair of prey of the little owl's guild in each competition treatment. b) Percentage of patches already used by prey of the little owl's guild in 2013 which were occupied in 2014 (the year of the experiment) in each call treatment. The number of patches per treatment is shown in bars.

Table 3. Sources of variation in the response variables of prey species of the little owl to the Little owls' Calls and Competition treatments. Results of: 1) Generalized Linear Models investigating Patch occupation in 2014 (the year of the experiment) and Patch re-occupation in 2014 of patches already occupied in 2013 in relation to Calls and Competition treatments; and 2) General Linear Model in which Patch occupation rate was the dependent variable and the Call and the Competition treatments and its interaction were explanatory fixed factors. For each whole model, sample size and Pearson χ^2 (for logistic regression models) or R2 (for lineal regression models) values are shown as measures of their relevance. Note that a non-significant Pearson χ^2 is indicative of no evidence of lack of fit of the model.

	Patch occu	patior	1	Patch oc	cupation	rate	Patch re-	occup	oation
	(N = 59 plots) Pearson χ^2_{52} = 53.50,			(N = 19 plots)			(N = 20 plots)		
				$R^2 = 0.17$			Pearson $\chi^2_{13} = 13.99$,		
	P > 0.1						P > =.1		
Parameter	χ2	df	Р	F	df	Р	χ2	df	Р
Call Treatment	2.21	2	0.330	0.49	2, 12	0.622	6.77	2	0.034
Competition Treatment	3.78	1	0.052	0.22	1,12	0.644	0.00	1	0.998
Call *Competition treatment	2.02	2	0.365	0.21	2,12	0.815	2.22	2	0.329
Number of nestboxes	3.84	1	0.050	0.24	1, 12	0.631	1.66	1	0.198

Discussion

The value of alarm calls

We found support for the idea that the informative value of alarm calls emitted by a bird species differs for species in different trophic levels in a food web. For species included in the same guild of the little owl, calls were very important, regardless of the level of competition. Thereby, informed patches were the preferred ones by competitors (Fig. 2a and 2b). Meanwhile, prey species of the little owl appeared to rely on calls of little owls as indicators of predators' stress to reoccupy patches already used in the precedent year because they preferentially re-settled down in patches where little owls were alarmed (Fig. 3b). Hence, these results would provide support for the idea that information on predation risk encoded in alarm calls can be a driver of the

structure of bird communities, through its effects on both individuals of the trophic guild of callers, i.e. competitors, and their prey.

There are many pieces of evidence showing that eavesdroppers get benefits by using information from alarm calls emitted by heterospecifics. In the short time, the benefits can come in the form of improved antipredatory responses (Seyfarth and Cheney 1990; Templeton and Greene 2007; Vitousek et al. 2007; Magrath and Bennett 2012). In the longer term, benefits can come by the choosing of safe habitats (Parejo et al. 2012) and by the enlargement of foraging niches (Ridley et al. 2014). Here, we have detected that species of the little owl's guild (little owls, scops owls and rollers) preferred to breed in patches where little owls' vocalizations were broadcasted, but they did not preferentially choose non-alarm patches. Little owls were expected to respond in this way, as previous experimental work in this community has shown that they were attracted to conspecifics even when they signal high local predation risk through alarm calls (Parejo et al. 2012). Regarding the diurnal roller, it could be argued that it does not distinguish between little owls' alarm and nonalarm calls. As top predators may be behaviourally flexible and adjust their activity rhythms to the rhythms of their prey (Penteriani et al. 2013), the alarm system of little owls against shared predators might be useless for a diurnal bird as the roller. Therefore, rollers could simply be attracted to little owls' calls as indicators of suitable places for competitors and, consequently, for them. However, rollers tended to choose non-alarm patches on their own (Supplementary Material Appendix 1, Fig. A1), indicating that they are able to decode little owls' calls and to choose the safer places. For scops owls, based on previous results in the area (Parejo et al. 2012), they should prefer the patches with lower predation risk indicated by little owls, however, here they seem to prefer alarm patches (Supplementary Material Appendix 1, Fig. A1). The difference between the two studies (Parejo et al. 2012) may reside on the fact that preference for breeding sites was differently measured. Indeed, in Parejo et al. (2012) preference was measured by using the earliest laying date of scops owls per patch. Here, however, as we aimed to measure preference by individuals from different species of the same trophic level, the use of the earliest laying date as a proxy of preference is unsuitable due to speciesspecific phenologies. Therefore, while in the first study we measured the preference of the best individuals immediately after the performance of the experiment, here we analyse the preference of all scops owls through all the breeding period. The effect of the latter experiment could have been diluted after a time, which might lead to the found preference for alarm patches whenever later individuals choose patches with low quality individuals to avoid competition.

Regarding prey species, we only found a response to little owls' calls when we analysed the reoccupation in the experimental year of occupied patches in t-1. This result means, first, that primary prev can distinguish between alarm and non-alarm calls of their predators and, second, that sites with stressed predators attract their prey. That is, predators surrounded by many enemies in a site may make the site attractive to prey because enemies of the enemies may be allies (Sabellis et al. 2001). This could be because predators under high risk of predation would be ineffective predators. Alarm calls as indicators of predation risk so far have largely been considered relevant only when are produced in response to threats that endanger the eavesdropper (Templeton and Greene 2007; Magrath et al. 2009). However, alarm calls may have a different meaning for different receivers, as it is shown above. Here we show that alarm calls may be important to assess local predation risk only for species that share predators with the callers. This happens even when little owls and their prey species share several predators (Fig. 1). However, as prey species of little owls are mainly predated by species from the little owl guild, including the little owl (Obuch and Kristin 2004; Marchesi and Sergio 2005; Tome et al. 2008; own unpublished data), alarm calls of the predator might

provide information about the stress of the predator. This may constitute an example of a top-down mechanism where the (simulated) occurrence of a top predator negatively affects the mesopredator and, in the end, favours the primary prey.

The importance of competition

For species of the little owl's guild, the competition treatment affected the responses to the call manipulation of individuals reoccupying previously used patches in 2013. At least 30% of captured individuals from the little owl's guild in 2014 were also breeding in the area in 2013. Therefore, many individuals occupying in 2014 previously occupied patches are probably familiar with the area. Results suggest that in informed environments, because they mainly chose informed patches, competition for nest-sites with both conspecifics and heterospecifics may have some influence on the structure of the communities of this group of species. Indeed, within informed patches, birds likely to have already bred in the area the year before the experiment chose alarmed patches when availability of holes was high, but trended to choose non-alarm patches when hole availability was low. This pattern might be given by the choice each time of only one commodity by individuals with previous experience in the area. That is, animals might opt by either avoiding competition (through the choice of alarm patches with low competition) or by avoiding predation risk (through the choice of non-alarm patches but with higher competition) in relation to their individual condition or personality. Supporting this argument, animal behaviour is claimed to be determined by individual state on the one hand (Dingemanse and Wolf, 2010), and, on the other hand, individual personality may affect risk-taking behaviour (e.g. Couchoux and Creswell, 2011). These results point out that information, more than competition for nest-sites influences decisions in this trophic level because competition only modulates responses in informed patches, which may be based on the size of these species because they are clearly stronger competitors than the smaller prey species. This seems to be the case at least under the competitive regime we have imposed with our experiment, which could in any case be not large enough to lead to the complete avoidance of competitors and hence to the discarding of call information (Parejo and Avilés 2016).

In contrast, prey species of the little owl tended to preferentially occupy patches with high availability of holes to breed, irrespective the information they conveyed. Secondary hole nesting birds are highly limited by hole distribution and availability to breed (Newton 1998), which may strongly determine their breeding process. Therefore, prey species may be strongly constrained to get a breeding site. Moreover, this response may be because competition for nest-sites also involves predation risk for prey because they compete for nest-sites in many occasions with their predators (Fig. 1). When competitors are also potential predators, it may be difficult to predict the spatial distribution of competitors (Morosinotto et al. 2017). Therefore, in this case results are difficult to interpret and could have been different if we had used calls of a non-competing predator whose calls would only inform on predation risk to prey species and not on competitive pressure. The trend of individuals of this trophic level for areas with low competition leads, however, to these areas to show the highest densities of birds (Fig. 3a), indicating that the manipulation induced a preference. Furthermore, the fact that mean laying dates of the three prev species are within or just after the experimental time (rock sparrow: 7th of May, great tit: 1st of May and spotless starling: 5th of May), leads us to think that the described distribution is mainly a result of the competition manipulation itself.

Conclusion

In conclusion, this study provides a first empirical evidence of the idea that the value of alarm calls as determinants of the spatial distribution of eavesdropping species in different tropic levels may depend on the level of niche overlap among callers and receivers (Parejo and Avilés 2016). The importance of heterospecific alarm calls for susceptible species seems to be determined by the number of limiting factors shared by the callers and the receivers. Thereby, species sharing predators and nest-sites with the callers seem to rely on any vocalization of their competitors to choose their breeding sites. Meanwhile, species sharing nest-sites with the callers and being predated by callers and by others species as well, seem to respond, at least in familiar environments, to their alarm calls when choosing their nesting sites. That is to say, for prey species, alarm calls of their predators seem to inform on predators' stress. Therefore, the consequences of alarm calls on prey distribution should be explored in each particular system to shed light on our understating of cascading effects through the use of social information in natural communities, which may have profound implications in food web dynamics. On the other hand, and also contrary to our expectations, competition for nest sites seemed not to modify the value of alarm calls when they inform on important predators. However, competition seemed to be an important factor of species distribution within a community when information on predation risk was not so relevant since the top predators that endanger the callers are less dangerous for the prey than for the mesopredators. Finally, one recommendation derived from our findings is that studies on community structure should not ignore species that emit alarm calls. Since animal communities are ideal information networks where individuals can gain information on danger from many different species (Parejo et al. 2012), because alarm calls are widespread in animals, social information encoded in alarm calls may greatly influence community structure through interspecific eavesdropping.

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Supplementary material Appendix 1

Additional analyses

Species-specific responses to treatments within each defined group (little owl guild or prey species of the little owl) were analysed when sample size was high enough to allow performing such analyses. This was only possible in the study of the effects of the experimental manipulations on patch occupation. To that aim, we performed a Generalized linear model (Genmod procedure in SAS), with binomial error structure and link function logit, to analyse the effect of the call and competition treatments on patch occupation by each species of the little owl guild during the experimental year. Also, the number of nestboxes per patch was introduced in the model as a covariable. The call treatment trended to determine occupation of Scops owls ($\chi^2 = 5.47$, df = 2, P = 0.06) and rollers ($\chi^2 = 5.59$, df = 2, P = 0.06) and did not affect little owls' patch occupation ($\chi^2 = 1.52$, df = 1, P = 0.22). Species-specific responses to the Call treatment are shown in Fig. A1.



Host nest site choice depends on risk of cuckoo parasitism in magpie hosts



Host nest site choice depends on risk of cuckoo parasitism in magpie hosts

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Abstract

Avian brood parasites impose large fitness costs to their hosts and, thus, brood parasitism has selected for an array of host defensive mechanisms to avoid them. So far most studies have targeted on anti-parasite defenses operating at the egg and chick stages and neglected defenses that may work prior to parasite egg deposition. Here, for the first time, we experimentally explore the possibility that hosts, as part of a front-line defense, might minimize parasitism costs through informed nest site choice based on perceived risk of cuckoo parasitism. We conducted a large-scale manipulation of visual and auditory cues potentially informing on the risk of great spotted cuckoo (Clamator glandarius) parasitism during the nest site choice period of the magpie (Pica pica) host to investigate its effect on host's nest settlement and individual year to year site fidelity. Early breeding magpies preferentially placed their nests in safe areas (i.e. in sites of low perceived risk of parasitism), and, this effect diluted with time elapsed since risk of parasitism was manipulated. Site fidelity of individual magpies decreased with risk of cuckoo parasitism, but only if they were not parasitized in the previous year. Our results constitute the first experimental evidence showing that hosts can minimize the costs of cuckoo parasitism through informed nest-site choice, calling for future consideration of defenses potentially operating prior to parasite egg deposition to achieve a better understanding of cuckoo-host coevolution.

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Introduction

Inter-specific avian brood parasitism occurs when a species, the parasite, lays eggs in the nest of another species, the host, which then raises the parasitic offspring. Brood parasitism often diminishes the reproductive success of the host, sometimes entailing the total failure of the breeding attempt (Payne 1977; Rothstein 1990). Thus, interspecific brood parasitism is a powerful selective force selecting for host-defenses against brood parasites. Host defenses may operate either before the parasite lays its eggs into the host's nest (e.g. mobbing behavior before laying, (Røskaft et al. 2002; Welbergen and Davies 2009), or after parasite egg laying (e.g. parasite egg discrimination and rejection (e.g. Davies and Brooke 1988; Soler and Møller 1990; Moksnes et al. 1991; Avilés et al. 2010; Spottiswoode and Stevens 2010) or nestling discrimination (Langmore et al. 2003; Grim 2007; Sato et al. 2010; Tokue and Ueda 2010).

Proactive avoidance of brood parasitism is a likely mechanism that may reduce the costs of parasitism and host-defense against cuckoos, by acting as a front-line defense (Feeney et al. 2012). Indeed, probabilities to protect the host's entire breeding attempt are higher when host defenses impede the parasite from laying the egg into the host nest than when prevention occurs after parasite's egg laying (Feeney et al. 2012). Thus, under high brood parasitism pressure, selection is expected to favor mechanisms allowing hosts to assess the risk of brood parasitism in advance and respond to it accordingly. Evidence that birds may proactively avoid risky breeding places came from experimental work showing that birds can rely on cues informing on predation risk when choosing their breeding locations (e.g. Eggers et al. 2006; Fontaine and Martin 2006a; Fontaine and Martin 2006b; Mönkkönen et al. 2009; Parejo and Avilés 2011). However, evidence that hosts of avian brood parasites may assess parasitism risk prior to choose their nest-sites is mostly correlative (reviewed in Kruger 2007), and, restricted to a handful of studies showing that some hosts locate their nests far from vantage points (Øien et al. 1996; Moskát and Honza 2000; Antonov et al. 2007; Patten et al. 2011; Welbergen and Davies 2012), or in non accesible places to brood parasites and/or predators (Kruger 2004; Avilés et al. 2005; Grim et al. 2011). Correlative studies however, do not exclude the possibility than noncontrolled environmental factors (e.g. food resources, predators and/or competitors) may indeed have shaped host nest-site choice. So far two studies have provided experimental support for habitat selection based on assessment of local parasitism risk. On the one hand, Forsman and Martin (2009) studied a bird host community parasitized by the generalist brown-headed cowbird Molothrus ater. These authors showed that hosts species, by settling down during or after the manipulation of parasitism risk, avoided the patches with simulated high risk of parasitism. However, potential hosts of cowbirds responded to the experiment as a group and not at the species level (except for one host species), raising the possibility that density-dependent processes may be at work (Forsman and Martin 2009). On the other hand, Tolvanen et al. (2017) have recently conducted a playback experiment to test for the capability of a bird community to perceive cues emitted by the common cuckoo Cuculus canorus in Finland. Density of open-nesting hosts as a whole, but not that of preferred hole nesting hosts, was lower in sites where cuckoo calls were emitted as compared to silent control sites (Tolvanen et al. 2017). However, as noted by the authors themselves, they did not include a control for the playback treatment, and it is possible that their results were the result of an increase in vocal activity by a species other than cuckoos at a particular site.

In this study we aim to demonstrate for the first time that hosts assess parasitism risk in advance and select nest sites with low risk. We experimentally increased great spotted cuckoo *Clamator glandarius* (hereafter cuckoo) parasitism risk to determine its influence on nest settlement and site fidelity of its main host, the European magpie *Pica pica* (hereafter magpie). We predicted i) that magpie hosts perceive the risk of being parasitized and choose nest sites with low risk of parasitism and, ii) that between-year site fidelity of individual magpies would decrease with an increase in risk of parasitism (e.g. Kolecek et al. 2015). Because previous experience with cuckoos is likely to influence future nest-site choice of individual hosts (Hoover 2003; Hoover and Robinson 2007; Pasinelli et al. 2007; Fuller 2012), it may affect their perception of risk. Indeed, experimental work has shown that previous experience with parasites may exacerbate host sensitivity to novel parasite cues in great reed warbler *Acrocephalus arundinaceus* hosts (Hauber et al. 2006), suggesting that experience may modulate anti-parasite defenses against parasites. Therefore, it is reasonable to expect that our second prediction was more likely fulfilled by the subset of individuals that had experienced cuckoo parasitism in the previous breeding season.

Finally, our experimental setup also allows us to conclude about the temporal value of cues of avian brood parasites. The informative value of cues is likely to depend on synchrony between cue production and the time when the observer can collect the information (Nocera et al. 2006). Perceived risk of parasitism was manipulated prior to the first magpie reproductive event in the population, hence we predicted iii) that the effect of the experiment diluted along the breeding season, and therefore that late breeding magpies disregarded cues on risk of parasitism.

Methods

Study area and system

The study was conducted in La Calahorra (37° 10′ N, 3° 03′ W, Hoya de Guadix, Southern Spain) during the breeding seasons of 2012 and 2013. This

is a patchy area of about 12 km² where groves of almond trees *Prunus dulcis*, in which magpies preferentially build their nests, are very common (Molina-Morales et al. 2012; Molina-Morales et al. 2013). Magpies are territorial, sedentary, and socially monogamous long-lived passerines (Birkhead 1991).

In our study area, magpies lay one clutch during April-May, and are the main host of the great spotted cuckoo (Soler 1990). Cuckoo parasitism increases over the season and varies between years (see Molina-Morales et al. 2013). Cuckoo parasitism severely reduces magpie reproductive success through early hatching of cuckoo nestlings and effective competition for parental care with magpie nestlings (e.g. Soler and Møller 1996; Soler et al. 1996). Consequently, parasitism has selected for host recognition and rejection of cuckoo eggs, which in turn has selected for punitive cuckoo behaviors promoting parasitism acceptance ("mafia behavior" *sensu* Soler et al. 1995). Thus, the interaction between magpies and great spotted cuckoos is considered an example of antagonistic coevolution (Soler and Soler 2000).

Experimental manipulation of cuckoo parasitism risk

We conducted a large-scale manipulation of visual and auditory cues potentially informing on risk of cuckoo parasitism during the nest site choice period of magpies in 2013.

The experiment started 15 days before the first magpie egg was laid, and hence potentially when magpies were still evaluating breeding territories. The experiment consisted on broadcasting 6 hours of great spotted cuckoo calls together with the presentation of a great spotted cuckoo dummy at 9 randomly selected fixed points (i.e. Risky points) of the study area. As a non-harmful control we emitted Hoopoe *Upupa epops* calls and placed a hoopoe dummy at 10 randomly selected fix points (i.e. Non-risky points) in the area (electronic Supplementary Material Figure S1). Treatments were applied in the morning during 15 days (from 21 March to 5 April), in alternate days in

risky and non-risky points. We chose the hoopoe as a non-harmful control for the experiment because it poses no threat to magpies and breeds regularly in sympatry with magpies; therefore, magpies are familiar with its presence in the study area. Dummies (5 great spotted cuckoos and 4 hoopoes) were handmade out of plaster and painted by a specialist (http://www.replicaanimal.com/) to resemble real great spotted cuckoos and hoopoes in color, size and shape (electronic Supplementary Material Figure S2). They were made in a standing position and have proved to elicit real responses in magpie hosts (e.g. Avilés, Bootello et al. 2014).

Dummies were placed on the top of a 50 cm height camouflaged box containing a speaker connected to an mp3 player that broadcasted the corresponding species' call. The calls consisted of three different tracks of great spotted cuckoo calls and four hoopoe calls (Llimosa et al. 1999) with two and three 1-min silence tracks, respectively, that were randomly selected and played continuously during the experiment. This produces unique assortments of calls for each nest and treatment due to their randomized presentation and combination with silence tracks, thus minimizing the risk of pseudo replication (e.g. Ghalambor and Martin 2000; Parejo et al. 2012). The natural location of breeding avian predators has been proposed to create predation risk gradients for their prey, giving rise to a predation risk landscape (Thomson et al. 2010). Analogously, our manipulation created a parasitism risk landscape that simulated a natural situation where avian brood parasites are likely to act more intensively in concrete areas of the population. A recent study has provided evidence of spatially structured cuckoo parasitism in our population (Molina-Morales et al. 2013), therefore, our manipulation of risk of cuckoo parasitism is likely to induce biologically relevant effects in magpies.

Data collection and variables

As part of a long-term project magpie nests from this population are monitored every year from 1 March to the beginning of July, which allowed us to locate every magpies breeding attempt. Nests were found by careful inspection of all trees in the area, and GPS positioned. For each nest we recorded: i) distance in meters to the nearest risky point (i.e. cuckoo stimulus), and ii) distance in meters to the nearest non-risky point (i.e. hoopoe stimulus). We calculated the difference between the distance to the nearest risky point and the nearest non-risky point for each nest as a measure of the spatial distribution of the host relative to risk of cuckoo parasitism (hereafter parasitism risk proximity). Thus, nests with positive values will be those located farther away from a risky stimulus and closer to a non-risky stimulus.

Also, magpies in this population are routinely banded with unique combinations of color plastic leg bands (e.g. Avilés, Bootello et al. 2014; Avilés, Molina-Morales et al. 2014; Molina-Morales et al. 2014; Molina-Morales et al. 2016). Some individuals were marked at the nest but most already showed adult plumage when caught (Birkhead 1991), and, therefore, their exact age was unknown. Thus, for the analyses, we assigned a relative age (i.e. value of 1 for naïve or 2 for adult) to differentiate categorically between naïve individuals (i.e. up 2 years old when normally this species begin reproducing (Birkhead 1991) and adult individuals (i.e. 3 years old or more) (see Molina-Morales et al. 2016). Aiming to study individual magpie's site fidelity in response to risk of parasitism, we identified the adults breeding in each nest by observing all nests with telescope from a hide located around 100 m away. We did so in 2012 and 2013 (i.e. the year when we performed the experiment), which allowed us to assign marked birds to each nesting attempt and test i) whether site fidelity to breeding territories (i.e. staying vs. leaving the population) was influenced by perceived risk of parasitism in

2013. Some individuals here considered as leaving the population may indeed be dead or not detected in 2013. However, apparent survival in our population is as high as 83.0% (\pm SE = 2.8, Martinez et al. unpublished data), suggesting that, here, non-detected individuals would be mostly dispersers. Nests were visited at 5 days intervals, except during egg laying when nests were visited every 2-3 days to detect cuckoo parasitism. Nests were categorized as parasitized if at least one cuckoo egg was detected in the nest. Although magpie nests were not visited daily, magpies in our study area only eject about 5% of real cuckoo eggs (see Soler et al. 1995), and so the risk of not detecting parasitized nests (because magpies rejected the cuckoo eggs before our next visit) is negligible. In addition, since cuckoo eggs can easily be differentiated from magpie eggs (see Soler et al. 2003), we are confident that parasitism instances were accurately assessed.

Ethical statement

The Consejería de Medio Ambiente y Ordenación del Territorio (Junta de Andalucía) authorized the fieldwork of the present study (projects CGL2011-27561/BOS and CGL2014-56769-P; license code: P06-RNM-01862).

Statistical analyses

Analyses were performed in SAS, version 9.4. (SAS Institute 2012). Variables were standardized prior to run the analyses.

The effect of cuckoo parasitism risk on host distribution was studied by using a General Linear Model on parasitism risk proximity as dependent variable. As our expectation was that the effect of experiment diluted over the season (see Introduction), we used laying date as a predictor in the model. Residuals of the general linear model did not significantly depart from normality (Kolmogorov-Smirnov test for normality, P > 0.20).

In addition, to study magpie site fidelity in response to risk of parasitism, we first performed a Generalized linear model (with Binomial error distribution and a logit link function) in which probability of finding one individual magpie in the population from 2012 to 2013 was modelled in relation to parasitism experienced in 2012 (i.e. parasitized vs. non-parasitized nest) as a categorical fixed factor, distance of each individual nest in 2012 to the nearest risky point in 2013 (distance to risky point) as a continuous predictor, as well as the interaction between these two factors. Relative age (naïve vs. adult bird) was also introduced in the model to control for possible age effects. In addition, we also entered conspecific density estimated as the average distance from each nest to the two nearest conspecific nests because a previous study showed that conspecific density influenced magpies breeding dispersal in the population (Molina-Morales et al. 2012). Only a small fraction of nests had both social parents ringed in 2012 (n = 8 nests) whereas most nests (n = 43 nests) had only one social parent ringed. Thus, only one randomly selected identified individual per nest was considered in the analyses, irrespective of its sex or any other individual characteristic. Once we had detected an interactive effect of distance to risky point and previous parasitism experience on probability of finding one individual in consecutive years (see Results), we explored in two separate Generalized linear models (one for previously parasitized individuals and another for individuals without a previous experience of parasitism) how this probability associates with the distance to a risky point.

Results

Cuckoo parasitism risk and nest settlement by magpie hosts

As expected, early, but not late, host breeders located their nests farther from cuckoo risky than from non-risky points (Beta (lower CL, upper CL) = -0.33 (-0.60, -0.07), F_{1,52} = 6.58, P = 0.01, Figure 1).



Figure 1.Distance to cuckoo (filled boxes) and hoopoe stimulus (open boxes) (median \pm quartile, boxes; and range, whiskers) of early and late magpie breeders (n= 54 nests).

Cuckoo parasitism risk and individual host site fidelity

Probability of finding individual magpies in consecutive years in our population depended on the interaction between perceived risk of parasitism and its previous experience with cuckoos (Table 1). Fidelity decreased with the increase of risk of cuckoo parasitism in individuals that had not experienced parasitism in the previous season ($X_{I}^{2} = 5.35$, P = 0.02, n = 27

individuals, Figure 2 a). Meanwhile, magpies that had experienced parasitism were not influenced by ulterior information about risk of parasitism in their breeding sites ($\chi^2_1 = 2.14$, P = 0.14, n = 17 individuals, Figure 2 b).

Table 1. Result of Generalized linear model (binomial errors, logit link) to study factors affecting site fidelity (i.e. probability of finding one individual magpie.

Site fidelity	Coefficient	SE	X^2	DF	р
n= 44 magpies					
Intercept	2.14	0.78			
Age	1.77	1.57	1.61	1	0.20
Distance to risky point	-0.003	0.001	5.12	1	0.02
Parasitism	-2.53	1.24	4.98	1	0.03
Distance to risky point*Parasitism	0.005	0.002	7.03	1	0.008



Distance to risk of parasitism (metres)

stimulus and previous parasitism experience. (a) Individuals that were not parasitized in 2012 (n= 27 individuals); (b) Individuals parasitized Figure 2. Individual site fidelity (i.e. probability of finding one individual magpie host in two consecutive years) in relation to cuckoo in 2012 (n= 17 individuals).

Discussion

Previous studies had suggested that hosts of avian brood parasites would prefer habitats with low risk of parasitism when building their nests (Øien et al. 1996; Moskát and Honza 2000; Antonov et al. 2007; Forsman and Martin 2009; Patten et al. 2011; Tolvanen et al. 2017). However, this largely assumed contention needed strong experimental support. Here we show, for the first time, that magpie hosts can perceive the risk of cuckoo parasitism through visual and auditory cues and respond accordingly modifying their nest site choice. As expected, the effect of fear to parasitism on host nest-site choice diluted with time elapsed since risk of parasitism was manipulated. Importantly, population patterns of nest site choice in relation to parasitism risk were supported by individual-based analyses of site fidelity. These results, thus, constitute the first experimental evidence of effects of risk of parasitism in host nest-site choice at an individual level.

As expected, early magpie hosts settled farther from risky cuckoo points, whereas late ones settled randomly with respect to risk of suffering cuckoo parasitism. One likely explanation to the pattern would be that late magpies disregarded of risk of parasitism cues emitted long before their reproduction because they do not provide reliable information about parasitism risk at the time of breeding (Nocera et al. 2006). Alternatively, it could be argued that late breeding magpies were not in their breeding territories at the time we manipulated cuckoo parasitism risk and therefore they could not detect induced variation in cuckoo parasitism risk. This possibility, however, is unlikely because magpies are detected in the area prospecting and building their nests before the laying period (Pers. Obs.). Another explanation is that the diluted effect was a by-product of habitat saturation, and, thus, that late breeder magpies had a lower chance to find suitable free space to breed. In this scenario, early individuals can assess the quality of their habitat and gain advantage compared to late ones, which

would be relegated to unoccupied areas (i.e. high risk of parasitism and/or low quality). Indeed, older magpies tend to reproduce earlier than younger ones (Birkhead 1991). This diluted effect would thus add to the classical relationship between individual quality and timing of birds' breeding season (Verhulst and Nilsson 2008), leading to early breeders to be settled in the best areas to breed. Irrespective of the mechanism behind this pattern, our results emphasize the importance of fear to suffer cuckoo parasitism during nest-site choice, and suggest that magpie hosts are able to perceive parasitism risk through visual and acoustic cues and proactively select areas, which are far from risk of parasitism to locate their nests.

Interestingly, magpie responses to risk of parasitism in terms of site fidelity were contingent on previous experience with cuckoos. Indeed, individuals that had previously experienced cuckoo parasitism disregarded for cues informing hosts about risk of parasitism, whereas individuals that had not been parasitized in the precedent breeding season were more faithful to their nesting sites if they were far from parasitism risk (Figure 2). These results are thus contrary to our a priori expectation (prediction ii) that previous experience with parasites will exacerbate host sensitivity to novel parasite cues. Why fear to cuckoos appeared to be less if magpies had suffered cuckoo parasitism is intriguing and could be explained by reliability of informative cues. Previous breeding experience in a particular habitat may influence return rate and breeding dispersal distances in birds (Hoover 2003; Pasinelli et al. 2007). More generally, when an individual possess own information about a resource, it should preferentially use it compared with external information (Templeton and Giraldeau 1996; Nordell and Valone 1998; Kendal et al. 2004). Information reliability is an important factor determining relevance of information, so that how consistently the information is related to something relevant in the environment, i.e. reliability (sensu Searcy and Nowicki 2005), may determine which information is attended for decision-making (Dunlap et al. 2016; Blumstein et al. 2004; Searcy and Nowicki 2005). In this context, previously parasitized magpies should balance information about a real parasitism event and a possible future event based on local parasitism risk. However, it is not easy to grasp why already parasitized individuals disregarded information about cuckoo risk as this may constitute reliable, although not sure, information about a known cost of parasitism. One possible explanation is that the absence of an effect of risk of parasitism for individuals that were already parasitized was due to an early dispersal event of these individuals out of the population (see main effect of parasitism in Table 1). In this vein, while nonparasitized magpies would make settlement decisions based on local abundance of parasites, once an individual faced cuckoo parasitism, it will preferentially disperse out of the population (Kolecek et al. 2015), and, therefore, will have a very low chance of being exposed to risk of cuckoo parasitism in the year of the experiment.

On the other hand, we failed to find an effect of relative age of individuals on their fidelity to their breeding area. Naïve individuals, due to their inexperience, are usually less competitive for breeding territories than older ones (Nordell and Valone 1998). Therefore, naïve individuals are expected to make a worse assessment of resource quality. In our study, however, we did not detect any effect of age on the response to parasitism risk on individual fidelity to breeding areas. Perhaps our relative estimation of individual age is under this lack of response because we only could distinguish between young and adult birds and the effect of age is probably more progressive.

Conclusion

Our experiment demonstrates that hosts can use cues informing about risk of cuckoo parasitism to proactively choose their nest site, and that the use of such cues by individual hosts is contingent on previous experience with

cuckoo parasites. Hence, our results highlight the importance of considering the fear of suffering cuckoo parasitism in future studies assessing habitat choice by cuckoo hosts. More broadly, habitat assessment of cuckoo parasitism risk may constitute a front-line defense against brood-parasites that may lead to hosts to successfully breed in presence of parasitism (Feeney et al. 2012), worth exploring in other avian-brood parasite systems. Finally, our study illustrates the importance of addressing nest-site choice by hosts on marked individuals with a known history of their interaction with the parasite, as current nest-site choice patterns may reflect the effect of parasitism in the past.

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Data accessibility statement Analyses reported in this article can be reproduced using the data provided by Expósito-Granados et al. (2017). Expósito-Granados, M, Parejo, D, Martínez, JG, Precioso, M, Molina-Morales, M, Avilés; JM. 2017. Data from: Host nest site choice depends on risk of cuckoo parasitism in magpie hosts. Behavioral Ecology. DOI: http://dx.doi.org/10.5061/dryad.5595b.

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Supplementary Material

Host nest site choice depends on risk of cuckoo parasitism in

magpie hosts

Supplementary Fig. S1. Map of the study area with the 19 experimental points. Experimental treatments are: Risky points (filled boxes); Non-risky points (open boxes). Open circles represent all GPS positioned nests of 2013.



Supplementary Fig. S2. Dummies presented at the experimental points of the study area. (a) Great spotted cuckoo used in risky points; (b) Hoopoe used in non-risky points.



Capítulo 4

Sex-specific parental care in response to predation risk in the European Roller, *Coracias garrulus*



Sex-specific parental care in response to predation risk in the European Roller, Coracias garrulus.

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Abstract

Sub-lethal effects of predation constitute an important part of predation effects, which may modulate prey population and community dynamics. In birds, the risk of nest predation may cause a reduction in parental activity in the care of offspring to reduce the chance of being detected by predators. In addition, parents may modify their parental food allocation preferences within the brood in response to predation risk. Our aim in this study is to evaluate the effects of risk of nest predation on parental care and within nests food allocation in the European Roller (Coracias garrulus), an asynchronously hatching bird. We manipulated brood predation risk by placing a snake model that simulates the most common nest predator in the Mediterranean region. Our results show that males but not females increased their provisioning rate when they were exposed to the model and that despite this, nestlings' body mass decreased in response to this temporary increase in predation risk. We did not find evidence that parents changed their food allocation strategy toward senior or junior nestlings in their nests in response to predation risk. These results show that the European roller modifies parental care in response to their perception of predation risk in the nest and a sex-specific sensitivity to the threat, which suggests a different perception of offspring reproductive value by parents. Finally, our results show that changes in parental behaviour in response to nest predation risk might have consequences for nestling fitness prospects.

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Introduction

Predation is a major evolutionary force shaping life history and behaviour in prey populations (Caro 2005). Beyond its lethal effects, animals must also cope with the threat of predation at some point in their life (Elton 1927). Risk of predation could also have important indirect or sub-lethal effects which may promote the evolution of plasticity in behavioural and reproductive traits (reviewed in Creswell 2008; Lima 2009; Martin & Briskie 2009), and that ultimately might induce changes at the level of population and community (Creel & Christianson 2008).

In birds, nest predation is the main cause of reproductive failure (Nice 1957; Martin 1993; Thompson 2007). Hence, during the breeding season, birds may adopt different strategies to cope with the risk of nest predation. For instance, birds may modify their habitat choice to avoid environments with a high risk of predation (Fontaine & Martin 2006a; Thomson et al. 2006a; Parejo & Avilés 2011; Parejo et al. 2012b). Also, birds can plastically modify their reproductive traits such as clutch size, tending to save energy for future breeding opportunities (Slagsvold 1984; Martin et al. 2000a; Ferretti et al. 2005; Eggers et al. 2006; Thomson et al. 2006b).

Predation risk can cause a reduction in parental activity during incubation (Martin & Ghalambor 1999; Ghalambor & Martin 2002; Fontaine and Martin 2006b) and in the care of offspring (Ghalambor & Martin 2000, Martin et al. 2000a; Eggers et al. 2005; Fontaine & Martin 2006b; Parejo et al. 2012a; Ghalambor et al. 2013) to reduce the chance of being detected by predators (Martin et al. 2000b; Ghalambor & Martin 2002). Some species, however, provide less but bigger food loads in risky situations (Martin et al. 2000a; Eggers et al. 2008), which partly alleviates the negative effect of a decrease in provisioning rate on offspring quality (Ydenberg 1994). Also, some studies have shown that adults of some species might increase nest attendance (Hakkarainen et al. 2002; Fontaine & Martin 2006b), and

provisioning (Martin et al. 2000a; Thomson et al. 2010) when their nests are in risk, which may vary between open and cavity-nester species (Martin & Li 1992; Martin 1995). By increasing provisioning rates, parents may reduce nestling begging that might attract predators (Briskie et al. 1999), or prioritize faster nestling growth (Cheng & Martin 2012).

A less studied aspect of parental care in birds in relation to predation risk is parental food allocation within broods. Analyses of parental preference within avian broods have often revealed unequal distribution of food among siblings (Kilner 1995, 1997; Price & Ydenberg 1995; Cotton et al. 1999; Smiseth & Amundsen 2002; Smiseth et al. 2003). However, there is very little information about how predation risk might modify parental food allocation within the brood. One study by Tilgar and colleagues (2011) has shown that, in the Pied Flycatcher (*Ficedula hypoleuca*), parents preferred to feed big *versus* small chicks under control conditions, whereas they feed at random when predation risk at the nest was increased. This would suggest that parents in a high risk environment could feed suboptimally at least part of their brood (Tilgar et al. 2011). In great tits (Parus major), however, females ignored the lightest nestlings under increased perceived predation risk, suggesting that the perception of predators may facilitate brood reduction mechanisms (Moks & Tilgar 2014). However, further studies with other species are needed, particularly in birds with a high degree of hatching asynchrony, in order to generalize about a possible role of nest predation risk on parental food allocation strategies.

In this study we experimentally increase nest predation risk aiming to determine its influence on i) parental care and ii) food allocation within broods of the European Roller (*Coracias garrulus*) (here after roller). A previous study has shown that rollers avoid breeding sites with high predation risk (Parejo & Avilés 2011). Also, rollers decrease nest attendance in response to odour cues potentially emitted by nestlings (Parejo et al.

2012a). We specifically predicted that i) parents would decrease provisioning rates which ii) may potentially affect body mass gain of nestlings in the nest in response to predation risk. In testing these two predictions we specifically will examine sex-specific sensitivity to predation risk as recent evidence suggests that social and genetic benefits of parental care might differ for females and males (Schroeder et al. 2013), which may promote that risk of predation was differently perceived by males and females (e.g. Moks & Tilgar 2014). Concerning food allocation strategy among siblings, we expected iii) that parents changed their feeding preferences. Whether parents under stress will bias toward senior or junior nestlings will depend on whether rollers prioritize feeding nestlings with more options to survive (i.e. senior nestlings) (Clutton-Brock 1991), or feeding as fast as possible to avoid predation risk and, thus, abandoning any feeding strategy (e.g. Tilgar et al. 2011).

Methods

Study system

The roller is a migratory, cavity-nesting and socially monogamous bird that lays one clutch per year of about 5 eggs (Cramp 1998; Avilés 2006). Both sexes participate in incubation and feeding tasks (Cramp 1998). Incubation takes 20d and begins before clutch completion, leading to asynchronous hatching and a size hierarchy between siblings in the nests (Parejo et al. 2007).

Study area

The study area is located in the surroundings of Baza (37 $^{\circ}$ 18' N, 3 $^{\circ}$ 11' W), in south-eastern Spain. Since 2007 adult and fledgling rollers have been

metallic and coloured ringed for identification. In this area rollers breed in cork nest boxes (base and roof surface 24 x 24 cm, a height of 40 cm and an opening of 6 cm in diameter) which are mostly attached to trees (mainly holm oak, *Quercus ilex*) (see Rodríguez et al. 2011 for details). The most frequent predators of rollers in nest boxes in this area are the ladder snake (*Rhinechis scalaris*) and the garden dormouse (*Eliomys quercinus*) (Parejo et al. 2012a). Nest predation rate in the population considering sure predated nests was around 22% for 8 years of monitoring (Parejo & Avilés own data).

Data collection

The study was conducted during the breeding season (from early May to late July) of 2011. Nest boxes were first visited weekly to record roller nest box occupation (i.e. at least one egg was detected in the nest-box). Subsequently, we visited the nest boxes every second day to calculate laying date, clutch size and hatching date of each nestling. Each hatched nestling was individually identified by marking their feet and legs with a felt-tip marker pen. Offspring within each nest were classified according to the hatching date in two groups of siblings: the first group included all siblings hatched on the first or second day (senior nestlings hereafter) and the second group that included siblings that were hatched 3 or more days after the date of the first chick hatched (junior nestlings hereafter). This differentiation is based on the pattern of incubation of the species, which normally begins to incubate after laying the third egg, so that the first 3 nestlings usually hatch in 1 to 2 days and the other ones hatch on successive days generating a patent hierarchy of size between siblings (Parejo et al. 2007). Therefore, the inclusion of hatching rank in our analyses will allow us to check if the nestlings' size influences parental food allocation in the nests.

Capítulo 4

Experimental manipulation of predation risk

Predation risk was increased by exposing all nests to one snake model the day of hatching of their last egg during 90 min (i.e. experimental time). The snake model was placed on the ground at a maximum distance of 5.0 m from the vertical line of the nest-box to the ground, so that it might be visible to any feeding roller approaching the nest. We used five different snake models in the experiment that were rubber-made and resembled a snake in a basking position. Habituation to snake models is expected to play a minor role because snakes are cold-blooded predators which show a static behaviour during long time while basking (Parejo & Avilés 2011). The ladder snake is the most common predator of eggs and nestling rollers in the south of Spain (Parejo et al. 2012a) and, previous experimental evidence has shown that rollers perceive our rubber snake models as a predator when they are searching for territories (Parejo & Avilés 2011). In each nest we filmed parental behaviour before (pre-treatment time) and after experimentally increasing predation risk on the same day. Thus, each nest was first filmed in a control non-predation risk situation and afterwards under experimental conditions.

Parental behaviour at the nests was filmed with video cameras (Sony DCR -SR32) that were camouflaged and placed at a distance of 5-10 m from the hole of the nests. Recordings of both, control and experimental periods lasted about 90 minutes (pre-treatment time: mean \pm SE = 97.72 \pm 1.52 min; experimental treatment time: mean \pm SE = 91.66 \pm 1.85 min), and were performed in the morning (average start time of the recordings: pre-treatment: 9:40 h; experimental treatment: 10:42 h). Previous experimental work in our population has shown that three hours is time enough to detect changes in parental provisioning (Parejo et al. 2012a) and body mass gain of nestlings (Avilés et al. 2011) in response to stimulus that parents may perceive at their nests.

The day of the experiment, nestlings were weighted to the nearest 0.50 g with a Pesola spring balance before start of filming, at the end of the pretreatment time and again at the end of experimental period, which allowed us to estimate body mass change of every nestling during the pre-treatment and the predation risk increased experimental time. Previous studies have shown that body mass gain is a reliable predictor of parental preference in altricial birds (Heeb et al. 2003; Bize et al. 2006). Furthermore, a pilot study in which we studied in rollers the relationship between body mass gain and feeds received by individual nestlings revealed that body mass gain can be used as a reliable indicator of parental food allocation in rollers (Supporting information).

From the recordings we estimated the parental visit rate (i.e. number of visits to the nest-box per hour), and provisioning rate (i.e. the number of visits with feed per hour) during the pre-treatment and experimental time. Preliminary analyses in which we compared parental behaviour between the 30 minutes period immediately after the first visit of one of the two adults and the 90 minutes experimental time revealed no differences in visit and provisioning rates, suggesting that parental habituation to the snake model was unlikely (see Supporting information).

In total we filmed 20 nests. Two were excluded from analyses because they were not visited at all by any adult roller during the pre-treatment time. One more nest was excluded for visit and provisioning analyses due to malfunction of video-camera during the experimental time. In addition, although adults were ringed, parental identification on films was only possible in 9 out of 17 nests due to impossibility to recognize coloured rings in 8 out of 17 nest's recordings. Thus, we used data from 17 nests for most analyses, but we evaluated differences in provisioning rate between female and male rollers using data recorded at 9 nests.

Our experimental setup has some limitations worth mentioning. Due to the small sample size of reproductive pairs in our study population we opted not to include a second group of nests in which parents were exposed to a non-harmful control stimulus after the pre-treatment time (see for instance Ghalambor et al. 2013). Thus, any parental response to predation risk in our experiment might be due to a methodological artefact derived from the order in which we conducted the recordings (i.e. first pre-treatment time and then experimental time). However, this seems unlikely because: 1) in a pilot study in which 4 nests were exposed to a non-harmful hoopoe Upupa epops dummy after a pre-treatment time, we did not find differences in parental attendance (i.e. visit (Wilcoxon matched pairs tests: Z = 1.46, p =0.14) and provisioning (Wilcoxon matched pairs tests: Z = 1.10, p = 0.27)) rates or brood body mass rate (Wilcoxon matched pairs tests: Z = 1.10, p =0.27) between pre-treatment and non-risky experimental treatment periods. Moreover, when we deeply explore the results of the pilot study, we found higher brood body mass gain for experimental non-harmful time (mean \pm SE = 0.94 \pm 1.33 g) than for pre-treatment time (mean \pm SE = -0.20 \pm 0.96 g), suggesting that the decrease in mass from pre-treatment to risky treatment do not reflect an initial compensatory body mass gain in nestlings after the fasting night. However, this evidence should be taken cautious because only four nests were used and thus cannot be discarded that we failed to detect differences in parental care due to low power; 2) Furthermore, a recent study in the same population showed that rollers did not modify their parental activity after a pre-treatment time when their nests were exposed to control odour cues (Parejo et al. 2012a). 3) Finally, a recent comparative study found lack of difference in parental care between pre-stimulus and a non-harmful control stimulus time consistently for all 10 bird tested species (Ghalambor et al. 2013). Therefore, we are confident that any change in parental behaviour of rollers in response to our experimental setup would be attributable to increased predation risk.

Ethical note

The experimental procedure did not affect the survival of the birds in any of the nests used for this study. Capture of adult for ringing was also non-harmful for rollers. Data was collected under license of the Junta de Andalucía, Spanish region in which the study was done (Reference number: SGYB/FOA/AFR/CFS). Therefore, data collection complies with the current laws of Spain, where the study was performed.

Statistics

We tested for differences in visit and provisioning rates and total body mass gain of nestlings of a nest (i.e. body mass gain per brood) between control predation free time (pre-treatment) and predation-increased experimental time using non-parametric Wilcoxon Matched Pairs Tests. In addition, for the subset of nests in which parental identification was possible we use Fisher exact tests to compare probabilities of nest provisioning by each sex in relation to predation risk. In this subsample we also run Wilcoxon Matched Pairs Tests to check for sexual differences in provisioning rate in control and predation-increased experimental time.

To analyse whether parental food allocation in the nests varies with predation risk we built a repeated measured linear mixed-model (MIXED SAS procedure). The model included body mass gain of each nestling as dependent variable, treatment (i.e. control *versus* predation risk increased time) as within-subject factor and nestling hatching rank (i.e. senior *versus* junior siblings) as a fixed factor. The model also included as covariates the initial body mass of the nestlings, their hatching date and nest brood size because the reproductive value of roller offspring varies throughout the breeding season and adult behaviour could be altered depending on brood value. The nest was included as a random intercept to control for the dependence of the siblings of the same nest. The interaction between the nest and the treatment and the interaction rank*treatment were included in the analysis to assess differential effects of the treatment on different nests and whether treatment effects differed between senior and junior siblings of each nest as would be expected whether predation risk affected allocation rules in roller nests. The model was fit using SAS 9.3, with type III tests of fixed effects. Degrees of freedom for fixed effects were estimated using the Kenward-Roger approximation and significance of covariance parameters was tested with Wald *Z* tests (SAS Institute Inc. 2013).

Results

Nest predation risk and parental care

Rollers visited and fed at similar rates during the pre-treatment period and after increasing predation risk (Wilcoxon matched pairs tests: Visit rate: Z = 1.40, p = 0.16; Provisioning rate: Z = 0.62, p = 0.53; N = 17 nests). When we targeted the subset of nests in which parental identification was possible, we found the probability that a male provisioned in a nest increased with predation risk (3 out of 9 males fed only during the pre-treatment time *versus* 8 out of 9 during the experimental time; Fisher exact text p = 0.049). However, probability that a female provisioned did not change with predation risk (6 out of 9 females fed during the pre-treatment and the experimental time; Fisher exact text p = 0.69). Analysis of provisioning rate by sex revealed that attending males and females provisioned at a similar rate during pre-treatment time (Wilcoxon matched pair test: Z = 0.52, p = 0.60, N = 9), but that males provisioned significantly more than females when risk of predation at the nest was increased (Wilcoxon matched pairs test: Z = 2.10, p = 0.04, N = 9) (Fig. 1).

Body mass gain per brood was significantly higher during the pretreatment time than after increasing predation risk (Wilcoxon matched pairs test: Z = 2.02, p = 0.04, N = 18 nests, Fig. 2).



Fig 1. Provisioning rate (median \pm quartile and range) of female and male rollers in reponse to predation risk at the nest. Provisioning rate was first measured in control conditions and afterwards under experimental predation risk increased conditions. N=9 nests.



Fig 2. Differences in brood body mass gain rate (median \pm quartile and range) during the control and the predation risk-increased treatment. N= 18 nests.

Nest predation risk and food allocation within the brood

Parents did not preferentially bias their feeds toward senior or junior nestlings in their nests (Rank order effect: Table 1). Furthermore, parental preferences for senior *versus* junior nestlings were not significantly affected by increasing predation risk at the nest (Interaction Rank order*treatment: Table 1) once the significant effect of brood size and treatment were considered (Table 1).

Table 1. Results of repeated measures linear mixed-effect model to analyse body mass gain of senior and junior nestling rollers in relation to predation risk at the nest. Analyses were based on 93 nestlings from 18 nests. Significant terms are highlighted in bold type. Degrees of freedom for fixed effects were estimated using the Kenward-Roger approximation.

		Random effects			
Term	Covariance	SE	Ζ	Р	
	Parameter				
Nest	0.11	0.24	0.46	0.32	
Nest*Treatment	0.03	0.33	0.10	0.46	
Residual	4.10	0.48	8.55	<.0001	
		Fixed effects			
Term	Coefficient	SE	F	df	Р
Initial nestling body mass	-0.01	0.01	0.80	1,96	0.37
Hatching date	-0.04	0.04	0.77	1,25	0.39
Brood size	0.45	0.19	5.84	1,18	0.03
Treatment	0.77	0.50	5.15	1,15	0.04
Rank order	0.30	0.58	0.26	1,133	0.61
Treatment*Rank order	-0.11	0.62	0.03	1,166	0.86

Discussion

Our results indicate that rollers show sex-specific sensitivity to increased predation risk at their nest. Although we found no changes in total visit and provisioning rates, males were more likely provisioning their nestlings, and did it more frequently than females when predation risk of nests was increased with the presentation of a snake. Furthermore, we found that nestling body mass gain was lowered when the risk of nest predation increased. Our results agree with previous findings showing no changes (Tilgar et al. 2011) or even an increase (Hakkarainen et al. 2002) in parental brood provisioning when nests are exposed to nestling, but not to adult, predators.

Males, but not females, modified their parental behaviour in response to nest predation risk. In birds, the contribution of each sex during the breeding

Capítulo 4

season might vary due to the different value given by each sex to their offspring (Milch et al. 2000) based on differences in benefits of parental care for females and males (Schroeder et al. 2013). In this vein, previous studies have found that when the extra costs of renesting are considerably higher for females than for males, females invest more than males in nest defensive behaviours (Rytkönen et al. 1993). In agreement, a previous study in Great Tits (Parus major) has shown that females, the sex investing more in reproduction, but not males, modified their parental behaviour in response to changes in predation risk of the nests (Moks & Tilgar 2014). Here we have found that male rollers, but not females, increased nest provisioning in response to snake presentation. Male rollers undertake the high costs of incubation with females (Cramp 1998), and thus at the time of the experiment the offspring reproductive value will be high for males. Another possibility is that the lower female sensitivity to predation risk was due to the fact that females are the sex responsible for warming the brood at the time of experiment. In fact, in a total of 16 nests recorded in which identified adults exhibited warming brood behaviour, 13 of them corresponded to females versus 3 males, which gives support to this possibility.

Surprisingly, nestlings' body mass decreased despite male rollers increased nest provisioning in response to predation risk. Several nonmutually exclusive explanations are possible for this result: Predation risk might affect the quality and not the quantity of food loads (Eggers et al. 2008; Tarwater et al. 2009). Male rollers might, during risky period, be feeding with non-suitable prey for nestlings, such as has already been shown in other species (Thomson et al. 2010), or might promote that females relaxed their search for suitable prey, for instance hunting in the closeness of nests to favour defensive behaviours.

Alternatively, parents might be inefficiently delivering the carried food items into nestling gapes due to stress, and these ones would fall down to the bottom of the nest. Also, it cannot be discarded that chicks might be perceiving predation risk through their parents' behaviour (Platzen & Magrath 2004) with the consequence of a poor assimilation or digestion of food. Unfortunately, this possibility cannot be tested because we used body mass gain as a correlate of parental preference and therefore feeds and/or nestling begging inside the nests in relation to our experiment were not recorded. However, irrespective of the mechanism behind, our results suggests that alteration of provisioning rates of adult rollers in response to nest predation risk could have fitness consequences in rollers through a reduction in body mass gain.

Rollers did not show a preference by feeding senior versus junior nestlings in their nests, and, parents did not modify this food allocation pattern when nest predation risk was increased. This result is intriguing as previous studies in which food delivery to senior and junior nestlings was studied in control and predation risk situations revealed changes in parental allocation strategies. For instance, great tit females change their preferences between senior and junior nestlings when the predation risk was high (Moks & Tilgar 2014). Also, in Pied Flycatchers (Ficedula hypoleuca), parents preferred to feed senior versus junior nestlings under control conditions, whereas they feed at random when predation risk at the nest was increased (Tilgar et al. 2011). Preferential feeding of senior nestlings over junior ones are expected to be more pronounced under food limitation conditions (Smiseth et al. 2003). In 2011, spring climatic conditions influencing insect phenology and abundance in the Mediterranean region, were exceptionally good (see Parejo et al. 2015 in press for details). Thus, it is possible that the good environmental conditions prevailing during the study season made unnecessary to favour senior over junior nestlings due to the availability of food. Alternatively, given that in this study it is not possible to ascertain the sex responsible of individual feeds, we cannot discard that males and females

actually would have modified their allocation rules among their nestlings but we had not detected it.

Previous comparative studies have shown that the risk of nest predation is higher for open- than for hole-nesting birds, which would predict a low influence of risk of predation on provisioning behaviour for hole-nester species (Martin & Briskie 1999). Here we have shown that in hole-nesting rollers, changes in parental provisioning mirrored changes in predation risk suggesting that whenever the predation risk stimulus is large enough, it may render changes in parental behaviour for hole-nesters too.

Conclusions

Summing up, our results demonstrate that the risk of nest predation affects parental behaviour in rollers through changes in the relative contribution of sexes in nest provisioning, and that changes in parental behaviour in response to nest predation risk might have profound consequences for nestling fitness prospects.

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Conflict of interest The authors declare that they do not have conflict of interest.

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Supporting information

Sex-specific parental care in response to predation risk in the European Roller, *Coracias garrulus*.

Mónica Expósito-Granados, Deseada Parejo and Jesús M. Avilés.

Body mass gain as a correlate of parental preference in rollers

To check whether the body mass gain of the nestling rollers might function as an indicator of parental food allocation (i.e. provisioning rate to each chick), we recorded food allocation of nestlings with micro cameras (KPC- S500, black and white CCD camera, Esentia Systems Inc., Baton Rouge, LA, USA) hidden in the roof of the nest in 9 nests (N = 39 nestlings) during a pilot study. A multimedia hard disc recorder (EMTEC, Gennevilliers, France) and a 3-inch portable monitor were used to check the correct positioning of the micro camera. We used combinations of Tipp-ex dots on top of nestlings' heads to identify them in the recordings. Nestlings were weighed before and after recording, so we could get the weight gain of each chick in the time of the recording.

A linear mixed-model (MIXED SAS procedure) on nestling body mass gain rate in which number of feeds was entered as a predictor together with brood size and initial body mass of nestlings, and nest was included as a random intercept revealed that provisioning rate to each chick was significantly related to body mass gain, therefore suggesting that body mass gain can be used as a reliable estimator of parental preference in roller nests (see Table).

		Random effects			
Term	Covariance	SE	Ζ	Р	
	parameter				
Nest	0.11	0.13	0.83	0.20	
Residual	0.27	0.07	3.46	0.0003	
		Fixed effects			
Term	Coefficient	SE	F	df	Р
Brood size	-0.02	0.16	0.02	1,28	0.89
Initial nestling's body mass	-0.02	0.01	10.22	1,28	0.003
Provisioning rate per nestling	0.30	0.12	6.43	1,28	0.017

Parental habituation to the snake model

To control for a possible parental habituation to the predator stimulus we compared a period of 30 min after the first visit of one of the two adults with the total period of the recording during the experimental time. We tested for differences in visit and feeding rates between both periods (30 min *vs* Total) of the experimental time, using non-parametric Wilcoxon Matched Pairs Tests. We found no differences in visit and provisioning rates (Wilcoxon matched pairs test: Visit rate: Z = 1.03, p = 0.30, N = 17; Provisioning rate: Z = 0.77, p = 0.44, N = 17). Therefore, parental habituation to the snake model seems unlikely.

Capítulo 5

An experimental test of host's life history traits modulation in response to cuckoo parasitism risk



An experimental test of host's life history traits

modulation in response to cuckoo parasitism risk

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Abstract

Hosts can counteract parasites through defences based on resistance and/or tolerance. The mechanistic basis of tolerance, which involve defensive mechanisms minimizing parasite damage after a successful parasitic attack, remains poorly explored in the study of cuckoo-host interactions. Here, we experimentally explore the possibility that the risk of great spotted cuckoo Clamator glandarius parasitism may induce tolerance defences in magpie Pica pica hosts through plasticity in life-history traits. We predict that magpies exposed to auditory cues indicating high parasitism risk will more likely exhibit resistance and/or modify their life-history traits to minimize parasitism costs (i.e. tolerance) compared to magpies under low parasitism risk. We found that manipulating the perceived parasitism risk did not affect host resistance (i.e. rejection of parasitic eggs) nor host life-history traits. Unexpectedly, host's egg volume increased over the season in nests exposed to auditory cues of control non-harmful hoopoes Upupa epops. Our results do not provide support for inducible defences (either based on resistance or tolerance) in response to risk of parasitism in magpie hosts. Even so, we encourage studying plastic expression of breeding strategies in response to risk of cuckoo parasitism to achieve a better understanding of the mechanistic basis of tolerance defences.

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Introduction

Parasitism is a strong selective agent shaping host evolution (Price 1980). Plant and animal hosts can minimize the negative effects of parasites through defences based on resistance and/or tolerance (Simms and Triplet 1994; Stowe et al. 2000; Rausher 2001; Núñez-Farfán et al. 2007; Svensson and Råberg 2010). Resistance involves physiological or behavioural defensive mechanisms that minimize the likelihood of being parasitized whereas tolerance involves defensive mechanisms reducing the negative effect of the parasite after a successful attack (Svensson and Råberg 2010). Contrary to resistance, tolerance diminishes the impact of parasitism without necessarily causing a negative effect on the parasite (Miller et al. 2006; Svensson and Råberg 2010). Although plant biologists have long discriminated between resistance and tolerance when studying plant defences (Råberg et al. 2009; Svensson and Råberg 2010), studies of animal enemy-victim interactions on tolerance defences are still scarce (Råberg et al. 2009; Svensson and Råberg 2010; Medina and Langmore 2016), particularly those investigating the mechanisms behind tolerance (but see Rohr et al. 2010).

In birds, interspecific brood parasitism is a reproductive strategy in which the parasite (e.g. cuckoo) lays eggs in the nest of another species, the host, which carries out the parental duties from incubation to offspring feeding (Rothstein 1990; Davies 2000). Brood parasitism often imposes large fitness costs to hosts, for example, due to egg removal or breakage during egg laying by the parasitic female, eviction of host eggs and/or nestlings from the nest by the parasite chick, and starvation of host's offspring when the parasite chick monopolizes parental feeds (Payne 1977; Rothstein 1990). As a consequence, natural selection has favoured the evolution of certain behaviours preventing effective parasitism by cuckoos (i.e. resistance mechanisms) ranging from mobbing cuckoos before laying (e.g. Røskaft et al. 2002; Welbergen and Davies 2009; Campobello and Sealy 2011), to

discrimination and rejection of cuckoo eggs (e.g. Davies and Brooke 1988; Soler and Møller 1990; Avilés et al. 2010; Spottiswoode and Stevens 2010), or nestlings (e.g. Langmore et al. 2003; Sato et al. 2010).

Beyond defences based on resistance, avian brood parasites may have selected for host tolerance as well (Svensson and Råberg 2010). Defences based on resistance can be costly because hosts may incur in recognition and rejection errors (Lotem et al. 1995) or because parasites may destroy host eggs as a punishment to host resistance (Soler et al. 1995a; Hoover and Robinson 2007). This scenario is likely to promote the evolution of tolerance defenses, which are by definition less costly (Medina and Langmore 2016). Theoretical reviews have largely emphasized the need of simultaneously studying resistance and tolerance when assessing host defences against cuckoo parasites (Svensson and Råberg 2010; Medina and Langmore 2016). So far only the study by Soler et al. (2010) has explicitly tested the adaptive value of tolerance while accounting for resistance in the Great spotted cuckoo Clamator glandarius-Eurasian magpie Pica pica host system. Operational tolerance (i.e. the slope of the regression between the number of cuckoo eggs in a clutch and the number of host produced fledglings) was larger in highly parasitized magpie populations, suggesting that tolerance may have evolved as an adaptive response to great spotted cuckoo parasitism (Soler et al. 2010).

However, empirical studies about the mechanisms of tolerance are lacking, possibly due to the more subtle and less detectable manifestation of tolerance mechanisms at the population level. Several indirect sources of evidence suggest that some hosts may reduce the costs of parasitism (i.e. show some degree of tolerance) by, for example, increasing clutch size (Soler et al. 2001; Cunningham and Lewis 2006) or laying less eggs but in several nesting attempts (Brooker and Brooker 1996; Hauber 2003; Anderson et al. 2013). Although the evolutionary causes of these different strategies remain poorly studied, the use of one strategy or another appears to be contingent on whether brood parasites are raised together with host nestlings or alone (Medina and Langmore 2016). Furthermore, these studies were not designed to detect tolerance, and given their correlative nature they do not allow discriminating if changes in host life-history traits in response to parasitism reflected a micro-evolutionary process or were due to phenotypic plasticity.

Plastic responses regarding resource allocation to environmental stress are among the most commonly studied tolerance mechanisms in plants (reviewed in [(Strauss and Agrawal 1999; Fornoni 2011)), but their role is still poorly understood in animal enemy-victim interactions. Variable risk of predation can induce plasticity in life-history traits in birds (Cresswell 2008; Lima 2009; Martin and Briskie 2009; Lamanna and Martin 2016). Also, cuckoo hosts can flexibly change resistance defences (e.g. nest defence (Welbergen and Davies 2009; Feeney and Langmore 2015), egg rejection (Davies and Brooke 1988; Moksnes et al. 1993; Bártol et al. 2002; Welbergen and Davies 2012) and nestling rejection (Sato et al. 2010)) as a response to the risk of brood parasitism. However, it remains unknown whether variable risk of cuckoo parasitism may also induce plasticity in host life-history traits, and whether that plasticity may help somehow to minimize the costs of cuckoo parasitism.

In this study, we investigated host defences in a cuckoo-host system, the Eurasian magpie - Great spotted cuckoo, hereafter cuckoo, at the intrapopulation level. Theoretical work has suggested that this would be a suitable system for the evolution of defensive mechanisms based on tolerance (Svensson and Råberg 2010; Medina and Langmore 2016) given that in this system rejection costs are particularly high due to the retaliatory behaviour of the brood parasite. Here we experimentally modified the risk of cuckoo parasitism during laying and incubation of magpies and measured their response in terms of egg rejection (i.e. as a proxy of resistance) and plasticity of life-history traits (i.e. number of eggs and their volume) that may subsequently influence host fitness (i.e. tolerance).

Previous theoretical studies had suggested the existence of a trade-off between resistance and tolerance mechanisms, so that if one of the two defences evolves, then the other would be less likely to do it (Svensson and Råberg 2010). However, empirical evidence suggests that this would not apply to our study system as resistance does not covary with tolerance across magpie populations (Soler et al. 2010). We predict that individual magpie hosts under high risk of suffering brood parasitism will be more likely to exhibit resistance (i.e. more prone to reject cuckoo eggs) and/or tolerance (i.e. more prone to modify their life-history traits to minimize the costs of raising cuckoos) compared to individuals under low risk of parasitism. In birds, females may modify their breeding investment to buffer variable environmental conditions affecting their offspring prospects. This can be achieved through changes in number and volume of eggs (Krist 2011), and therefore it is worth exploring whether a modification in perception of risk of parasitism may induce different host breeding investment (i.e. clutch size and egg volume).

Methods

Study area and system

The study was conducted in La Calahorra (37° 10′ N, 3° 03′ W, Hoya de Guadix, Southern Spain). This is a patchy area of approximately 12 km² where groves of almond trees *Prunus dulcis*, in which magpies preferentially build their nests, are very common (Molina-Morales et al. 2012; Molina-Morales et al. 2013). Magpies are territorial, sedentary, and socially monogamous long-lived passerines (Birkhead 1991). In our population, the great spotted cuckoo is a non-mimetic egg and specialist parasite of the
magpie, although can sporadically parasite other corvids as the Hooded crow *Corvus corone* (Soler 1990). Great spotted cuckoo females usually destroy magpie eggs while laying and multiple parasitism is frequent (Soler 2016). Once hatched, the parasite chicks do not evict the magpie nestlings, but due to their shorter incubation length and faster development of the cuckoo, magpie nestlings rarely fledge from parasitized nests (Soler et al. 1996). The natural parasitism rejection in magpie hosts is about 5% (Soler et al. 1995a), lower than that for experimental models of cuckoo eggs (see Molina-Morales et al. 2014). The percentage of parasitized nests in our population (i.e. parasitism rate) greatly varies between years (range 15.9 - 65.6%, Molina-Morales et al. 2016).

Experimental manipulation

We conducted a manipulation of cuckoo parasitism risk during the laying and incubation periods of magpies in the year 2014 (mid-March to April). The breeding season of this year lasted from the first egg laid on mid-March to end of June when the last nestlings fledged. At the beginning of the breeding season magpie nests were located by careful inspection of all trees in the area, and GPS positioned. In total, 75 nests were found, but 21 nests were discarded from the experiment because the laying activity had already finished when they were found. The remaining 54 nests were found before clutch completion (mean \pm SE clutch size when found was 3.13 \pm 0.23 eggs (range: 1-7), final clutch size of magpies in our population: Parasitized nests $(\text{mean} \pm \text{SE}) = 6.56 \pm 1.11$ (range: 4-9); Non-parasitized nests (mean $\pm \text{SE}$) = 6.85 ± 0.76 (range: 5-9), (Molina-Morales et al. 2013)). Each nest was randomly assigned to one of the following treatments: i) "increased parasitism risk": perceived risk of cuckoo parasitism was increased by broadcasting cuckoo calls in the surroundings of the nest (n = 23 nests), ii) "no parasitism risk": calls of Hoopoes *Upupa epops* were played as a control for the effect of the playback (n = 14 nests); and iii) "control": we visited the surroundings of the nest as often as for treatments i) and ii), but did not play any call (n = 17 nests).

Portable amplified speakers (MOLGAR 3" 20W 4 ohm) connected to digital audio players (takeMS MP3 Player "Deseo") were used to broadcast calls. The calls consisted of three different tracks of great spotted cuckoo calls and four hoopoe calls from different individuals extracted from Llimosa et al. (1999) and three 1-min silence tracks, respectively, that were randomly selected and played continuously using the random function in the audio players during the experiment. This produces unique assortments of calls by their randomized presentation and combination with silence tracks for each nest and treatment, thus minimizing the risk of pseudoreplication (Eggers et al. 2006; Parejo et al. 2012a). Treatments were applied three times during two hours every third day from the day each nest was found. Some nests were found at the very beginning of laying whereas others did not, and, hence, every nest might have received either 1 or 2 playback sessions during laying. However, the number of eggs laid at the time we started our experiment did not vary among treatments (see below Variables and Experimental Randomization). The minimum distance between two magpie nests in this study year was 350 m, thus minimizing the possibility that magpies others than the tested perceived the stimulus.

Previous experimental studies have shown that birds have the potential to perceive threats based on acoustic cues emitted by predators (e.g. Eggers et al. 2006; Emmering and Schmidt 2011), and even avian brood parasites (*Chalcites* cuckoo species (Kleindorfer et al. 2013) and Brown-headed cowbird (*Molothrus ater*) (Forsman and Martin 2009)), which justifies the only use of vocal cues for manipulating parasitism risk. Furthermore, male cuckoos perch and call close to host nests to attract magpie attention; meanwhile female cuckoos make a silent approach to the nest to lay (Arias-

de-Reyna 1998). Therefore, continuous broadcasting of cuckoo calls close to a nest is likely to be perceived as a real parasitism challenge for magpies. We chose the hoopoe as a "no parasitism risk" control because hoopoe *a priori* poses no threat to magpies and lives in sympatry with them.

We opted to manipulate risk of parasitism once laying was started in the knowledge that in birds, females have the potential to modify their investment on eggs (i.e. hormone composition and egg size) in response to a sudden change in environmental conditions during laying (Saino et al. 2005; Saino et al. 2010a; Saino et al. 2010b; Parejo et al. 2012b).

Estimation of host resistance

On the first visit to each nest, we introduced a plaster model egg that resembled a cuckoo egg in appearance, size and mass (see details in (Molina-Morales et al. 2012)). Previous studies have shown that rejection of model eggs provides a reliable estimate of host defences based on resistance (Davies et al. 1996; Soler et al. 2010). We determined level of resistance as a categorical variable with two levels: rejecter, if the model egg disappeared from the nest, and acceptor, if the model egg was still in the nest after 6 days. Although rejecter magpies reject experimentally added eggs within the first 72 hours (Avilés et al. 2004), we chose here a longer response period to avoid modifying magpie perception of risk of parasitism if we had removed accepted eggs at day three, as this might have affected life-history traits of those pairs that would have not yet complete the clutch.

Variables and experimental randomization

For all nests we recorded number of laid eggs (before and after the experiment), and egg volume (before and after the experiment). 10 nests were discarded because either have unusual small clutch sizes (3 or less eggs) which suggested partial predation (n = 6 nests), or were totally predated (n = 6).

4 nests). We measured the length and width of each egg with a digital calliper; egg volume was calculated using the formula Volume = Length * Width² * 0.515 (Hoyt 1979).

To test whether the experiment was fully randomized with respect to magpie pair quality we checked for differences before the experiment in magpie laying date (a proxy of individual quality in magpies (Soler et al. 1995b)) and average magpie egg volume already laid between our three treatments using one-way ANOVAs. Neither laying date ($F_{2,51} = 0.33$, P = 0.72) nor the average egg volume recorded before the experiment ($F_{2,45} = 0.81$, P = 0.45) differed among treatments, suggesting that our experiment was properly randomized regarding magpie quality. Additionally, we tested for differences in number of laid eggs the day of the experiment (i.e. before any effect of broadcasting) using a generalized linear model with a Poisson distribution. Number of laid eggs before the experiment did not differ between the treatments ($F_{2,51} = 0.13$, P = 0.88), suggesting that treatments were evenly established regarding magpie laying sequence.

Statistical analyses

Analyses were performed in SAS, version 9.4.

To study the effect of risk of parasitism on magpie resistance, we ran a generalized linear model (GENMOD procedure in SAS) in which probability of egg rejection was entered as a binary dependent variable (rejection *vs.* acceptance; link function: logit) and treatment as fixed factor with three levels (increased parasitism risk, no parasitism risk, control). Laying date ($1 = 1^{st}$ March) was fitted as a covariate to control for possible differences in magpie quality. Additionally, we fitted the interaction of laying date and treatment. Given that previous work has shown that egg rejection behaviour increased with age in female magpies (Molina-Morales et al. 2014), we

conducted a second model including a subset of 23 females of known age. In this model, age was entered as a fixed factor.

To check for the effect of the parasitism risk on magpie life-history traits, we tested for differences among treatments in number of eggs laid after the experiment (Poisson distribution, link = log) and in mean egg volume after the experiment (normal distribution, link = identity) using a generalized linear model (GENMOD procedure in SAS) and a general linear model (GLM procedure in SAS), respectively. Again, treatment, laying date, and the interaction between the two were fitted as predictors. Graphical inspection of residuals plots indicated that the error distribution of the data was modelled correctly and did not depart from Poisson and normal model assumptions, respectively. To improve the interpretability of regression coefficients laying date and egg volume were mean-centred.

Ethical statement

Consejería de Medio Ambiente y Ordenación del Territorio (Junta de Andalucía) authorized the fieldwork of the present study (projects CGL2011-27561/BOS and CGL2014-56769-P; licence code: P06-RNM-01862). Spanish law does not require ethical approval for this specific study from an Institutional Animal Care and Use Committee (IACUC). Experimental manipulation of perceived parasitism risk did not affect the natural rate of nest abandonment of the species suggesting that our experimental procedure has a negligible negative effect on magpies.

Results

Risk of parasitism and host resistance

Magpies rejected 20 out of 54 (37.03 %) model eggs; however, the risk of cuckoo parasitism did not influence egg rejection behaviour once we control for laying date (rejection rates were 34.78%, 35.71% and 41.17 % for the increased risk of cuckoo parasitism, no-risk of cuckoo parasitism and control treatments, respectively(Treatment effect: $\chi^2_2 = 0.14$, P = 0.93; laying date effect: $\chi^2_1 = 0.19$, P = 0.67; Treatment*laying date: $\chi^2_2 = 0.58$, P = 0.75). A second model only with the subset of females of known age confirmed this pattern (Treatment effect: $\chi^2_2 = 0.36$, P = 0.84; laying date effect: $\chi^2_1 = 0.65$, P = 0.42; Treatment*laying date: $\chi^2_2 = 1.57$, P = 0.46; Age effect: $\chi^2_1 = 0.53$, P = 0.47).

Risk of parasitism and host life-history traits

Magpies did not modify their investment in number of eggs after the experiment, and this pattern did not vary over the season (Table 1a). However, there was a significant effect of treatment on average magpie egg volume, which changed over the season (Table 1b). Egg volume after the experiment increased over the season in nests exposed to the hoopoe treatment, whereas it did not vary in nests exposed to the cuckoo treatment or in control nests (Fig 1). The interactive effect of treatment and laying date on egg volume remained robust when we excluded the control group of nests in which host life-history traits could not be measured after 25th April due to logistic problems (see Fig 1; Laying date*treatment: $F_{1,26} = 6.68$, Coefficient (low CL, high CL) = 0.0003 (-0.002, 0.003), P = 0.01; Treatment: $F_{1,26} = 6.91$, Coefficient (low CL, high CL) = -0.59 (-1.06, -0.13), P = 0.01). However, this pattern vanished when we excluded the late extreme value in

host egg volume in the hoopoe treatment (Fig 1), which nonetheless cannot be considered a statistically significant outlier (Grubb's test: G = 2.62, P > 0.05, n = 43).



Figure 1. Seasonal variation in volume of magpie eggs laid after the experiment in relation to risk of parasitism at the nest. Dashed lines represent the 95% confidence limits for the regression line (solid line). Egg volume and laying date were centred for the analysis but not in the figure to allow a biological interpretation of measurements. The arrow indicates an *a priori* outlier.

Table 1. Variation in magpie life-history traits in relation to risk of cuckoo parasitism at the nest. Results of general and generalized linear models of number and volume of magpie eggs laid after the experiment in relation to risk of cuckoo parasitism. Significant terms are highlighted in bold.

(a)

0	
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Number of eggs (n= 44 nev	sts)							Egg volume (n= 43 nests)							
Term		Coefficient	Lower CL	Higher CL	F	DF	d	Term		Coefficient	Lower CL	Higher CL	F	DF	р
Intercept		0.85	0.48	1.21	4.71	40	<0.0001	Intercept		-0.28	-0.79	0.12	-1.42	37	0.16
Laying date		-0.02	-0.05	0.001	3.69	1,40	0.06	Laying date		0.002	-0.06	0.07	6.06	1,37	0.02
Treatment	Hoopoe	0.50	0.03	96.0	2.46	2,40	0.09	Treatment	Hoopoe	0.17	-0.44	0.79	1.68	2,37	0.20
	Cuckoo	0.39	-0.05	0.83					Cuckoo	0.46	-0.06	0.99			
	Control*								Control*						
Laying date*treatment	Hoopoe	-0.06	-0.14	0.02	1.12	2,38	0.34	Laying date*treatment	Hoopoe	0.12	0.02	0.21	4.65	2,37	0.02
	Cuckoo	-0.03	-0.10	0.04					Cuckoo	0.003	-0.08	0.08			
	Control*								Control*						

CL, 95% confidence level.

* Reference level in the treatment contrast parametrization of the model matrix.

Discussion

Recent theoretical work has emphasized the importance of integrating the study of resistance and tolerance defences to better understand the evolution of animal enemy-victim interactions, including those between avian brood parasites and their hosts (Råberg et al. 2009; Svensson and Råberg 2010; Medina and Langmore 2016). Despite this, empirical studies focusing on tolerance defences in cuckoo-host systems are surprisingly scarce and, so far only confined to investigate operational tolerance across different host populations (Soler et al. 2010). Furthermore, the mechanistic basis of tolerance still remains unstudied in the framework of cuckoo-host interactions.

Here we explore the possibility that tolerance may be expressed at the population level through plasticity in host life-history traits. However, we found that manipulating the perceived risk of cuckoo parasitism, did not affect the rejection behaviour of parasitic eggs (a reliable indicator of host resistance against cuckoo parasites) nor host life-history traits which are candidate to reflect tolerance. However, our experimental set-up revealed plastic expression of magpie host life-history traits in response to experimental treatments at the nest. Magpies did not modify the number of eggs laid but egg volume.

Magpies did not change their resistance against cuckoos in response to the risk of parasitism at their nests. Previous studies had reported the existence of plastic resistance (rejection and mobbing behaviour) in hosts of the European cuckoo *Cuculus canorus* in response to risk of parasitism (Davies and Brooke 1988; Moksnes et al. 1993; Lindholm 2000; Bártol et al. 2002; Čapek et al. 2010; Welbergen and Davies 2012; Thorogood and Davies 2013). Plastic resistance was explained as an adaptive response to the high cost of rejecting and mobbing in populations where risk of parasitism greatly varied in space and time (Welbergen and Davies 2009). Our findings, however, agree with Soler et al. (2000), who found that placing a live cuckoo close to a magpie nest did not modify the rate of ejection of cuckoo model eggs. Experimental results in the magpie-cuckoo system documented that cuckoos destroy magpie eggs after realizing that magpies have rejected their eggs (Mafia hypothesis, (Soler et al. 1995a)). Therefore, one likely explanation for the lack of effect of risk of parasitism on egg rejection is that the costs of rejecting when there is constant presence of cuckoos in the surroundings are greater than those of accepting the parasitic egg. However, this would predict a stronger response in hoopoe and control nests, which is not the case. Another possibility is that most of our tested magpies could have been young individuals, which, in contrast to adult females, may still have not learnt to reject cuckoo eggs or perceive parasitism risk and respond accordingly (Molina-Morales et al. 2014). This possibility, however, is unlikely because most females of known age (78.26% of N = 23 females) during the experimental year were adults (older than two-years old). Within this sample, female mean age was 4.04 years old (range: 1-8). Moreover, our results confirmed the absence of a treatment effect on rejection once we control for female age. Alternatively, it could be argued that our playback experiment may have failed to increase perceived risk of parasitism but, instead, worked as an additional non-important parasitic stimulus added to the introduced model egg. This could have been the case if magpies were already aware of having been (artificially) parasitized, which would just make cuckoo calls an extra threat. On the other hand, it is possible that the stimulus needed to provoke a response is the association of call and cuckoo presence together but not cuckoo call (as in this study) or cuckoo presence (as in (Soler et al. 2000)) separately. However, it is unlikely that magpies disregarded call information as they did react to hoopoe calls (see below).

Magpies did not modify their life-history traits (number and egg volume) in response to the risk of cuckoo parasitism. Unexpectedly, they

modified the volume of their eggs in response to hoopoe calls, which is intriguing given previous work showing that hoopoes were considered as a non-harmful threat by magpies (Avilés et al. 2014). Egg volume increased over the season in nests exposed to hoopoes but not in nests exposed to cuckoos or in control nests. One first explanation for the absence of an effect of cuckoo calls could be that magpies might have already perceived their nests as being parasitized because we introduced a model cuckoo egg, and hence, they might disregard cuckoo calls. Another possibility could be that broadcasting was performed too late in the laying of the host with no time to induce a physiological response. However, these two possibilities seem very improbable because magpies responded to hoopoe calls.

Regarding the unexpected effect of hoopoe calls on egg volume, several explanations are possible. First, although hoopoes do not predate on magpie nests it is possible that hoopoe calls emitted close to the nest would have been perceived as a predation threat by magpies because hoopoes calls may draw the attention of predators to the nest (Fontaine and Martin 2006). In this scenario high quality breeders, which reproduce early in the season, reduced their investment in current reproduction by laying smaller eggs when perceiving a potential risk, as they could still save some energy for a replacement clutch (Hua et al. 2014). Alternatively, hoopoe calls may have attracted territorial hoopoe males in the surroundings of magpie nests. Indeed, we detected in some instances the presence of active singing males in the neighbourhood of nests where hoopoe songs were broadcasted (Pers. Obs.). The unusual and active presence of hoopoe males (in addition to calls) close to their nests, could have led to changes in their life-history traits due to the additional source of stress. Also, it could be the case that late breeding magpies associated hoopoe presence with factors other than risk, as highquality habitats and/or food abundance. Last, but not least, given the low sample size in the hoopoe treatment, it cannot be discarded that this pattern emerged randomly and corresponded with a false positive result or a methodological artefact due to an unexpected effect of the used control stimulus (Grim et al. 2005).

Conclusions

We have theoretically introduced and experimentally tested for the first time the possibility that hosts may tolerate cuckoo parasitism through plastic expression of reproductive traits. The reasoning for this novel hypothesis is based on overwhelming evidence documenting plastic responses in resource allocation in response to environmental stress in plants (reviewed in (Strauss and Agrawal 1999; Fornoni 2011)), and a large body of empirical work showing that birds may plastically modify their breeding strategies in relation to perceived risk of predation at their nests (Fontaine and Martin 2006; Hua et al. 2014; Lamanna and Martin 2016). However, we did not find that magpies modified their breeding strategy in response to risk of parasitism. Although our results do not support the tolerance hypothesis, we emphasized the need of studying plastic expression of breeding strategies in response to different sources of environmental stress in hosts of avian brood parasites, and, their fitness consequences for the parasites. In this sense, it seems advisable to study the same reproductive couple throughout a season or the same individuals in different seasons (i.e. using longitudinal studies) to achieve a better understanding on how hosts may tolerate cuckoo parasitism.

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Fear of cuckoos does not affect magpie host offspring physiology



Fear of cuckoos does not affect magpie host offspring physiology

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Abstract

There is ample evidence that brood parasites impose direct fitness costs to their hosts. However, risk of brood parasitism may also induce sub-lethal effects on hosts by modifying their physiological state and that of their offspring, a possibility that has never been investigated. Here, we test whether fear of Great Spotted Cuckoo Clamator glandarius by European magpie *Pica pica* host parents may translate into phenotypic changes in offspring. We manipulated the parasitism risk in magpie nests and measured its effect on the physiology of nestlings in terms of corticosterone response, immune function (agglutination and immunoglobulin Y levels) and weight. Cuckoo parasitism risk experienced by magpie parents did not translate into high levels of corticosterone or immunological depression of magpie host nestlings. However, nestlings in control nests had lower agglutination titers than nestlings of parents exposed to cuckoo or hoopoe calls, possibly due to an increase in parental attentiveness in response to the manipulation. Future studies on cuckoo-host interactions should further investigate if stress induced by risk of brood parasitism or exposure to experimental stimulus may influence host offspring phenotypes through changes in corticosterone response or immunological levels.

Manuscrito enviado a revista.

Introduction

Interspecific avian brood parasitism is a reproductive strategy in which the parasitic bird lays its eggs in the nest of a host species and leaves incubation and parental care of the resulting offspring to an unrelated foster parent (Rothstein 1990; Davies 2000). Parasitism can sometimes reduce the reproductive success of the host, and, sometimes, can cause the total failure of its breeding attempt (Payne 1977; Rothstein 1990; Davies 2000). Thus, brood parasitism is a powerful selective agent acting on host anti-parasite defenses, which at the same time have selected for further trickeries on the parasite side, which makes the study of avian brood parasites and their hosts an ideal system to study co-evolution (Feeney et al. 2014; Soler 2014).

A growing line of enquiry suggests that high costs imposed by brood parasites on their hosts may have selected for hosts that capable of assessing the chance of being parasitized *via* cues informing on parasite presence and/or abundance. Hosts are able to preventively assess the threat of parasitism near their nests based on visual or auditory cues and to respond accordingly modifying their habitat choice (Forsman and Martin 2009; Expósito-Granados et al. 2017a; Tolvanen et al. 2017), incubation behavior (Øien et al. 1996), and defensive behaviors (i.e. egg rejection (Moksnes et al. 1993; Brooke et al. 1998)). However, although brood parasites are likely to be a major indirect source of environmental stress for hosts, we lack an understanding of the impacts of risk of brood parasitism on physiology.

Hormones released from the hypothalamic-pituitary-adrenal (HPA) axis (Romero 2004) play a substantial role managing the homeostatic energy balance of vertebrates (Sapolsky et al. 2000; Landys et al. 2006). When individuals are challenged by stressor agents, the HPA axis is activated resulting in an increase in the level of circulating plasma glucocorticoids. In the short term, glucocorticoids can promote prompt physiological and

behavioral changes that help escaping from unexpected survival challenges (Sapolsky et al. 2000). However, when perturbations last longer or are repeated (i.e. chronic stress), maintaining high baseline levels of glucocorticoids may be harmful because they can suppress the innate and acquired immune system (e.g. Buehler et al. 2008; Stier et al. 2009; Chin et al. 2013), and negatively impact on fitness (Zanette et al. 2011; Boonstra 2013). In addition, the effects of stressful events may project into the next generation, so that offspring of stressed parents can show increased levels of glucocorticoids transferred via maternal effects (Groothuis and Schwabl 2008; Sheriff and Love 2013). A previous study has shown that host nestlings raised in artificial broods together with parasitic nestlings, had higher corticosterone levels than those raised without parasitic nestlings (Ibáñez-Álamo et al. 2012a), which would suggest that sharing the nest with a brood parasite is a potential physiological stressor for the host offspring. Also, it has been shown that parasitized parents had significantly higher stress-induced levels of corticosterone than non-parasitized parents, which influenced parental care in Rufous and white wren Thryophilus rufalbus hosts parasitized by American striped cuckoo Tapera naevia (Mark and Rubenstein 2013). However, the physiological implications for the host offspring of risk of cuckoo parasitism experienced by its parents remain largely unknown.

In this study, we perform a manipulation of the risk of Great Spotted Cuckoo *Clamator glandarius* (hereafter cuckoo) parasitism in nests of European magpie host *Pica pica* (hereafter magpie) and measure the effect on the physiological state (corticosterone response, immunity and body mass) of host nestlings. This will allow us to test, for the first time, if risk of cuckoo parasitism experienced by host parents may translate into phenotypic changes in their offspring. We measured concentrations of corticosterone (Romero 2004) in plasma (hereafter abbreviated pCORT) and in feathers (hereafter abbreviated fCORT) as indices of stress response. Plasma CORT

characterizes the current state of the HPA axis (see more details in Romero 2004) whereas fCORT represents the whole amount of CORT secreted during the growth of the feather (Bortolotti et al. 2008, 2009; Lattin et al. 2011; Jenni-Eiermann et al. 2015), and, hence, in nestlings it will represent the state of the HPA axis over the nest-bound period. Activation of the HPA axis is likely to down-regulate the immune function (Martin 2009) and growth of nestlings (Hayward and Wingfield 2004). Hence we also measured red blood cell agglutination as a proxy of natural antibody (herefafter abbreviated NAb) levels (an index of innate immunity), immunoglobulin Y (hereafter abbreviated IgY) levels (an index of acquired immunity) and body mass in magpie host nestlings to assess the effect of risk of parasitism on the immunological status and weight of nestlings. We expect to find: 1) more stressed host nestlings in nests exposed to cuckoo playbacks (i.e. higher levels of CORT), and 2) nestlings with a worse immunology and lower body mass in nests exposed to high risk of cuckoo parasitism as compared to control ones.

Methods

Study area and system

The study was conducted in La Calahorra (37° 10′ N, 3° 03′ W, Hoya de Guadix, Southern Spain). This is a patchy area where groves of almond trees *Prunus dulcis*, in which magpies preferentially build their nests, are very common (Molina-Morales et al. 2012; 2013). Magpies are territorial, sedentary, and socially monogamous long-lived passerines (Birkhead 1991), and are the main host of the great spotted cuckoo in Southern Spain (Soler 1990).

Experimental manipulation of perceived risk of parasitism

In the year 2014 we conducted a manipulation of perceived risk of cuckoo parasitism in 55 nests during the egg-laving and early incubation period (mean \pm SE clutch size when found was 3.20 ± 0.24 eggs; final clutch size of magpies in our population: Parasitized nests (mean \pm SE) = 6.56 \pm 1.11; Nonparasitized nests (mean \pm SE) = 6.85 \pm 0.76 (Molina-Morales et al. 2013) Nests were randomly assigned to one of the following three treatments: i) "increased parasitism risk": perceived risk of cuckoo parasitism was increased by broadcasting cuckoo calls in the surroundings of the nest, ii) "no parasitism risk": calls of Hoopoes Upupa epops were played as a control for the effect of the playback; and iii) "control": we visited the surroundings of the nest as often as for treatments i) and ii), but did not play any call. 31 out of 55 experimentally manipulated nests could not be used to assess nestling physiology because they were naturally parasitized by great spotted cuckoos (n = 16 nests), and, thereby nestlings starved, or because they were predated (n = 9 nests) or abandoned (n = 4 nests) at rates within the natural values for this species (Martínez 2016), or because they failed for unknown reasons (n =2 nests). Thus, final sample size in this study was 24 nests (Experimental treatments: Cuckoo: n = 9; Hoopoe: n = 8; Control: n = 7) with 111 nestlings.

We used portable amplified speakers (MOLGAR 3" 20W 4 ohm) connected to digital audio players (takeMS MP3 Player "Deseo") to broadcast calls. To avoid familiarity effects broadcasted calls came from individuals from foreign populations, and consisted of three different tracks (each with duration of 30 sec) of great spotted cuckoo calls (i.e. increased parasitism risk treatment) and four hoopoe calls (i.e. no parasitism risk) extracted from Llimosa et al. (1999) combined with two and three one-minute silence tracks, respectively. These tracks were randomly selected using the "track repeat" and "random track selection" functions, which assures an unique assortment of calls for each nest and day of treatment,

minimizing the risk of pseudoreplication by not using the same auditory stimulus in different nests (Ghalambor and Martin 2000; Parejo et al. 2012). Calls were broadcasted three times during 2 hours every third day from the day each nest was found. Therefore, every nest received a total of 3 playback sessions of which 1 or 2 were broadcasted during laying. The minimum distance between two magpie nests in the study year was about 198 m, which, given that neighbor magpie territories are rarely synchronous and that we did not put recordings in nests neighboring any nest where we had already broadcasted that day, diminishes the chance that magpies others than the tested one perceived the stimulus.

Previous experimental studies have shown that hosts of avian brood parasites (Forsman and Martin 2009; Kleindorfer et al. 2013), including magpies (Expósito-Granados et al. 2017a, b), can assess risk at their nests based on acoustic cues. In this species cuckoo males perch and call close to host nests to attract host attention; meanwhile females make a silent approach to the nest to lay (Arias de Reyna 1998). Therefore, broadcasting of cuckoo calls near a nest is likely perceived as a real parasitism threat by magpies. Hoopoes were chosen as a suitable control in the knowledge that they pose no threat to magpies and live in sympatry with them in the study area (e.g. Avilés et al. 2014).

Treatments were properly randomized regarding magpie quality (i.e. laying date and number of eggs laid before the experiment did not differ between experimental groups).

Nestlings' sampling

We sampled blood from nestlings in two different days in order to minimize detrimental effects of bleeding on nestlings. At the age of 12 days chicks were bled (225μ L per chick) to obtain innate (NAb and complement) and acquired (IgY) immunity measurements. When 15 days old, nestlings were

bled again to obtain CORT measurements (stress-induced pCORT). Sampling was always done in the morning (8:30 to 11:30 a.m.) to avoid a possible problem of circadian CORT changes (Breuner et al. 1999). Twenty to 30 min after accessing the nests, a blood sample was taken (about 225μ L per chick) to obtain stress-induced pCORT. At the second sampling day we also collected the octave primary major covert feather of each nestling and stored it in a dark box at ambient temperature until laboratory analyses of fCORT.

Blood was collected from the brachial vein using a 25 gauge needle and heparinized microhaematocrit capillary tubes (75mm long, inner diameter 1.07-1.23mm) to carefully transfer it into a 1.5 mL tube and immediately deposit it in a refrigerator. Plasma and red blood cells were separated by centrifugation the same day at 13300 rpm for 5 min. All samples were stored in a -20°C freezer until the end of fieldwork when they were kept in a -80° C freezer until laboratory analyses. Nestlings were weighted at age of 12 days to the nearest 1 g using a 300 g Pesola spring balance.

Corticosterone assay

Corticosterone concentrations were determined at the Centre d'Études Biologiques de Chizé (France) following the procedure of Lormée et al. (2003) for steroid hormones. Briefly, an ethyl ether extraction technique was used to extract CORT from plasma (Lormée et al. 2003) and a methanolbased extraction technique was used to extract CORT from feathers (Bortolotti et al. 2008). Plasma and feather CORT extracts were measured using radioimmunoassay with a highly cross-reactive rabbit anti-mouse antibody from Sigma (C8784). Corticosterone antiserum cross-react with other hormones: 11-deoxycorticosterone 20%, progesterone 15.7%, 20 α hydroxyprogesterone 8.8%, testosterone 7.9%, 20 β -hydroxyprogesterone 5.2%, cortisol 4.5%, aldosterone 4.4%, cortisone 3.2%, androstenedione

2.6%, 17-hydroxyprogesterone 1.8%, 5 α -dehydrotestosterone 1.4%, androsterone <0.1%, estrone <0.1%, estriol <0.1%. Duplicate aliquots of the extracts were incubated overnight at 4°C with 3H-corticosterone and antiserum. The bound and free corticosterone fractions were separated by adding dextran-coated charcoal. Once centrifuged, the bound fraction was counted in a liquid scintillation counter. If samples were not assayed the same day, they were frozen at -20° until analysis. The lowest detectable corticosterone level was 0.28 ng/ml. Intra and inter-assay coefficients of variation for plasma assays were 7.09 and 15.50%, respectively. For feather assays intra and inter-assay coefficient were 8.06 and 13.67 %, respectively.

Immune assays

Assessment of innate humoral immunity was made by using the protocol described in detail by Matson et al. (2005). Briefly, this assay is based on NAb mediated complement activation and red blood cell agglutination. The agglutination reaction measures the interaction between NAb and antigens and the lytic reaction measures the amount of hemoglobulin released from the lysis of foreign erythrocytes. Quantification of agglutination and lysis was done by assessing the dilution stage (using numeric scores on a scale from 1 to 12) at which these two reactions stopped against the same amount of rabbit blood cell suspension on digitalized images (for further details on the method, see Matson et al. 2005; Parejo et al. 2007). Titres that showed intermediate agglutination or lysis values were assigned the lower score. There was no lytic activity in most of the nestlings sampled (98 over 106 nestlings) indicating low lytic activity against the concentration of rabbit blood used as detected in other nestlings' species (Arriero et al. 2013). Therefore, for analyses we only targeted on agglutination activity (multifactorially associated with circulating NAbs) after excluding 5 nestlings without agglutination activity.

Assessment of acquired immunity was made by measuring the levels of nestlings' IgY using an ELISA (enzyme-linked immunosorbent assay following the protocol described in Martínez et al. (2003). IgY is an antibody in birds that can neutralize pathogens (Demas et al. 2011). Polystyrene 96well plates (Maxi-sorp, Nunc, Rochester, NY, USA) were covered overnight (4°C) with serial dilutions of serum (100µl) in carbonate-bicarbonate buffer (0.1 M, pH = 9.6) in order to determine the linear range of the sigmoid curve (Plasma dilution of 1/8000 to suit for magpie chicks). Wells were washed three times with 200 µl per well of PBS,1% BSA, 0.05% Tween buffer. Then, plates were blocked with defatted milk diluted in PBS-Tw buffer for 1 h at 37°C (200µl). Anti-chicken conjugate (Sigma A-9046) at 1/250 dilution was added in PBS-Tw and incubated for 2 h at 37°C (100µl). Finally, we added a substrate comprising ABTS and concentrated hydrogen peroxide for 1h at 37°C to measure the absorbance at 405 nm with a spectrophotometer. All plasma samples were run in duplicate, and the mean IgY absorbance value was used in the analyses. The average CV between duplicates was 5.36 %.

Ethical statement

The Consejería de Medio Ambiente y Ordenación del Territorio (Junta de Andalucía) authorized the fieldwork of the present study (projects CGL2011-27561/BOS and CGL2014-56769-P; licence code: P06-RNM-01862). Spanish law does not require ethical approval for this specific study from an Institutional Animal Care and Use Committee (IACUC). Experimental manipulation of parasitism risk did not affect the natural rate of nest abandonment of the species. The number of fledglings did not vary between treatments ($F_{2, 20} = 0.68$, p = 0.52) nor was nest success influenced by the experimental manipulation ($\chi^2_2 = 0.15$, p = 0.93) in the 23 nests with hatchlings that were not cuckoo parasitized or predated, suggesting that our experimental procedure has a negligible effect on magpies.

Data Analysis

Analyses were performed using SAS v.9.4 statistical software (SAS Institute, Cary, NC, USA).

Sample sizes may vary amongst analyses because some physiological data were unavailable (e.g. CORT levels under the threshold for detection or absence of immunological activity) and because, due to logistical problems, not all information could be collected for all nestlings or broods.

Linear Mixed Models (LMM hereafter, MIXED procedure in SAS) were performed to test for differences among nestlings from the three experimental groups in CORT levels (stress-induced pCORT and fCORT), immunity (i.e. NAb and Ig Y levels) and body mass at the age of 12 days. Dependent variables in each of the models were, respectively, pCORT (Normal distribution, link= identity), fCORT (Normal distribution, link= identity), agglutination score (Poisson distribution, link = log), Ig Y level (Normal distribution, link = identity) and body mass at age 12 of nestlings (Normal distribution, link = identity). In order to account for differences in parental qualities in the response to our experiment, we included laying date as a covariate in the models, in the knowledge that laying date is a proxy of parental quality in magpies (Birkhead1991). Finally, the nest was introduced as a random intercept in these analyses to control for the non-independence of nestlings that were raised in the same nest.

All continuous variables were centered by subtracting their mean value from each value to improve the interpretability of regression coefficients. We also log-transformed levels of IgY to fit them to a normal distribution.

Results

Risk of cuckoo parasitism and nestlings CORT response

Corticosterone levels measured in nestlings' plasma and feathers (pCORT and fCORT) did not differ between the treatments of induced experimental risk of cuckoo parasitism (Table 1). Laying date had no effect on the CORT response of nestlings either (Table 1).

Table 1. Results of LMMs to test the effect of risk of cuckoo parasitism on corticosterone response (i.e. fCORT and pCORT). Laying date
was also included in the models to account for differences in parental quality.

$\begin{array}{c c c c c c c c c c c c c c c c c c c $							pCORT			
Coefficient Lower CL 0.36 -0.48 ate 0.06 -0.02 t Cuckoo -0.52 -1.59	89 nestlings)					(u =	99 nestlings	()		
t 0.36 -0.48 late 0.06 -0.02 mt Cuckoo -0.52 -1.59	Higher CL	DF	F/Z	d	Coefficient	Lower CL	Higher CL	DF	F/Z	d
date 0.06 -0.02 ant Cuckoo -0.52 -1.59	1.21	19	0.9	0.38	-0.5	-9.01	8.01	19	-0.12	0.9
ent Cuckoo -0.52 -1.59	0.14	1,66	2.25	0.14	-0.28	-1.08	0.51	1,76	0.5	0.48
	0.55	2,66	0.54	0.59	-1.29	-12.05	9.48	2,76	0.34	0.71
Hoopoe -0.48 -1.64	0.67				3.15	-8.49	14.78			
Control*										
			1.55	0.06					1.55	0.06

* Reference level in the treatment contrasts parametrization of the model matrix. CL, 95% confidence level.

Risk of cuckoo parasitism and nestlings' immunity and weight

Agglutination titer of nestlings differed among treatments (Table 2). Nestlings in control nests had lower agglutination score than nestlings in nests where parents were exposed to cuckoo and hoopoe calls (Fig. 1). However, laying date had no effect on nestlings' agglutination score (Table 2). On the other hand, IgY levels and nestling body mass did not vary in relation to treatment or in relation to laying date. However, the three measurements were dependent of the nest in which the nestling was being raised (Table 2, Table 3).



Fig. 1 Immunological state of nestlings (agglutination score) in relation to risk of brood parasitism. Agglutination score was calculated at the titer where agglutination stopped. For each treatment, the smallest box in the plot represents the mean, larger box the standard error and whiskers the standard deviation about the mean.

Table 2. Results of LMMs to test the effect of risk of cuckoo parasitism on immunity (i.e. agglutination and Ig Y levels) of nestlings. Laying date was included in the models to account for differences in parental quality. Significant terms are highlighted in bold.

			Ag	glutination sc	ore				Į	g Y levels			
	_		(u	= 100 nestling	gs)				(u =	68 nestlings)			
Term		Coefficient	Lower CL	Higher CL	DF	F/Z	d	Coefficient	Lower CL	Higher CL	DF	F/Z	d
Intercept		1.79	1.53	2.04	17.2	14.48	<.0001	0.13	-0.03	0.28	17	1.74	0.1
Laying date		-0.01	-0.03	0.02	1,14.65	0.46	0.51	-0.01	-0.02	0.01	1,47	1.24	0.27
Treatment	Cuckoo	0.36	0.02	0.69	2,13.52	4.22	0.04	-0.14	-0.32	0.04	2,47	1.65	0.2
	Hoopoe	0.46	0.11	0.82				-0.16	-0.35	0.02			
	Control*												
Nest	_					1.7	0.04					1.76	0.04

* Reference level in the treatment contrasts parametrization of the model matrix. CL, 95% confidence level.

	weig	nt at 12 days old			
	(n =	= 101 nestlings)			
Coefficient	Lower CL	Higher CL	DF	F/Z	р
5.03	-8.02	18.07	18	0.81	0.43
0.37	-0.78	1.53	1,79	0.41	0.52
-9.56	-25.56	6.45	2,79	0.83	0.44
oe -9.3	-26.49	7.88			
ol*					
				2.25	0.01
	Coefficient 5.03 0.37 -9.56 oe -9.3 ol*	Coefficient Lower CL 5.03 -8.02 0.37 -0.78 00 -9.56 -25.56 0e -9.3 -26.49	Weight at 12 days of $(n = 101 \text{ nestlings})$ Coefficient Lower CL Higher CL 5.03 -8.02 18.07 0.37 -0.78 1.53 00 -9.56 -25.56 6.45 0e -9.3 -26.49 7.88	Keight af 12 days old (n = 101 nestlings) Coefficient Lower CL Higher CL DF 5.03 -8.02 18.07 18 0.37 -0.78 1.53 1,79 00 -9.56 -25.56 6.45 2,79 0e -9.3 -26.49 7.88	Weight at 12 days old $(n = 101 \text{ nestlings})$ Coefficient Lower CL Higher CL DF F/Z 5.03 -8.02 18.07 18 0.81 0.37 -0.78 1.53 1,79 0.41 00 -9.56 -25.56 6.45 2,79 0.83 0e -9.3 -26.49 7.88 2.25

Table 3. Results of LMMs to test the effect of risk of cuckoo parasitism on body mass at age of 12 days of nestlings. Laying date was included in the models to account for differences in parental quality. Significant terms are highlighted in bold.

* Reference level in the treatment contrasts parametrization of the model matrix. CL, 95% confidence level.

Discussion

Our experimental manipulation of risk of cuckoo parasitism showed no effect on the corticosterone response of nestlings (concentrations of fCORT and pCORT). One first explanation to this lack of effect of risk of parasitism on corticosterone response could be that our playback experiment may have failed to increase the perceived risk of parasitism. However, recent studies in this population showed that magpies can assess and modify their nest-site choice in response to risk of parasitism (i.e. when they were exposed to call and decoy of cuckoo) (Expósito-Granados et al. 2017a). Thereby, it is possible that the stimulus needed to induce a response is the association of the call and cuckoo presence together, but not only the cuckoo call (Expósito-Granados et al. 2017b). That is to say that magpies perceived the stimulus of risk of parasitism, but that this was not enough to modify important traits (Expósito-Granados et al. 2017b) or affect the hormonal levels of nestlings either through hormone deposition in eggs or through the change in parental care of chicks. Supporting this explanation, our experiment was performed when around half of host eggs were already laid, and hence, hormones
deposited in eggs before manipulation. Unfortunately, we did not sample hormones from eggs and did not identify the egg from which each chick was born. Therefore, we could not test for differences in hormones between eggs laid before/after the experiment. Finally, another explanation to these results could be that the effect of risk of cuckoo parasitism loses strength over time. The manipulation of the perceived risk of parasitism was done during the egg-laying and incubation periods to accurately simulate when cuckoo parasitism occurs naturally. In this way, the possible effects would be weakened over the season and may be imperceptible at the time we sampled magpie nestlings. Finally, it could be argued that our sample size is not large enough to allow detecting statistically significant differences among treatments. Indeed, power calculations for our analyses assuming an $\alpha = 0.05$ showed a low power (ranging from 0.10 to 0.13) to detect low effect sizes (0.10), a moderate power (ranging from 0.42 to 0.59) to detect medium (0.25)effect sizes, and a high power (ranging from 0.83 to 0.95) for detecting large effect sizes (0.40) (Cohen 1992). This allows us to infer that the experimental manipulation of perceived risk of parasitism does not exert a large effect and probably not a medium effect either on nestling's physiology. Subtle effects of our experiment, however, may be unnoticeable with our sample sizes.

Regarding the effect of risk of cuckoo parasitism on nestlings' immunology and weight, we failed to find any effect of the treatment on Ig Y levels and weight of nestlings. However, nestlings raised in cuckoo- exposed nests showed higher agglutination score than those raised in control nests and similar to those nestlings raised in hoopoe-exposed nests (Table 2, Fig.1). The most obvious explanation to this intriguing pattern may be that our manipulation had an effect on parental nest attendance, which collaterally affected nestlings' immunology. Parents may increase nest attendance if they perceive our manipulation as a threat due to our visits to the surroundings of nests to connect and leave the speakers. Previous studies have found that avian parents, under stressful challenges at their nests, may increase nest attendance and provisioning (Hakkarainen et al. 2002; Thomson et al. 2010). This seems to be the case here, which may have indirectly induced a better condition of nestlings (i.e. high agglutination score) in manipulated nests. Alternatively, this pattern may suggest that our manipulation could be diminishing the presence of predators (Ibáñez-Álamo et al. 2012b) in nests exposed to cuckoo or hoopoe, so that owners of these nests may be less stressed and/or more attentive to their offspring. However, irrespective of the mechanism modulating agglutination of nestlings, it seems not to be powerful enough to affect the survival of nestlings (see *Ethical statement*).

Conclusions

We suggest that apart from the well-known lethal effect of brood parasites on their hosts (Davies 2000), risk of cuckoo parasitism might potentially induce sub-lethal effects on hosts (e.g. Brooke et al. 1998; Expósito-Granados et al. 2017a; Øien et al. 1996; Tolvanen et al. 2017), even by promoting changes in the physiological components of the host phenotype, as has been profusely documented for risk of predation (e.g. Creel et al. 2007; Cresswell 2008; Dunn et al. 2010; Sheriff and Thaler 2014). Our study has experimentally tested for the first time the possibility that the effect of a stressful parasitism challenge suffered by adults could be projected into the next generation causing the phenotypic modulation of physiological traits of the host's offspring. However, our results do not show conclusive trans-generational physiological effects of risk of cuckoo parasitism, therefore we encourage future studies investigating the relative importance of maternally transferred hormones and pre- and post-natal parental behaviors in shaping host offspring phenotypes in relation to variable risk of parasitism.

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Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Conflict of interest The authors declare no competing interests.

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Physiological effects of human disturbance differ between diurnal and nocturnal insectivorous birds



Physiological effects of human disturbance differ between diurnal and nocturnal insectivorous birds

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Abstract

Human activities are altering ecosystems and threatening the well-being of living animals. The study of the stressful effects of human disturbances on animal physiology can provide fundamental insights for wildlife conservation. In this study we assess whether human activities (i.e. agricultural practices and habitat transformation for agriculture or urban development) are perceived as stressors by two declining insectivorous birds, the European Roller Coracias garrulus and the European Scops Owl Otus scops differing in their activity rhythms. We hypothesize that nocturnality may buffer the impacts of human activity, which mostly takes place during the day, on the strictly nocturnal scops owl. We quantified a set of physiological indicators in nestlings of both species in a Mediterranean ecosystem where habitat quality varies within a human alteration gradient. We found that roller chicks reared on cereal crops, where diurnal human activity is intense, were more stressed during feathering, but showed a better condition at fledging, possibly as a consequence of higher food availability in those areas. In addition, nestling rollers showed higher stress induced response in less anthropic, but shrubby areas, which could be related to an increased risk of predation or changes in parental care. Meanwhile, scops owl nestlings reared in highly urbanized areas and close to roads, where nocturnal anthropic activity is high, showed higher stress induced response than those reared in less urbanized areas. Our results suggest that susceptibility to human disturbance may vary between species, probably due to the effect of the activity rhythm and the daily pattern of variation in human disturbance.

Manuscrito.

Introduction

Over the last few decades, human activities have altered the earth's ecosystems and threated the well-being of wildlife (Jetz et al. 2007). Anthropogenic perturbations are diverse and threat habitats at different intensity and frequency, constituting a stressor for wildlife (Benítez-Díaz and Bellot-Rojas 2007). For instance, human-induced forest fragmentation decreases food availability (Zanette et al. 2000), urban development reduces habitat suitability for some species (Alonso et al. 2003, 2004, Martín 2008), and agriculture exposes animals to disturbance caused by farming activities (Sastre Olmos et al. 2009). All these human-induced impacts lead wildlife to react against as facing predation risk (Frid & Dill 2002) by changing its distribution and density (Gomes et al. 2008; Chávez-Zichinelli et al. 2013), behaviour (e.g. Mougeot and Arroyo 2017) and physiology (e.g. Chávez-Zichinelli et al. 2010; Fokidis and Deviche 2011; Chávez-Zichinelli et al. 2013; Tarjuelo et al. 2015). Furthermore, susceptibility to human habitat alterations may be species-specific (Samia et al. 2015). Therefore, management plans for species of special conservation interest should be designed on the basis of knowledge about the effects of human activities (Ramírez Sanz et al. 2000; Cooke et al. 2013).

In particular, theoretical studies have suggested that an understanding of the specific physiological mechanisms underlying conservation problems might be critical for conservation biologists and managers given that physiological tools are useful for identifying the optimal range of habitat and stressor thresholds for species with conservation concern (Cooke et al. 2013). Under both short- and long-term unpredictable alterations in their environment, individuals initiate a stress response resulting in an activation of the hypothalamic-pituitary-adrenal (HPA) axis and then in an increase in the level of circulating plasma glucocorticoids (Romero 2004). Released hormones manage the homeostatic energy balance and allocation of resources between vital processes and threat events in vertebrates (Buchanan 2000; Sapolsky et al. 2000; Landys et al. 2006). In the face of short-term perturbations in environmental circumstances, glucocorticoids can promote prompt physiological and behavioural changes that help escaping from unexpected survival challenges (Sapolsky et al. 2000). However, when perturbations continue over the long-term (i.e. chronic stress), the maintenance of high baseline levels of glucocorticoids may impair immunocompetence (Martin 2009), reproductive functions (Wingfield et al. 1997; Sapolsky et al. 2000), and, ultimately, affect fitness (Boonstra 2013).

In this study we aim to test whether human activities associated with agriculture or urban development can be viewed as environmental stressors for two hole-nesting birds: the European Roller (hereafter Roller) Coracias garrulus and the Eurasian Scops Owl (hereafter Scops owl) Otus scops. The Roller and the Scops Owl are medium-sized (141 g and 91g, respectively) migrant insectivorous birds (Cramp 1998). The two species are obligate secondary cavity-nesters (i.e. they depend for nesting on holes excavated by woodpeckers and other birds in trees (Avilés and Folch 2004)) and share potential preys (Cramp 1998) and predators (authors unpublished data). However, while the roller is a diurnal species, the scops owl is a strictly nocturnal species with peaks of hunting and nestling feeding after sunset (Mikkola 2014). Populations from both species have declined in the last few decades although currently they are considered to be of Least Concern (Bird International 2018). The main threatening factors for the two species are habitat loss caused by agricultural intensification (Avilés and Folch 2004; Martínez et al. 2007) and the use of pesticides that can affect their potential prey and favour the accumulation of polluting residues, respectively (Avilés and Parejo 2004). Beyond their direct effects, agricultural intensification leads to the increase in human activities in cultivated lands, which may increase the encounter rate between humans and wild animals and, therefore, their potential negative impacts on animals (Steven et al. 2011). Also,

agriculture or urban development leads to the construction of linear infrastructures as roads which may cause detrimental habitat loss, fragmentation, degradation and noise for wild animals. Moreover, deforestation and the plantation of young trees in agricultural lands is one of the factors promoting the reduction of cavities, negatively affecting both species (Avilés and Parejo 2004; Köning et al. 2008; Rodríguez et al. 2011). Although all these factors are recognized as potential stressors, direct measurements of physiological stress in wildlife in relation to gradients of agricultural and human activity are scarce.

Here we aim to identify the stressor effects of habitat alterations on reproductive rollers and scops owls by measuring a wide set of physiological traits in nestlings from both species occupying a Mediterranean ecosystem that vary in habitat quality along a gradient of human alteration. Specifically we quantified corticosterone concentration in plasma (hereafter CORT) and feathers (hereafter fCORT), which allowed us to characterize the current state of the HPA axis (see more details in Romero, 2004) and the state of the HPA axis over the nest-bound period (Bortolotti et al. 2008; Bortolotti et al. 2009; Blas et al. 2005), respectively. In addition, aiming to assess the stressful effect of human disturbance on fitness related traits we also measured the immunological status (both innate (i.e. agglutination activity) and acquired immune response (i.e. immunoglobulin Y (hereafter IgY) levels and Tlymphocyte immune response) and weight of nestlings from both species. We predict that nestlings reared in more transformed habitats should show higher corticosterone concentrations than nestlings reared in more natural habitats. This is based on the knowledge that more intensely cultivated habitats have reduced availability of medium size Arthropods (i.e. Coleoptera, Arachnidae and Ortopetera) (Avilés and Costillo 1998), which are main prey of rollers and scops owls during reproduction (Parejo et al. 2013; Avilés and Parejo 1997), and produce high encounter rate between humans and parent birds, hence, increasing disturbance (e.g. Walker et al. 2005; Fokidis and Deviche 2011; Chávez-Zichinelli et al. 2013). Moreover, human alterations and circulating levels of corticosterone in acute and chronic stress response can affect birds' health (Sapolsky et al. 2000; Dhabhar 2002) by down-regulating the immune function (Martin 2009), and growth of nestlings (Hayward and Wingfield 2004). Hence, we also expected worse immunological status and a lower weight in nestlings raised in more transformed habitats. However, due to the different activity rhythms of the two species, we predict species-specific response to different human stressors. Indeed we expect a weaker effect of agricultural intensification on the scops owl (nocturnal species) than on the roller (diurnal species), because farming takes place during the day, but a stronger effect of roads and motorways on the nocturnal than on the diurnal species, because of intense traffic also at night.

Methods

Study area

The study was conducted in the breeding season (April– July) of 2013 in south-eastern Spain (37818° N, 3811° W). The area is an extensive agricultural landscape with scattered Holm Oak *Quercus ilex* and crossed by numerous dry riverbeds (*ramblas*). The area covers approximately 756 ha of holm oak wooded landscape where 443 cork oak nest-boxes (roof surfaces of 24 x 24 cm, 40 cm height and an opening of 6 cm diameter) were held on trees. At least once a week, nest boxes were checked to determine occupancy and to record reproductive parameters. A nest-box was considered to be occupied if at least one egg was laid in it. The year of study, 28 nests of rollers were initiated and physiological measurements were taken from 60 nestlings from 16 nests that escaped from predation. For scops owls, 21 nests were initiated and physiological measurements were taken from 74 nestlings from 18 available nests that escaped from predation.

Environmental data

We used R software version 3.3.3 (raster Package v 2.6-7 (Hijmans et al. 2017)) to process environmental data based on aerial photographs and 2003 Vegetation Cover and Land Use Databases for the Province of Granada that were freely available from Junta de Andalucía (Consejería de Medio Ambiente, Junta de Andalucía 2003, Junta de Andalucía 2006). For each nest-box, we derived the following variables: (1) distance to the nearest road (m), (2) distance to the highway (m), (3) distance to the nearest building (m) and (4) altitude above sea level (m). The study area is crossed by a heavily used highway and other minor roads that could also potentially disturb animals (Reijnen and Foppen 1994, Fig. 1). Buildings are isolated farmhouses, most of them abandoned, and one small village. Also, in order to qualify variability in human pressure/alteration, we estimated the percentage of the surface with human presence as urban surface, bare ground, the surface occupied by grassland / herb crop (i.e. cultivated areas), almond grove, pine plantation, scrubland and surface occupied by riparian surfaces (i.e. watercourses) within a circular area with a radius of 100 m centred on each nest box (Rodriguez et al. 2011). Preliminary analyses revealed that some environmental variables were highly inter correlated, so we simplified the data by performing a principal components analysis (PCA) on the set of environmental variables. The first three PCA axes explained 25.63, 14.32 and 12.67% of the variance, respectively (Table 1). The first PCA axis loaded positively distance to highway, road and buildings and surface of herb crops (i.e. positive loadings) and negatively tree groove plantations (i.e. almond trees) and bare surface (Table 1). Thus, roller and scops owls breeding events with positive scores for the first axis occurred in cultivated areas with low surface of tree plantations and far away from the highway. The second axis loaded positively for surface of scrublands, hence roller and scops owls breeding events with positive PC2 score occurred in low human disturbed areas (Table 1). The third axis classified nest-boxes according to the density of pine plantations vs density of watercourses (i.e. riparian areas) (Table 1). Factor scores derived from the PCA for each occupied roller and scops owls breeding attempt were used in subsequent analyses.

Fig. 1. Study area and population distribution of rollers and scops owls.



Table 1. Loadings of the environmental variables in the three principal components (n = 443 nestboxes). Important loadings (>0.50) within each component are depicted in bold.

Variable	Factor 1:	Factor 2	Factor 3:
Distance to road	0.57	0.52	0.09
Distance to highway	0.78	0.03	-0.13
Distance to building	0.54	0.49	-0.07
Urban surface	-0.42	-0.11	-0.25
Bare surface	-0.55	0.07	0.06
Riparian surface	-0.09	-0.002	0.58
Surface of herb crop	0.71	-0.66	0.08
Surface of treegroove	-0.64	0.16	0.46
Surface of pine plantation	-0.32	0.45	-0.74
Surface of scrublands	0.22	0.61	0.42
Altitude	0.16	-0.09	-0.13

Bird capture

Blood from nestlings of the two species was extracted in two different days but at similar stage of nestling development for both species. At the first sampling day (15 days age of the first chick born for rollers and 12 days age of the last chick born for scops owls) chicks were bled (225μ L per chick) to obtain innate (agglutination activity) and acquired (IgY) immunity measurements. At the second sampling day (20 days age of the first chick born in rollers and 15 days age of the last chick born in scops owls), nestlings were bled again to obtain CORT measurements (stress-induced plasma CORT). Sampling was always done in the morning (8:30 to 11:30 a.m.) to avoid a possible problem of circadian CORT changes (Breuner et al. 1999). At the second sampling day we also collected the third primary major covert feather of each nestling and stored it in a dark box at ambient temperature until laboratory analyses of fCORT. Blood was collected from the brachial vein using a 0.5 x 16 mm needle and heparinized capillary tubes to carefully transfer it into a 1.5 mL tube. Blood was refrigerated and plasma and red blood cells were separated by centrifugation the same day at 13300 rpm for 5 min. All samples were stored in a -20°C freezer until the end of fieldwork when they were kept in a -80° C freezer until laboratory analyses. Nestlings were weighed at age of 21 days to the nearest 1 g using a 300 g Pesola spring balance.

Corticosterone assay

Corticosterone concentrations were determined at the Centre d'Études Biologiques de Chizé (France) following the procedure of Lormée et al. (2003) for steroid hormones. Briefly, an ethyl ether extraction technique was used to extract CORT from plasma (Lormée et al. 2003) and a methanolbased extraction technique was used to extract CORT from feathers (Bortolotti et al. 2008). Plasma and feather CORT extracts were measured using radioimmunoassay with a highly cross-reactive rabbit anti-mouse antibody from Sigma (C8784). Duplicate aliquots of the extracts were incubated overnight at 4°C with 3H-corticosterone and antiserum. The bound and free corticosterone fractions were separated by adding dextran-coated charcoal and after centrifugation, the bound fraction was counted in a liquid scintillation counter. Samples were assayed the same day or were frozen at -20° until analysis. The lowest detectable corticosterone level was 0.28 ng/mL. Intra and inter-assay coefficients of variation for plasma assays were 6.29 and 15.72%, respectively. For feather assays intra and inter-assay coefficient were 8.95 and 14.05 %, respectively.

Immune assays

Assessment of innate humoral immune responsiveness was made by using the standard protocol based on Natural Antibody (hereafter NAb) mediated complement activation and red blood cell agglutination (Matson et al. 2005). The agglutination responsiveness represents the interaction between NAb and antigens. Quantification of agglutination was done by assessing the dilution stage (on a scale from 1 to 12 titres) at which this reaction stopped against the same amount of rabbit blood cell suspension on digitalized images (for further details on the method, see Matson et al., 2005; Parejo et al. 2007). This assay determines the values of circulating NAbs by measuring red blood cell agglutination. We did not consider lytic activity, which can be feasibly determined by this assay because it was not variable in nestlings of the two species.

Assessment of acquired immune responsiveness was made by measuring the levels of nestlings' IgY following the protocol described in Martínez et al. (2003). IgY is an antibody in birds that can neutralize pathogens (Demas et al. 2011). Polystyrene 96-well plates (Maxi-sorp, Nunc, Rochester, NY, USA) were covered overnight (4°C) with serial dilutions of serum (100 μ l) in carbonate-bicarbonate buffer (0.1 M, pH = 9.6) in order to determine the linear range of the sigmoid curve (Plasma dilution of 1/4000 to suit for chicks from both species). Wells were washed three times with 200 µl per well of PBS,1% BSA, 0.05% Tween buffer. Then, plates were blocked with defatted milk diluted in PBS-Tw buffer for 1 h at 37°C (200µl). Antichicken conjugate (Sigma A-9046) at 1/250 dilution was added in PBS-Tw and incubated for 2 h at 37°C (100µl). Finally, substrate comprising ABTS and concentrated hydrogen peroxide for 1h at 37°C were added and measured the absorbance at 405 nm with a spectrophotometer. All plasma samples were run in duplicate, and the mean of IgY absorbance value was used in the analyses.

Finally, to assess cell-mediated immunity in birds we measured the T-lymphocyte immune responsiveness (hereafter PHA response) by injecting phytohemagglutinin (PHA) (Cheng and Lamont, 1988). At the age of 21

days, the thickness of the right and left wing webs (patagium) of nestlings was measured by a pressure-sensitive caliper, a spessimeter (Baxlo 3000), to the nearest 0.01 mm. The left wing web was injected with 0.2 mg of PHA (Sigma, L-8754) in 0.04 ml of PBS. The right wing web was injected with 0.04 ml PBS only. Twenty-four hours later we re-measured the thickness of each wing patagiums at the inoculation sites, marked the previous day with a permanent marker. The PHA response is the difference in patagium thickness between day 2 and day 1 for the PHA-inoculated patagium minus the difference in wing web thickness between day 2 and day 1 for the PBS-inoculated patagium (Saino et al. 1997).

Statistics

Analyses were performed using SAS v.9.4 statistical software (SAS Institute, Cary, NC, USA).

Sample sizes may vary amongst analyses because some physiological data were unavailable (e.g. CORT levels under the threshold for detection or absence of immunological activity) and because, due to logistical problems, not all information could be collected for all nestlings or broods.

We run General Linear Models (GLM procedure in SAS) to test for the effect of human disturbance on average values per nest of stress-induced CORT levels, fCORT levels, Ig Y levels, PHA response and weight of roller and scops owl nestlings. All variables were centred prior to analyses and standard model validation graphs (Zuur 2009) revealed that model assumptions of homogeneity of variance and normality of residuals were fulfilled.

In addition, we run Poisson regressions (GENMOD procedure in SAS, link= log) to test for effects of human disturbance on innate immune responsiveness of nestlings by using agglutination scores per nest as a dependent variable. In all models (both GLM and Poisson regressions), we

entered the three PCA factor scores derived from the PCA on environmental variables for each occupied breeding attempt as explanatory variables. In addition, to control for possible differences in seasonality of perturbations or declining of resources, laying date was included as a further covariate in the models. Finally, brood size was entered as a covariate in the knowledge that it is a good correlate of individual quality in birds. Due to low sample size we disregarded testing interactive effects in the models. Model simplification was performed following backward stepwise elimination of non-significant terms from the initial models.

Results

Effect of habitat features on nestlings' corticosterone responses

Stress-induced CORT levels varied in both species in relation to different components of habitat features. In Rollers, stress-induced CORT levels varied according to PC2 scores (Table 2), meaning that nestlings raised in nests located in very natural areas (i.e. a high percentage of shrub cover) had higher levels of stress induced CORT than those raised in nests located in areas with low shrub cover (Fig. 2a). On its side, stress-induced CORT levels of scops owl chicks were almost significantly associated with PC1 scores (Table 2): chicks from nests located in areas with high density of tree plantations and close to the highway had higher stress induced CORT levels that those from nests located in areas with high surface of grassland / herb crop (Fig. 3).

Concerning fCORT, levels in roller chicks were almost significantly associated with PC1 scores (Table 2). Nests placed in cultivated areas with low density of tree groove and farther to the highway had higher values of fCORT concentration than those nests located close to the highway but with low percentage of cultivated surface (Fig. 2b). fCORT levels of scops owl chicks were not significantly associated with any of PC scores from the PCA on environmental variables (Table 2).



Fig. 2. Mean stress induced corticosterone (a) and feather corticosterone (b) of roller nestlings in relation to second (a) and first (b) PC scores of a PCA on habitat features (see table 1).



Fig. 3. Mean stress induced corticosterone of scops owl chicks in relation to PC1 scores of a PCA on habitat features (see table 1).

(i.e. mean stress ind	uced CORT an	nd fCORT pe	r nest). Signifi	cant ter	rms are	highlig	ghted in bold.		,			
		Stress	Induced CORT					. –	Feather CORT			
Coraciae aarmine		u	= 14 nests						n = 16 nests			
Coracias garraias	Coefficient	Lower CL	Higher CL	DF	F	р	Coefficient	Lower CL	Higher CL	DF	F	р
Intercept	0.14	-3.22	3.50	12	0.09	0.93	-0.84	-0.94	-0.74	14	-18.28	<.0001
Laying date	-0.01	-0.62	0.59	1,7	0.00	0.97	0.001	-0.01	0.01	1,11	0.10	0.76
Brood Size	-0.35	-3.74	3.04	1,9	0.05	0.82	0.01	-0.10	0.11	1,9	0.01	0.91
PC1	1.98	-1.20	5.16	1,11	1.88	0.20	0.10	0.00	0.20	1,14	4.76	0.05
PC2	4.88	1.41	8.35	1,12	9.37	0.01	0.01	-0.11	0.13	1,10	0.02	06.0
PC3	1.22	-3.88	6.31	1,10	0.28	0.61	0.08	-0.05	0.22	1,13	1.71	0.21
Ofue come		n	= 18 nests						n = 15 nests			
cdore ento	Coefficient	Lower CL	Higher CL	DF	F	р	Coefficient	Lower CL	Higher CL	DF	F	p
Intercept	0.38	-3.01	3.77	16	0.24	0.82	96.0	0.64	1.28	13	6.50	<.0001
Laying date	-0.07	-0.68	0.53	1,12	0.07	0.80	0.02	-0.02	0.07	1,12	1.46	0.25
Brood Size	1.47	-2.46	5.39	1,14	0.64	0.44	0.03	-0.47	0.53	1,10	0.02	06.0
PC1	-3.39	-6.85	0.08	1,16	4.29	0.05	0.01	-0.41	0.43	1,9	0.00	0.97
PC2	2.85	-2.37	8.06	1,15	1.35	0.26	0.27	-0.18	0.72	1,13	1.65	0.22
PC3	-0.60	-4.29	3.09	1,13	0.12	0.73	-0.10	-0.41	0.22	1,11	0.46	0.51

Table 2. Results of GLMs testing for the effect of habitat features (i.e. PC scores of a PCA on habitat variables) on stress hormone measures

CL, 95% confidence level.

Effect of habitat features on the nestlings' immunity

Nestlings' innate immune responsiveness (i.e. agglutination) (Table 3), and nestlings' acquired immunity (i.e. Ig Y and PHA response) (Table 4) of rollers and scops owls did not significantly vary in relation to any of PC scores from the PCA on environmental variables.

Table 3. Results of GLZs testing for the effect of habitat features (i.e. PC scores of a PCA on habitat variables) on immune innate response (i.e. agglutination) of roller and scops owl chicks. Significant terms are highlighted in bold.

			Agluttination			
Conscience commuters			n = 16 nests			
Coracias garruius	Coefficient	Lower CL	Higher CL	DF	F	р
Intercept	2.29	2.12	2.46	14	28.16	<.0001
Laying date	-0.005	-0.02	0.01	1,11	0.28	0.61
Brood Size	-0.03	-0.22	0.15	1,10	0.17	0.69
PC1	-0.04	-0.25	0.18	1,9	0.15	0.70
PC2	0.10	-0.08	0.28	1,14	1.36	0.26
PC3	0.06	-0.21	0.33	1,13	0.22	0.65
Otus soons			n = 18 nests			
Olus scops	Coefficient	Lower CL	Higher CL	DF	F	р
Intercept	2.38	2.23	2.54	16	32.19	<.0001
Laying date	0.004	-0.02	0.03	1,15	0.15	0.70
Brood Size	0.004	-0.19	0.20	1,13	0.00	0.97
PC1	0.002	-0.18	0.18	1,12	0.00	0.98
PC2	0.03	-0.21	0.27	1,14	0.07	0.79
PC3	-0.03	-0.18	0.12	1,16	0.14	0.71

CL, 95% confidence level.

		d	0.12	0.81	0.41	0.54	0.30	0.96		d	0.09	0.26	0.20	0.75	0.68	0.20
		F	1.64	0.06	0.73	0.39	1.15	0.00		F	-1.87	1.49	1.86	0.11	0.18	1.88
		DF	14	1,10	1,13	1,12	1, 14	1,9		DF	10	1,8	1,10	1,7	1,6	1,9
PHA response n = 16 nests	n = 10 nests	Higher CL	0.26	0.02	0.08	0.21	0.08	0.31	n = 12 nests	Higher CL	0.12	0.01	0.29	0.12	0.25	0.21
H		Lower CL	-0.04	-0.02	-0.19	-0.12	-0.23	-0.30		Lower CL	-1.36	-0.03	-0.07	-0.16	-0.35	-0.05
		Coefficient	0.11	-0.002	-0.05	0.05	-0.08	0.01		Coefficient	-0.62	-0.01	0.11	-0.02	-0.05	0.08
		р	<.0001	0.07	0.23	0.25	0.73	0.97		p	0.0001	0.65	0.09	0.96	0.45	0.12
		F	-12.18	3.75	1.61	1.50	0.13	0.00		F	4.93	0.21	3.29	0.00	0.60	2.64
		DF	13	1,13	1,12	1,11	1,10	1,9		DF	16	1,13	1,15	1,12	1,14	1,16
Ig Y levels $n = 15$ nests	SISOU $CI = II$	Higher CL	-0.02	0.001	0.01	0.01	0.005	0.01	n = 18 nests	Higher CL	0.04	0.002	0.02	0.01	0.02	0.02
		Lower CL	-0.04	-0.0001	-0.002	-0.002	-0.01	-0.01		Lower CL	0.02	-0.001	-0.002	-0.01	-0.01	-0.003
		Coefficient	-0.03	0.0004	0.003	0.003	-0.001	0.0002		Coefficient	0.03	0.0004	0.01	0.0003	0.01	0.01
	Coracias garrulus	0	Intercept	Laying date	Brood Size	PC1	PC2	PC3	Ottic coone	sectors smice	Intercept	Laying date	Brood Size	PC1	PC2	PC3

Table 4. Results of GLMs testing for the effect of habitat features (i.e. PC scores of a PCA on habitat variables) on acquired immunity (i.e.

CL, 95% confidence level.

Effect of habitat features on nestlings' weight

Weight of roller nestlings positively associated with PC1 scores (Table 5), meaning that nests located in areas with a high percentage of grassland/ herb crop and more distant from the highway had heavier chicks than those located close to the highway with lower surface of cultivated land (Fig. 4).

No relationship was found between the mean weight of scops owl chicks and PCA components of habitat, laying date or brood size (Table 5).



Fig. 4. Mean weight of roller nestlings in relation to PC1 scores of a PCA on habitat features (see table 1).

Table 5. Results of GLMs testing for the effect of habitat variables of PCA analysis on weight of roller and scops owl nestlings at 20 day aged. Significant terms are highlighted in bold.

			Weight				
			n = 15 nests				
Coracias garrulus	Coefficient	Lower CL	Higher CL	DF	F	р	
Intercept	36.55	31.12	41.97	13	14.56	<.0001	
Laying date	0.24	-0.34	0.82	1,11	0.81	0.39	
Brood Size	-2.10	-8.96	4.76	1,10	0.46	0.51	
PC1	6.07	0.79	11.34	1,13	6.17	0.03	
PC2	0.15	-7.63	7.93	1,8	0.00	0.97	
PC3	1.13	-8.18	10.43	1,9	0.07	0.79	
			n = 14 nests				
Otus scops	Coefficient	Lower CL	Higher CL	DF	F	р	
Intercept	-42.54	-56.61	-28.47	11	-6.66	<.0001	
Laying date	-0.11	-0.57	0.35	1,10	0.27	0.61	
Brood Size	1.34	-2.17	4.85	1,11	0.71	0.42	
PC1	-1.17	-4.24	1.90	1,11	0.71	0.42	
PC2	-1.68	-8.07	4.71	1,9	0.35	0.57	
PC3	-0.19	-4.37	3.99	1,8	0.01	0.92	

CL, 95% confidence level.

Discussion

We found an association between nestling physiology of two declining insectivorous and secondary hole-nesting birds breeding in a Mediterranean landscape and habitat features identifying gradients of habitat alteration due to farming activities and urbanization.. However, the found relationships between habitat features and nestlings' physiology were different for rollers and scops owls.

Firstly, roller nestlings from nests in more natural areas (i.e. high shrub cover), showed a higher response to induced stress. The explanation to this non-expected result may be related to risk of predation by natural predators. Indeed, in our study area, ladder snakes (Zamesis scalaris) and garden dormouse (Eliomys quercinus) predate on nesting birds (authors, unpublished data) and both predators are associated with scrublands. Ladder snakes usually inhabit scrublands (Pleguezuelos 2017) from where they can more easily access trees. On its side, dormouse nest box occupancy in the year of study was positively associated with high percentage of shrub surface (Mean $(\pm SE)$ percentage of shrub surface around: Non-occupied boxes =7.82 ($\pm SE$) versus Occupied boxes= 12.20 (\pm SE); ANOVA: F = 5.40, df = 1, 441, p = 0.02). These two pieces of evidence would suggest that scrublands are under higher predation risk, which may have led to changes in parental attentiveness that had translated into higher levels of corticosterone in chicks (e.g. Sheriff and Love 2013). The possibility of higher corticosterone levels helping to scape predation can be discarded because chicks cannot escape from the nest at this age. Alternatively, this habitat could be unsuitable for rollers due to their preference to perch and hunt in open areas (Avilés and Costillo 1998; Rodríguez et al. 2011), which could lead parents to reduce their attentiveness promoting higher CORT concentrations in it. Finally, it could be argued that good quality parent rollers avoided areas with scrubland, which is unlikely because laying date (i.e. a correlate of quality in rollers (Avilés et al. 1999)) did not relate with habitat features in our sample (Table S2, Supplementary Material).

All the other relationships between nestlings' physiology and habitat features concerns with PC scores of the first PCA factor, which put in front cultivated areas with cereals and highly human-used areas due to the closeness to the highway. However, results were distinct for the two species, confirming our expectation that susceptibility to human habitat alterations is species-specific (Samia et al. 2015).

In one hand nestling rollers seemed to be more stressed during feathering when being raised in cereal crops where land working was intense

during daylight, but, however, showed a better condition at fledging. Two explanations to this pattern are possible. Previous studies have shown that breeding rollers settle preferentially in areas with low tree groove surface (Rodríguez et al. 2011), which is inversely related to cultivated land surface (Table 1). Cultivated areas may be preferred because they provide parents with good feeding opportunities (as shown by the higher weight of nestlings growing there), and despite the high disturbance produced by the working of land (as shown by the high fCORT concentration). Hence, the found results may be due to the high abundance of insects that the intensification of farming practices promotes (Avilés and Parejo 2004), possibly also favouring the increase in feeding rates. Alternatively, the found pattern may be due to feeding compensation of stressed, highly demanding chicks. This possibility rests on the untested assumption that increased levels of CORT in nestling rollers were related to begging (e.g. Kitaysky et al. 2001; 2003). That is, in shrubby areas roller nestlings beg more and therefore receive more food (greater weight). Previous studies have shown that parents of different bird species (Zanette et al. 2011), including the European roller (Expósito-Granados et al. 2016), may compensate the harmful consequences of stressful stimulus by increasing their feeding rates.

For scops owls, we found that nestlings raised in highly urbanized areas near important roads showed the highest response to induced-stress. Roads represent a threat to wildlife (Reijnen and Foppen 1994; Crino et al. 2011), and the highway crossing the area is one of the most important communication ways of the south of Spain, being highly used both during day and night. This is reflected in the higher capacity of nestlings of this nocturnal species to mount a stress response when living near the highway, suggesting that parents might transmit the effect of the traffic to their nestlings. Nevertheless, this stress seems not to be translated into other different fitness proxies as immunology or body weight of nestlings before fledgling, perhaps because the most harmful effects of the highway are during daylight, when scops owls are inactive. Alternatively, we cannot discard that scops owls have a lower susceptibility to human disturbances.

Correlative studies like this do not allow establishing causal-effect relationships and cannot exclude the possibility that uncontrolled environmental factors may have influenced the physiological traits of nestlings. Although we can reasonably discard a general issue of low sample size, because our analyses showed significant results for both species, it could be argued that the found patterns might be the result of differential predation in relation to the different habitat features considered in our study. However, logistic regression revealed that there is not a relationship between the probability of nest predation for scops owl nests and the PC scores of PCA on environmental variables (Table S1, Supplementary Material). On the other hand, probability of predation of roller nests was associated with the scores of the third PCA factor, but not to PC1 or PC2 scores which are the ones related to nestling roller physiology. Nests located in riparian areas and with low surface of pine plantations had more probabilities to be predated (Fig. S1, Table S1, Supplementary Material). This result suggests that predation of roller nests is related to habitat features and one part of the roller population would not be represented in our analyses. However, it seems unlikely that this result will affect evidence obtained in relation to the other PCA factors. Alternatively, our results may be affected by a differential distribution of individuals across considered habitats features according to their quality. However, this possibility seems unlikely because laying date and number of fledglings (as correlates of individual quality) were significantly related to habitat features neither in rollers nor in scops owls (Table S2, Table S3, Supplementary Material).

In conclusion, this study provides evidence of physiological effects of human activity on two species inhabiting an area with a gradient of human

alteration. Susceptibility to human disturbance, however, varied between species, probably reflecting the effect of activity rhythms on susceptibility to daily variation in disturbance due to agricultural and human activities. Therefore, nocturnal rhythm of scops owls might help buffering the impact of human activity in their habitats. Future studies should consider the possibility that human disturbance may vary during the day and the fact that activity rhythm of species may buffer or exacerbated their effects. Experimental approaches are also necessary to understand how activity rhythm of the species can affect the susceptibility to human factors.

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Supplementary Material for:

Phiysiological effects of human disturbance differ between diurnal and nocturnal insectivorous birds

Figure S1. Probability of predation of roller nest in relation to third PCA factor.



Capítulo 7

Table S1. Results of Generalized Linear Models (Binomial distribution, link=logit) testing for the effect of habitat disturbances on probability of predation of roller and scops owl nest as a binary dependent variable. Non significant terms were removed following a backward procedure. Significant terms are highlighted in bold.

	Probability of predation n = 28 nests						
Coracias garrulus	Coefficient	Lower CL	Higher CL	DF	F	р	
Intercept	-1.72	-3.03	-0.41	26	-2.70	0.01	
PC1	0.78	-0.61	2.17	1,25	1.34	0.26	
PC2	-0.70	-2.45	1.05	1,24	0.68	0.42	
PC3	1.72	0.08	3.37	1,26	4.65	0.04	
	n = 21 nests						
Otus scops	Coefficient	Lower CL	Higher CL	DF	F	р	
Intercept	-4.78	-10.61	1.06	19	-1.71	0.10	
PC1	-0.33	-4.20	3.54	1,17	0.03	0.86	
PC2	-2.95	-9.00	3.10	1,19	1.04	0.32	
PC3	1.75	-5.10	8.59	1,18	0.29	0.60	

Table S2. Results of General Linear Models (Normal distribution, link=identity) testing for the effect of habitat disturbances on laying date of roller and scops owl nest as dependent variable. Non significant terms were removed following a backward procedure. Significant terms are highlighted in bold.

	Laying date					
	n = 25 nests					
Coracias garrulus	Coefficient	Lower CL	Higher CL	DF	F	р
Intercept	-3.04	-7.70	1.62	23	-1.35	0.19
PC1	-2.12	-8.15	3.91	1,21	0.53	0.47
PC2	-4.29	-9.78	1.20	1,23	2.62	0.12
PC3	-1.81	-7.76	4.13	1,22	0.40	0.53
	n = 21 nests					
Otus scops	Coefficient	Lower CL	Higher CL	DF	F	р
Intercept	1.75	-1.74	5.25	19	1.05	0.31
PC1	-0.75	-4.86	3.35	1,17	0.15	0.70
PC2	-2.53	-7.48	2.42	1,18	1.15	0.30
PC3	1.85	-1.63	5.33	1,19	1.24	0.28

Table S3. Results of Generalized Linear Models (Poisson distribution, link=log) testing for the effect of habitat disturbances on number of roller and scops owl fledglings as dependent variable. Non significant terms were removed following a backward procedure. Significant terms are highlighted in bold.

1

	Number of fledglings						
	n = 21 nests						
Coracias garrulus	Coefficient	Lower CL	Higher CL	DF	F	р	
Intercept	0.87	0.55	1.19	19	5.74	<.0001	
PC1	-0.01	-0.37	0.34	1,17	0.01	0.93	
PC2	0.19	-0.14	0.52	1,19	1.43	0.25	
PC3	-0.08	-0.54	0.37	1,18	0.15	0.70	
	n = 20 nests						
Otus scops	Coefficient	Lower CL	Higher CL	DF	F	р	
Intercept	0.97	0.67	1.26	18	6.96	<.0001	
PC1	0.04	-0.30	0.37	1,16	0.05	0.82	
PC2	0.16	-0.25	0.56	1,18	0.65	0.43	
PC3	-0.05	-0.34	0.25	1,17	0.12	0.73	

Discusión General

DISCUSIÓN GENERAL

Esta tesis ofrece resultados novedosos que contribuyen a profundizar en el conocimiento de los efectos en tiempo ecológico tanto del riesgo de depredación como del parasitismo de cría (el miedo) en las aves, de gran interés tanto en el campo de la Ecología como del Comportamiento y con aplicación en la Conservación y Gestión Animal. Combinando aproximaciones experimentales (**capítulos 1-6**) y correlativas (**capítulo 7**) abordamos los efectos del miedo tanto desde un punto de vista poblacional o de comunidad (**capítulos 1, 2 y 3**) como a nivel individual (**capítulos 4-7**), a distintas escalas espaciales y considerando diferentes tipos de pistas que informan sobre el riesgo (i.e. olfativas, visuales y acústicas), ofreciendo así, un análisis holístico de los efectos del miedo sobre la ecología de la aves.

Nuestros resultados concuerdan con estudios previos que muestran que las aves usan diferentes pistas que informan sobre el riesgo de depredación (Eggers et al. 2006; Fontaine y Martin 2006a; Parejo y Avilés 2011; Parejo et al 2012b; Hua et al. 2013) y parasitismo de cría (Forsman y Martin 2009; Patten et al. 2011; Welbergen v Davies 2012; Tolvanen et al. 2017) a la hora de seleccionar sus hábitats de reproducción (antes de iniciar la reproducción, capítulos 1, 2 y 3). En el capítulo 1, nuestros resultados muestran por primera vez que el riesgo de depredación percibido a través de pistas olfativas puede modular la selección de lugares de reproducción. Aumentamos experimentalmente la percepción del riesgo de depredación a escala de parche mediante la aplicación de olores de depredador. Las parcelas con olor a depredador fueron menos ocupadas que las parcelas con un tratamiento sin olor de depredador y que las parcelas control. Se observó además que las aves se instalaban más tarde en parcelas con olor de depredador. Por otro lado, el tratamiento del olor influyó en la relación entre la abundancia de aves y roedores. Así, en parcelas sin riesgo de depredación se encontró una relación inversa entre la abundancia de aves y roedores que sugiere que ambos están en competencia, pero la abundancia aviar no aumentó cuando la abundancia de roedores era baja en aquellas parcelas con olor de depredador, sugiriendo que las aves evitaban estas parcelas. Estudios anteriores han mostrado que las aves pueden evaluar el riesgo de depredación en el nido a través de pistas olfativas (Roth *et al.* 2008; Amo *et al.* 2008; Parejo *et al.* 2012a; Stanbury y Briskie 2015). Sin embargo, nuestros resultados muestran por primera vez que el miedo a la depredación, inducida por el olor de depredador, afecta a las decisiones previas a la reproducción a través de cambios en la elección de los territorios, y que éstos tienen consecuencias ecológicas alterando la abundancia, composición y fenología de una comunidad compuesta por roedores y aves que utilizan las cavidades.

Las comunidades animales constituyen una excelente red de información social en la que los individuos pueden evaluar el riesgo de depredación a través de las llamadas de alarma de otras especies (Templeton y Green 2007; Vitousek et al. 2007; Parejo et al. 2012b). Descifrar esa información tiene un alto valor adaptativo porque muchas especies dentro de la comunidad pueden compartir depredadores. Aunque, las comunidades incluyen especies en diferentes niveles tróficos, y aún no se sabe cómo el nivel trófico del receptor influye en el valor informativo de una llamada de alarma. Por otro lado, el valor de la información social proporcionado por las llamadas emitidas dentro de una red trófica puede variar en relación al nivel de competencia entre emisores y receptores, como sucede con otras fuentes de información social (Seppänen et al. 2007; Parejo y Avilés 2016). Encontramos que los mesodepredadores, que comparten nivel trófico y depredadores con el mochuelo, que es la especie que emite las llamadas, interpretaron las llamadas de alarma de manera diferente a las especies presa, eligiendo preferentemente las zonas donde se producía cualquier tipo de llamada (alarma o contacto)(capítulo 2). Por el contrario, las especies presa seleccionaron parches donde los mochuelos (i.e. sus depredadores) emitían

llamadas de alarma, sugiriendo que interpretaban, en estos parches, que el riesgo de depredación era más bajo como consecuencia del miedo de los mochuelos ante su propio riesgo a ser depredados ("los enemigos de mis enemigos son mis amigos" *sensu* Sabellis *et al.* 2001). Finalmente, la competencia por el recurso nido afectó más a la selección de hábitat de las especie presa que a las especies del nivel trófico del mochuelo. En este caso la interpretación de los resultados puede ser difícil ya que para la especie presa, el competidor es también un potencial depredador (Morosinotto *et al.* 2017). En conjunto, los resultados de este capítulo muestran que el valor de las llamadas de alarma emitidas por una especie dentro de una comunidad difiere en función de las diferencias en nivel trófico entre el emisor y receptor (Parejo y Avilés 2016), y que el valor de dicha información esta modulado por los niveles de competencia por recursos clave como el nido.

De manera análoga, el riesgo de parasitismo de cría mostró tener efectos sobre la elección de lugares de nidificación por los hospedadores para minimizar los costes del parasitismo (capítulo 3). Hasta ahora, la evidencia de que los hospedadores de parásitos de cría podrían evaluar el riesgo de parasitismo antes de elegir sus lugares de reproducción era fundamentalmente correlativa (e.g. Øien et al. 1996; Moskát y Honza 2000; Antonov et al. 2007; Patten et al. 2011; Welbergen y Davies 2012), y los escasos estudios experimentales se han basado en sistemas que incluyen parásitos generalistas (Forsman y Martin 2009; Tolvanen et al. 2017). Encontramos que la manipulación experimental del riesgo de parasitismo percibido a través de señales visuales y acústicas del parásito tuvo un efecto en la elección de territorios de reproducción y fidelidad a los mismos en la urraca. Las urracas que se reproducen antes en la estación colocaron preferentemente sus nidos en áreas de bajo riesgo percibido de parasitismo, y este efecto se diluyó con el tiempo transcurrido desde la manipulación del riesgo, posiblemente porque, avanzada la temporada, esa señal ya no

Discusión General

constituiría una información fiable (Nocera *et al.* 2006). Asimismo, la fidelidad al territorio de individuos conocidos entre años disminuyó con el riesgo de parasitismo, pero sólo si no fueron parasitados el año anterior, mostrando una influencia de la experiencia previa de parasitismo en la evaluación del riesgo actual de parasitismo. Nuestros resultados constituyen la primera evidencia experimental de los efectos del riesgo de parasitismo de cría durante la elección de lugares de reproducción en una especie hospedadora que interactúa con un parásito especialista.

Una vez comienza la reproducción, el miedo a la depredación puede afectar a las estrategias reproductivas de las aves (Lima y Dill 1990; Lima 2009; Martin y Briskie 2009). Así, el riesgo de depredación de nidos puede causar una reducción de la actividad parental (e.g. Fontaine y Martin 2006b; Ghalambor et al. 2013; Peluc et al. 2008; Parejo et al. 2012a) para reducir la posibilidad de ser detectado por los depredadores (Skutch 1949; Martin et al. 2000a, b; Ghalambor y Martin 2002). Además, el riesgo de depredación podría modificar el valor que los padres otorgan a pollos grandes y pequeños en nidadas asincrónicas modificando sus preferencias de reparto de alimento (Tilgar et al. 2011). En el capítulo 4 estudiamos el efecto del riesgo de depredación de nidos percibido a través de pistas visuales sobre el cuidado parental y el reparto de alimento entre pollos en la carraca, un ave con marcada asincronía de eclosión. Los resultados mostraron que en nidos con riesgo de depredación alto los machos, pero no las hembras, aumentaron su tasa de aprovisionamiento al nido, y a pesar de esto, el peso de los pollos al vuelo fue menor que en nidos control. Sin embargo, los padres no modificaron su estrategia de reparto de alimento entre pollos grandes y pequeños en respuesta al riesgo de depredación. Estos resultados sugieren que las aves pueden modificar comportamientos asociados al cuidado parental en función del riesgo de depredación que perciben en el nido, y que la sensibilidad a dicho riesgo puede depender del sexo (Moks y Tilgar 2014). Además, nuestros resultados muestran que los cambios en cuidado parental en repuesta al riesgo llevan aparejados cambios en las perspectivas de éxito de la descendencia que podrían tener consecuencias en términos evolutivos puesto que el peso al vuelo es un predictor fiable de la probabilidad de supervivencia en aves.

Hasta donde sabemos, no existen aún evidencias empíricas que muestren cambios en rasgos reproductivos o en el comportamiento parental de los hospedadores en respuesta a un incremento del riesgo de parasitismo (Avilés 2017) pese a que esa posibilidad se haya sugerido en estudios teóricos recientes (Medina y Langmore 2016). Predijimos que los hospedadores de parásitos de cría que perciban un riesgo alto de parasitismo en su nido, podrían modificar plásticamente sus rasgos reproductivos y comportamientos de cuidado parental con posibles efectos sobre la supervivencia de su descendencia, del mismo modo que se ha demostrado con el riesgo de depredación en diferentes taxones (e.g. Zanette et al. 2011; Lamanna v Martin 2016; Dudeck et al. 2018). Así, introducimos por primera vez la posibilidad teórica de que el miedo al parasitismo percibido a través de señales acústicas pudiera tener efectos sobre comportamientos defensivos de resistencia (i.e. expulsión de huevo parásito) y/o sobre rasgos de historia vital (asimilable a mecanismos de tolerancia) en urracas (capítulo 5). No encontramos, sin embargo, evidencias que apoyen esta idea en el sistema críalo-urraca puesto que ni el comportamiento de expulsión de huevos ni los rasgos reproductivos de la urraca cambiaron en respuesta a un incremento experimental del riesgo de parasitismo en los nidos.

Por otro lado, existe un interés creciente por conocer los efectos fisiológicos del miedo a la depredación (e.g. Clinchy *et al.* 2013; Sheriff y Thaler 2014; Harris y Carr 2016). Sin embargo, hasta el momento no se han investigado los posibles efectos del riesgo de parasitismo que podrían manifestarse en términos del estado fisiológico de los hospedadores y de su

255

Discusión General

descendencia (**capítulo 6**). Encontramos que el riesgo de parasitismo que experimentaron las urracas en sus nidos no se trasladó en cambios en sus niveles normales de corticosterona o en estimadores del estado inmunológico en su descendencia. No medimos, sin embargo, el efecto más directo del experimento, que sería un efecto sobre los niveles de corticosterona o el sistema inmune en las urracas adultas. Sin embargo, los pollos que crecieron en nidos control tuvieron peores niveles inmunológicos (baja aglutinación) que los crecidos en nidos expuestos a cantos de críalo o de abubilla (un control de "no riesgo"), probablemente como consecuencia de un incremento del cuidado parental en respuesta a las manipulaciones en nidos con cantos.

Finalmente, el humano también puede ser percibido como un potencial depredador, por lo que los animales que se enfrenten a nosotros deben utilizar estrategias similares a las que usarían durante el encuentro con un depredador natural (e.g. Beale y Monaghan 2004). Las alteraciones humanas pueden tener consecuencias ecológicas (e.g. Frid y Dill 2002; Gomes et al. 2008) comportamentales (e.g. Mougeot y Arroyo 2017), y fisiológicas (e.g. Chávez-Zichinelli et al. 2013; Tarjuelo et al. 2015) en las aves. Con el objeto de abordar las consecuencias aplicadas en términos de conservación del estudio del efecto del miedo sobre las aves, evaluamos mediante un estudio correlativo si la actividad humana frecuente puede ser percibida como un agente de riesgo por dos especies de aves insectívoras en declive, la carraca y el autillo (capítulo 7). Ambas especies difieren en sus ritmos de actividad por lo que esperábamos que el carácter nocturno del autillo le hiciera menos vulnerable al impacto de la actividad agrícola que tiene lugar durante el día. Cuantificamos un conjunto de rasgos fisiológicos indicadores de estrés en pollos de ambas especies que habitan en un ecosistema Mediterráneo donde la calidad de hábitat varía dentro de un gradiente de alteración humana. Los resultados muestran que los pollos de carraca criados en cultivos de cereal, donde el laboreo diurno es intenso, estuvieron más estresados a largo plazo, pero que tuvieron mayor peso al vuelo, posiblemente como consecuencia de una mayor disponibilidad alimentaria local. Además, su respuesta al estrés inducido fue mayor en zonas menos antrópicas (más arbustivas). Por su parte, los pollos de autillo criados en áreas altamente urbanizadas y cerca de carreteras importantes, donde la actividad antrópica nocturna es alta, mostraron mayor respuesta al estrés inducido que los criados en zonas menos transitadas en ese periodo. Nuestros resultados sugieren que la susceptibilidad a la perturbación humana puede variar entre especies (e.g. Samia *et al.* 2015), probablemente por efecto de los ritmos de actividad y por la variación de las molestias durante el día que podrían atenuar o exacerbar los efectos fisiológicos (o de otro tipo) sobre las especies.

En resumen, los resultados de esta tesis en conjunto muestran un importante papel del riesgo de depredación y parasitismo de cría en tiempo ecológico sobre las decisiones previas a la reproducción así como efectos del riesgo de depredación percibido durante la reproducción mediante cambios en el cuidado parental. Encontramos también que la actividad humana puede ser percibida como un factor de riesgo modulando el estado fisiológico de especies en declive poblacional como consecuencia de la alteración de sus hábitats. Además nuestros resultados muestran que las aves tienen la capacidad de evaluar distintos tipos de pista que indican riesgo, así como efectos a nivel individual, poblacional y de comunidad del miedo a sufrir depredación o parasitismo de cría.

PERSPECTIVAS FUTURAS

A la luz de los resultados de esta tesis, cabe hacer una serie de consideraciones a tener en cuenta en estudios futuros y que pueden contribuir a profundizar en el conocimiento de la Ecología del Miedo en las aves:

1. De nuestros resultados (**capítulo 3**) se desprende la importancia de estudiar la selección de hábitat de nidificación por los hospedadores mediante el estudio de individuos marcados, con un historial conocido de su interacción con el parásito (Molina-Morales 2014), ya que los patrones actuales de selección de hábitat pueden reflejar el efecto del parasitismo en el pasado.

2. La ausencia de evidencia clara de un efecto del miedo al parasitismo de cría sobre rasgos reproductivos y fisiológicos en el sistema críalo-urraca (capítulos 5 y 6), sugeriría que esa posibilidad debería examinarse en otras poblaciones o sistemas parásito de cría-hospedador. En concreto, el alto porcentaje de parasitismo existente en nuestra población de estudio (Molina-Morales et al. 2013) puede ser el motivo de la ausencia de respuesta al riesgo durante la reproducción. Para un entendimiento más amplio de los posibles efectos del riesgo de parasitismo sobre los hospedadores, sería deseable estudiar las consecuencias del miedo al parasitismo en poblaciones con niveles más bajos de parasitismo, o que varíen temporalmente en su porcentaje de parasitismo. También sería interesante testar esta posibilidad sobre hospedadores con una historia evolutiva reciente con el parásito o en sistemas en los que el parasitismo no suponga el fallo completo del evento reproductivo, ya que, al compartir el nido con el parásito, existe un margen mayor para minimizar los costes del parasitismo a través de mecanismos de tolerancia (i.e. cambios comportamentales y/o de rasgos de historia vital).

3. El estudio de los efectos fisiológicos del riesgo de depredación y parasitismo de cría mediante una integración de diferentes medidas fisiológicas puede aportar grandes avances en la Ecología del Miedo (revisado en Sheriff *et al.* 2011; Zanette *et al.* 2014). Además, para estudiar los posibles efectos trans-generacionales del riesgo, sugerimos la toma de medidas fisiológicas tanto en adultos como en pollos.

4. Los resultados de la tesis en su conjunto sugieren que el estudio simultáneo de múltiples pistas que indican riesgo controlando por su persistencia temporal podría ofrecer una compresión mucho más profunda de los efectos del miedo puesto que las aves tienen la capacidad de evaluarlo en base a pistas de muy diversa naturaleza (i.e. percibidas a través de distintos canales de comunicación). En particular, el estudio controlado de los efectos de múltiples pistas sobre las aves cuando ofrecen información contradictoria podría proporcionar información única sobre el valor relativo de las distintas pistas.

5. Estudios a nivel comparativo que investiguen variaciones en la sensibilidad y respuesta al riesgo en grupos amplios de especies mediante el estudio de diferentes rasgos comportamentales, reproductivos y fisiológicos, son de gran importancia y pueden aportar evidencias sobre la evolución de distintos rasgos entre las especies (Ghalambor y Martin 2002; Ghalambor *et al.* 2013).

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Conclusiones/ Conclusions

CONCLUSIONES

1. La información olfativa sobre el riesgo de depredación puede afectar a la selección de territorios de reproducción en aves y tener consecuencias ecológicas alterando la abundancia, composición y fenología de las comunidades de las que las aves forman parte.

2. Dentro de una comunidad, las llamadas de alarma emitidas por una especie son percibidas e interpretadas en función de las diferencias en el nivel trófico entre emisor y receptor, de forma que la información es tanto más valiosa cuando el nivel de solapamiento entre las especies que interactúan es mayor. Además, el valor de la información sobre el riesgo proporcionado por esas llamadas está modulado por los niveles de competencia interespecífica por recursos clave como el nido.

3. Las urracas utilizan información sobre el riesgo de parasitismo que estiman en base a señales acústicas y visuales durante la elección de sus territorios de reproducción. No obstante, la importancia de la información sobre el riesgo presente está condicionada por las experiencias pasadas de parasitismo que ha sufrido un individuo.

4. En una de las especies estudiadas, la carraca, encontramos que el riesgo de depredación de nidos afecta al comportamiento parental durante la reproducción, lo que podrían tener consecuencias en términos de fitness, y que la sensibilidad a dicho riesgo podría diferir entre los dos sexos.

5. No encontramos evidencias de que el miedo a sufrir parasitismo de puesta con posterioridad al inicio de la reproducción influenciara el comportamiento, los rasgos de historia vital o la fisiología de los pollos de un hospedador del críalo europeo en una población con una elevada presión de parasitismo.

6. La alteración del hábitat por la actividad humana puede ser percibida como un factor de riesgo que puede generar cambios en el estado fisiológico de las especies silvestres, como mostraron nuestros resultados en dos especies de aves en declive poblacional. Además, la susceptibilidad a la perturbación humana puede variar entre especies, probablemente por efecto de los ritmos de actividad y por la variación de las molestias día lo largo del ciclo diario, lo que podría atenuar o exacerbar los efectos fisiológicos (o de otro tipo) sobre las especies.

7. Los resultados conjuntos de esta tesis sugieren que, el riesgo de depredación y parasitismo de cría juegan un importante papel modulador de las estrategias previas a la reproducción así como de rasgos comportamentales, reproductivos y fisiológicos en las aves.

8. Por último, nuestros resultados muestran que, la evaluación del riesgo de depredación y parasitismo es un proceso multifacético en el que las especies integran la información sobre el riesgo que perciben a través de distintos canales de información, con el fin último de reducir los efectos de dichas presiones selectivas.

CONCLUSIONS

1. The olfactory cues informing of risk of predation can affect the selection of breeding habitats and nest sites in birds and have ecological consequences by altering the abundance, composition and phenology of bird communities.

2. Within a community, alarm calls broadcasted by a species are perceived and interpreted based on differences in the trophic level between caller and receiver, so that information is all the more valuable when the level of overlap amog interacting species is greater. In addition, the value of the information about the risk provided by these calls is modulated by the levels of interspecific competition for key resources such as the nest.

3. Magpies use acoustic and visual cues informing of brood parasitism risk when choosing breeding habitats and nest sites. However, the importance of information about the current risk is conditioned by the past experiences of cuckoo parasitism suffered by an individual.

4. Risk of nest predation affects the parental behavior of European roller during reproduction, which could have consequences in terms of fitness. Furthermore, the sensitivity to risk could differ between parental sexes in this species.

5. We found no evidence that the fear of suffering cuckoo parasitism after the beginning of reproduction influenced the behavior, life-history traits or physiological status of magpie nestlings in a population exposed to high levels of cuckoo parasitism.

6. Habitat alteration caused by human activity can be perceived as a risk factor through changes in the physiological status of wild species, as our results showed for two insectivorous bird species under population decline. In addition, the susceptibility to human disturbance may vary between

species, probably due to the effect of the activity rhythms and the variation of the disturbances during the day, which could attenuate or exacerbate the physiological (or other) effects.

7. Overall, our results suggest that predation risk and inter-specific brood parasitism risk might play an important role modulating pre-breeding strategies as well as modifying behavioral, reproductive and physiological traits in birds.

8. Finally, our results show that the evaluation of predation and interspecific brood parasitism risk constitute a multifaceted process. During this process, the species integrate the information about the risk perceived through different information channels with the ultimate goal of reducing the effects of these selective pressures.

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