Costes reproductivos y de supervivencia del parasitismo de cría: un estudio a largo plazo

Marta Precioso Sáenz

Tesis Doctoral

Programa de Doctorado en Biología Fundamental y de Sistemas

UNIVERSIDAD DE GRANADA

UNIVERSIDAD
DE GRANADA

Facultad de Ciencias

Departamento de Zoología

Costes reproductivos y de supervivencia del parasitismo de cría: un estudio a largo plazo

Marta Precioso Sáenz

Tesis Doctoral

Granada, 2021

Editor: Universidad de Granada. Tesis Doctorales
Autor: Marta Precioso Sáenz
ISBN: 978-84-1306-973-9
URI: http://hdl.handle.net/10481/69873

Agradecimientos institucionales

La investigación que ha conducido a esta Tesis doctoral se enmarca dentro del proyecto "Patrones de parasitismo a lo largo de la vida y longevidad en urracas." (CGL2014-55362-P). También se ha contado con la financiación de un contrato predoctoral para la formación de doctores (BES-2015-075675).

Esta tesis se ha realizado en el Programa de Doctorado "Biología Fundamental y de Sistemas".

A mi madre

Índice

Resumen / Abstract

Resumen

Los parásitos de cría reducen el éxito reproductivo de sus hospedadores, por lo que ejercen en ellos fuertes presiones selectivas que favorecen la evolución de defensas para evitar el parasitismo. Esto, a su vez, selecciona contra-adaptaciones en los parásitos, por lo que parásitos y hospedadores se ven inmersos en una carrera de armamentos coevolutiva. La magnitud de los costes del parasitismo difiere en los distintos sistemas parásito-hospedador, y una estima precisa de estos costes debería considerar el efecto del parasitismo sobre el éxito reproductivo de los hospedadores a lo largo de toda su vida. Además, el parasitismo podría conllevar costes o consecuencias diferentes a las directamente relacionadas con la disminución en el éxito reproductivo de las puestas parasitadas, también denominados costes extra, como por ejemplo consecuencias sobre la supervivencia de los hospedadores, que podrían manifestarse a lo largo de la vida de los individuos. A pesar de ello, en casi ningún sistema se han explorado los costes del parasitismo a lo largo de la vida de los hospedadores, ni cómo estos costes se traducen en pérdidas en el éxito reproductivo total. La mayoría de estudios se han centrado en estimar los costes a corto plazo, por lo que, por lo general, no se conocen los costes reales que el parasitismo supone para la mayoría de los hospedadores, y por lo tanto, tampoco se conoce la verdadera magnitud de la presión que los parásitos ejercen sobre la evolución de sus defensas.

Esta tesis tiene como objetivo explorar costes o consecuencias del parasitismo a corto y medio plazo en el sistema formado por el críalo europeo (*Clamator glandarius*) y la urraca (*Pica pica*), su principal hospedador en Europa, determinando los costes extra y las consecuencias del parasitismo sobre la longevidad de las urracas y su éxito reproductivo a lo largo de la vida. En este sistema se han documentado los efectos negativos del parasitismo sobre el éxito reproductivo del hospedador y la existencia de mecanismos de defensa, como el reconocimiento y rechazo de huevos parásitos, pero hasta ahora no se han determinado los costes que supone el parasitismo a lo largo de la vida de los hospedadores.

Por un lado, se han estimado los costes reproductivos del parasitismo a corto plazo a través de dos aproximaciones diferentes: una aproximación comportamental y correlacional, y una aproximación molecular y experimental, utilizando la dinámica telomérica como biomarcador de los costes reproductivos (y del parasitismo). Por otro lado, se ha explorado la posible asociación del parasitismo con las trayectorias de supervivencia, la mortalidad y la longevidad de los hospedadores, a través de un estudio longitudinal con individuos marcados que han

sido monitorizados a lo largo de su vida y que fueron parasitados alguna vez o nunca durante sus eventos reproductivos.

Los resultados de esta tesis sugieren que, a corto-medio plazo, el parasitismo no supone un coste extra para los hospedadores comparado con el esfuerzo reproductivo invertido en criar nidadas no parasitadas. De hecho, es probable que en la mayoría de casos suponga un coste menor. También se pone de manifiesto que el parasitismo se traduce en un menor acortamiento de los telómeros en algunos individuos, probablemente los más jóvenes, lo cual podría relacionarse con una mayor probabilidad de supervivencia y longevidad en individuos parasitados en comparación con aquellos que no son nunca parasitados.

Además los resultados sugieren que estos costes a corto-medio plazo tienen consecuencias a largo plazo sobre la esperanza de vida y la longevidad de los hospedadores, ya que los análisis de las trayectoria de supervivencia y mortalidad muestran que los individuos que son parasitados al menos una vez a lo largo de su vida viven más tiempo que los individuos que no son nunca parasitados debido a que sufren una menor tasa de mortalidad.

Estas diferencias en longevidad entre individuos con distinto status respecto al parasitismo se traducen en que los individuos que son alguna vez parasitados tienen el mismo éxito reproductivo a lo largo de su vida que los individuos que no son nunca parasitados. Los individuos parasitados que viven más años son capaces de compensar las pérdidas de éxito reproductivo de los eventos reproductivos parasitados. Esto podría deberse no solo a un mayor número de intentos de cría, sino estar además mediado por el desarrollo del principal mecanismo de defensa frente al parasitismo en este hospedador (el reconocimiento y rechazo de huevos parásitos), que aparece a edades tardías.

Abstract

Brood parasitism reduces the reproductive success of their hosts, therefore exerting a strong selective pressure which favours the evolution of defences to avoid parasitism. This, in turn, selects for counter-adaptations in parasites, and thus parasites and hosts are immersed in a coevolutionary arms race. The extent of the costs of parasitism differs in distinct host-parasite systems, and an exact estimation of these costs should consider the effects of parasitism on the reproductive success of the hosts across their lifetime (LRS, lifetime reproductive success). Furthermore, brood parasitism could entail costs or consequences distinct to those directly related to the reduction of breeding success in the parasitized broods, also known as extra costs, such as consequences for host survival, which could manifest through the lifetime of individuals. Despite this, the lifetime costs of parasitism have not been explored in most of the systems and the consequences of these costs in terms of overall reproductive success have not been explored. The majority of studies have focused on estimating the short term costs and therefore, in general, we don't know the true costs of brood parasitism for the majority of hosts and thus we lack a precise estimation of the magnitude of the pressure that parasites exert on the evolution hosts' defences.

This thesis aims to explore short and mid-term costs or consequences of brood parasitism on the system formed by the great spotted cuckoo (*Clamator glandarius*) and the magpie (*Pica pica*), its main host in Europe, determining its extra costs (not directly related with the decrease of reproductive success) and the its effect on the longevity of magpies and their LRS. The negative effects of brood parasitism on the reproductive success of this host and the existence of defence mechanisms such as recognition and rejection of parasitic eggs have been documented in this system, but up to now, the lifetime costs of brood parasitism for hosts has not yet been determined.

On one hand, the short-term costs of brood parasitism have been estimated *via* two different approaches: a) a behavioural and correlational approach, and b) a molecular and experimental approach using telomere dynamics as a biomarker of the reproductive (and parasitism) costs. On the other hand, the possible association of parasitism with survival and mortality trajectories, and longevity of the hosts has been explored through a longitudinal study with marked individuals which have been monitored through their lives and which were either never or sometimes parasitized during their reproductive events.

The results of this thesis suggest that parasitism does not represent an extra cost for the hosts in the short-to-mid-term, compared to the reproductive effort

invested in raising non-parasitized broods. In fact, it is probable that in most of cases parasitism represents a smaller cost than a non-parasitized brood. It also brings to light that parasitism results in reduced telomere shortening in some (probably younger) individuals, which could be related to a greater probability of survival and longevity in parasitized individuals compared to those which are never parasitized.

It is suggested that these short -term costs have long-term consequences on life expectancy and host longevity given that the analysis of survival trajectories and mortality shows that those individuals which were parasitized at least once through their lives live longer than those individuals which were never parasitized, as they suffered a lower mortality rate.

These differences in longevity amongst individuals with distinct parasitism status imply that individuals sometimes parasitized have a similar LRS than those individuals never been parasitized during their lives. Parasitized individuals who live longer are able to compensate the loss of reproductive success from parasitized reproductive events. This maybe a consequence of a larger number of breeding events but could also be mediated by the development of this host's main defence against brood parasitism (recognition and rejection of parasitic eggs), which appears at older ages.

Introducción general

Introducción general

El parasitismo es un tipo de interacción biológica entre dos organismos en la que uno de ellos, el parásito, depende de otro, el hospedador, para sobrevivir y /o reproducirse, y esta interacción resulta en un perjuicio de mayor o menor entidad al hospedador. Dentro de este tipo de interacción se incluye una forma particular de estrategia reproductiva, en la que algunos individuos, los parásitos, consiguen que otros, los hospedadores, se encarguen de los cuidados parentales de la descendencia parásita, lo que se denomina parasitismo de cría. Los parásitos de cría reducen el éxito reproductivo de sus hospedadores y a su vez dependen de ellos para reproducirse, por lo que los rasgos de ambos coevolucionan como consecuencia de presiones selectivas recíprocas (Rothstein 1990, Davies 2000).

El parasitismo de cría ha evolucionado en diversos grupos animales, como en las Clases Aves (Payne y Sorensen 2005, Davies 2000) y Actinopterygii (Taborsky 2001, Blažek et al. 2018) del Filo Chordata, o en las Clases Insecta (González-Megías y Sánchez-Piñero 2003, Huang y Dornhaus 2008, Lhomme y Hines 2019) y Arachnida (Boulton y Polis 2002) del Filo Arthropoda; pero es probablemente en el grupo de las aves y en el de los insectos donde mejor se ha estudiado la interacción entre los parásitos de cría y sus hospedadores (Kilner y Langmore 2011). El parasitismo de cría puede ser intra-específico (o conespecífico), cuando el parásito y el hospedador pertenecen a la misma especie, o inter-específico, cuando pertenecen a distintas especies. Por otra parte, el parasitismo puede ser facultativo si el parásito además puede reproducirse de forma independiente, proveyendo de cuidados parentales a una parte de su descendencia pero no a otra parte; u obligado, si el parásito no cuida nunca de su propia descendencia.

Los parásitos de cría aviares ponen sus huevos en los nidos de sus hospedadores y éstos se encargan de la incubación de los huevos y del cuidado de los pollos parásitos hasta su independencia. El parasitismo de cría facultativo e intra-especifico se ha descrito en unas 256 especies de aves (Yom-Tov y Geffen 2017) mientras que el facultativo inter-especifico se ha descrito en unas 64-68 especies (Mann 2017). El parasitismo de cría inter-especifico obligado, por otro lado, está presente en 109 especies de aves repartidas por todo el mundo (Mann 2017) y ha evolucionado independientemente al menos siete veces en este grupo (Sorenson y Payne 2002, 2005): tres veces dentro del Orden Cuculiformes (Familia

Cuculidae; 65 especies); dos veces en el Orden Paseriformes: en la Familia Viduidae (20 especies) y en la Familia Icteridae (6 especies); una vez en el Orden Piciformes (Familia Indicatoridae; 17 especies); y una vez en el Orden Anseriformes (Familia Anatidae; 1 especie).

Los parásitos de cría obligados pueden ser generalistas, si parasitan a distintas especies hospedadoras (por ejemplo, el tordo cabeci-café (*Molothrus ater*) puede parasitar a más de 200 especies hospedadoras (Davies 2000)) o especialistas, si parasitan sólo a una especie. Incluso en algunos parásitos generalistas como el cuco común (*Cuculus canorus*) ha evolucionado el uso preferencial por parte de las hembras de una especie particular de hospedador, apareciendo así las denominadas "razas" o "gentes" que parasitan casi exclusivamente a una determinada especie (Davies 2000). Es en los sistemas en los que existe un parasitismo más específico donde la relación evolutiva entre parásito y hospedador va a ser más estrecha.

Interacciones ecológicas entre parásitos de cría y hospedadores

Los parásitos de cría reducen el éxito reproductivo de sus hospedadores en los eventos reproductivos parasitados de diferentes formas. En algunas especies parásitas, como el cuco común, la hembra retira un huevo del hospedador cuando pone su propio huevo (Davies 2000), mientras que en otras especies las hembras parásitas pueden depredar, picar o romper varios huevos del hospedador, como los tordos americanos del Género *Molothrus*, (Massoni y Reboreda 2002), o el críalo europeo, *Clamator glandarius* (Soler et al. 1997)); en ocasiones los huevos del hospedador pueden resultar dañados durante la puesta de los huevos parásitos, que a veces las hembras dejan caer sobre la puesta hospedadora (López et al. 2018), gracias a que a menudo los huevos parásitos tienen una cáscara especialmente resistente (Igic et al. 2011, Soler et al. 2019). Por otro lado, los huevos parásitos suelen ser de pequeño tamaño comparado con el tamaño corporal de los parásitos adultos (Krüger y Davies 2002, Krüger y Davies 2004) y tener un periodo de incubación menor que los de sus hospedadores (Payne 2005), por lo que los huevos parásitos eclosionan antes, reduciendo así el éxito de eclosión de los hospedadores. En algunos casos las hembras hospedadoras dejan de incubar cuando eclosionan los parásitos y en otros casos las especies parásitas han desarrollado diferentes mecanismos para eliminar la competencia con los pollos hospedadores. En la mayoría de las especies de cucos, por ejemplo, los pollos de

cuco recién eclosionados son capaces de expulsar cualquier otro huevo o pollo que se encuentre en el nido (Davies 2000, Payne 2005). En otras especies parásitas, como el cuclillo listado (*Tapera Naevia*) o los Indicadores de la miel del Género *Indicator*, los pollos recién eclosionados poseen unos ganchos en el pico con los que laceran y matan a los otros pollos que se encuentran en el nido (Davies 2000, Spottiswoode y Koorevaar 2012). Sin embargo, algunas especies parásitas no han desarrollado este tipo de adaptaciones y los pollos de parásitos y hospedadores pueden crecer juntos (cucos de los Géneros *Clamator* y *Scythrops* y algunas especies del Género *Eudynamys*, los tordos americanos del Género *Molothrus* o los miembros de la Familia *Viduidae*), aunque a veces los pollos parásitos poseen adaptaciones que los hacen más eficientes en monopolizar los cuidados parentales (Soler et al. 1995a, Tanaka y Ueda 2005) y los pollos hospedadores pueden llegar a morir de inanición (Davies 2000, Soler y Soler 1991).

Interacciones evolutivas entre parásitos de cría y hospedadores

La reducción del éxito reproductivo a causa del parasitismo ejerce importantes presiones selectivas sobre los hospedadores, por lo que la selección natural ha favorecido la aparición de adaptaciones en los hospedadores para evitar el parasitismo, que a su vez selecciona contra-adaptaciones en los parásitos, dando lugar a una carrera armamentística coevolutiva (Rothstein 1990). Diferentes rasgos de las historias vitales de parásitos y hospedadores se pueden modificar debido a las interacciones entre ambos. Esto, unido a la gran variedad de estrategias y adaptaciones que presentan los diferentes parásitos y sus hospedadores (Tabla 1), hacen de este tipo de sistemas un modelo excepcional para estudiar las interacciones coevolutivas (Soler 2014).

Las adaptaciones consecuencia de esta carrera coevolutiva aparecen en distintos momentos del ciclo reproductivo de parásitos y hospedadores. Antes de la puesta de los huevos los parásitos buscan y, posiblemente, seleccionan los nidos que van a parasitar. El emplazamiento y la conspicuidad de los nidos pueden favorecer que los parásitos los encuentren más fácilmente, por lo que habrá una presión selectiva para que los hospedadores construyan nidos de menor tamaño (Soler et al. 1999), que los oculten mejor (Muñoz et al. 2007) y estén alejados de posibles perchas utilizadas por los parásitos para observar a los hospedadores

(Moskát y Honza 2000, Antonov et al. 2007) o de zonas con mayor riesgo de parasitismo (Expósito-Granados et al. 2017).

La segunda línea defensiva de los hospedadores es la defensa activa del nido frente a los parásitos expulsándolos o atacándolos (Røskaft et al. 2002), reduciendo, en algunos casos, la probabilidad de ser parasitados (Welbergen y Davies 2009, Fiorini et al. 2009) o los efectos negativos del parasitismo (Gloag et al. 2013) y puede ser muy específica frente a los parásitos (Davies et al. 2003, Požgayová et al. 2009, Campobello y Sealy 2010). Los parásitos entonces tratarán de evitar a los hospedadores siendo más sigilosos (Honza et al. 2002), desarrollando tácticas de distracción mientras la hembra parásita pone los huevos (Davies 2000), o desarrollando un plumaje críptico (Krüger et al. 2007) o mimético al de algunos depredadores (Davies y Welbergen 2008, Gluckman y Mundy 2013), lo que previene del ataque de los hospedadores (Welbergen y Davies 2011). Otras estrategias que presentan los hospedadores que pueden reducir la probabilidad de ser parasitados pueden incluir criar en zonas con mayor densidad de conspecíficos, beneficiándose de un efecto dilución (Martínez et al. 1996, Massoni y Reboreda 2001) y/o de una defensa colectiva (Welbergen y Davies 2009), o sincronizar las fechas de puesta (Martínez et al. 1996).

La siguiente línea de defensa y, probablemente la mejor documentada, ocurre durante la fase de la puesta de los huevos y es el reconocimiento y rechazo de los huevos parásitos por parte de los hospedadores (Rothstein 1990). Los hospedadores que son capaces de reconocer los huevos extraños pueden expulsarlos del nido, enterrarlos dentro del nido o incluso abandonar toda la puesta (Davies 2000). La capacidad de reconocimiento de huevos parásitos puede estar basada en un proceso de aprendizaje (Rothstein 1975, Lotem et al. 1995) o en la detección de la discordancia entre los huevos parásitos y los del hospedador (Rothstein 1975, Marchetti 2000) o en ambos (Moskát et al. 2010, Stevens et al. 2013, Bán et al. 2013). Posteriormente se propuso la hipótesis del proceso de aprendizaje prolongado que propone que el aprendizaje se lleva a cabo tras varios intentos de cría (Stokke et al. 2007). Algunos parásitos, como el cuco común, han respondido a esta adaptación desarrollando una apariencia mimética de sus huevos (Brooke y Davies 1988). A su vez, en respuesta al mimetismo de huevos parásitos en algunos hospedadores se ha seleccionado la reducción de la variabilidad intra-puesta de la apariencia de sus huevos para facilitar el reconocimiento de huevos extraños (Stokke et al. 1999) lo que ha dado lugar a un aumento de la variabilidad

inter-puesta (Stokke et al. 2002), que dificulta el mimetismo de los huevos parásitos.

La carrera coevolutiva continúa también en otros estadios del ciclo reproductivo existiendo ejemplos de reconocimiento y rechazo de pollos parásitos por parte de los hospedadores (Grim 2011), con la consecuente evolución del mimetismo de determinados rasgos de los pollos hospedadores por parte de los pollos parásitos (Langmore et al. 2003, Hauber y Kilner 2007, De Mársico et al. 2012). Las principales adaptaciones y contra-adaptaciones descritas en los diferentes parásitos de cría y sus hospedadores están resumidas en la Tabla 1 (modificada de Davies (2011)).

Sin embargo, se ha observado que algunos hospedadores aparentemente carecen de defensas frente a los parásitos. Por ejemplo, en alrededor de un 39% de las especies hospedadoras conocidas no existe rechazo de huevos parásitos (Soler 2014). Pero además, en algunas poblaciones donde algunos individuos rechazan huevos parásitos, éste comportamiento no se generaliza en la población, a pesar de la gran ventaja que supone.

Es en este punto donde la carrera de armamentos coevolutiva no es suficiente para explicar la ausencia o la magnitud reducida de las defensas de determinados hospedadores, y surgen otras hipótesis evolutivas que tratan de hacerlo. La hipótesis del retraso evolutivo propone que parásitos y hospedadores no han pasado el suficiente tiempo interaccionando para que, o bien surjan variantes genéticas responsables de los mecanismos de defensa o bien éstas se expandan (Rothstein 1975, Davies y Brooke 1988, Hosoi y Rothstein 2000). Sin embargo, esta teoría es difícil de confirmar, y podría simplemente ser un paso previo a la carrera de armamentos coevolutiva (Stokke et al. 2005). Por otro lado, la hipótesis de la transmisión horizontal limitada propone que no todos los individuos de una población tienen la misma probabilidad de ser parasitados, por lo que sólo una parte de ellos sufrirá las presiones selectivas del parasitismo (Hauber et al. 2004a, Hoover et al. 2006, Molina-Morales et al. 2013). Por último, la hipótesis del equilibrio evolutivo plantea que existe un balance entre los costes y beneficios de aceptar y rechazar el parasitismo, y que en determinadas circunstancias los costes de rechazar el parasitismo pueden exceder los costes de aceptarlo (Rohwer y Spaw 1988, Lotem et al. 1992, Avilés et al. 2005, Kruger et al. 2011).

Tabla 1: Adaptaciones y contra-adaptaciones de parásitos de cría y sus hospedadores (modificado de Davies (2011)).

Línea de defensa	**Defensa del hospedador**	**Respuesta del parásito**
Antes de la reproducción	Nidificación lejos de perchas que pueden utilizar los parásitos	Monitoreo de hospedadores
	Nidos crípticos	
	Nidos falsos	
	Puesta impredecible	
	Comportamiento sigiloso	
Defensa del nido	Aumento de la vigilancia y expulsión y ataque a los parásitos	Comportamiento sigiloso
		Mimetismo con depredadores
	Anidar donde hay mayor densidad de conespecíficos/ o en colonias	Polimorfismos en color
		Distracción de los hospedadores mientras la hembra parasita
	Construcción de estructuras en los nidos que dificultan el parasitismo	Selección cuidadosa de los nidos
		Menor tamaño corporal
Rechazo de huevos	Deserción si hay parásitos	Puesta sigilosa y rápida
	Expulsión de huevos extraños	Mimetismo de los huevos del hospedador
		Huevos crípticos
		Huevos atractivos
		Huevos más resistentes
		Destrucción de la puesta si el hospedador rechaza el huevo parásito
	Modificación de la variabilidad de la puesta	Mimetismo de las modificaciones
		Elección de hospedadores con huevos silimares
Rechazo de pollos	Expulsión de pollos parásitos	Mimetismo de los pollos del hospedador
	Modificación de las características de los pollos	Mimetismo de las modificaciones
		Utilización de señales manipulativas para explotar a los hospedadores
Cualquier momento	Variación en la intensidad de las defensas dependiendo del riesgo de parasitismo	Aumento del comportamiento sigiloso
	Confianza en pistas que sugieran parasitismo	

Costes del parasitismo a corto/medio plazo: esfuerzo reproductivo

Además de la evidente reducción o pérdida de éxito reproductivo en los eventos parasitados, existen otros potenciales costes o consecuencias asociados al parasitismo que han recibido menos atención y que pueden tener una gran importancia en las interacciones entre parásitos de cría y sus hospedadores. El parasitismo puede acarrear efectos a corto y largo plazo en los hospedadores y afectar diferentes rasgos de sus historias vitales (Krüger 2007).

El parasitismo de cría puede promover cambios en el esfuerzo reproductivo invertido por los hospedadores: por ejemplo, una especie de tordo americano (el tordo cabecicafé (*Molothrus ater*)) es capaz de causar un aumento en las tasas de aprovisionamiento de sus hospedadores (Dearborn et al. 1998, Hoover y Reetz 2006, Grayson et al. 2013); mientras que otros parásitos inducen una reducción de las tasas de aprovisionamiento de los nidos en comparación con las tasas de aprovisionamiento a nidadas no parasitadas (Soler et al. 1995b, Požgayová et al. 2015, Samaš et al. 2018). Sin embargo, estos cambios en el esfuerzo reproductivo podrían estar mediados por el efecto del parasitismo sobre los tamaños de nidada: en el primer caso, los pollos parásitos son criados junto a pollos hospedadores y el tamaño de nidada es mayor en nidos parasitados que en nidos no parasitados; por el contrario, en el segundo caso, los pollos parásitos son criados de forma general solos en el nido (Davies 2000), con lo que el tamaño de nidada es mucho menor en nidos parasitados.

De forma similar, el parasitismo también es capaz de inducir cambios en la contribución relativa de machos y hembras, al menos en especies poligínicas. Así, los machos de carricero tordal (*Acrocephalus arundinaceus*) reducen sus tasas de visitas en nidos parasitados por el cuco común en comparación con los nidos no parasitados (Požgayová et al. 2015); pero, por otro lado, los nidos de tordos alirrojos (*Agelaius phoeniceus*) parasitados por tordos cabecicafé tienen mayor probabilidad de recibir cuidado por parte de los machos que los nidos no parasitados (Grayson et al. 2013). No obstante, se conoce muy poco acerca de cómo el parasitismo podría afectar a las estrategias vitales de hembras y machos en la mayoría de las especies hospedadoras.

Se ha mostrado que los cambios en la inversión parental de los hospedadores debidos al parasitismo pueden modificar los costes de la

reproducción y acarrear efectos a más largo plazo en su reproducción futura y en sus perspectivas de supervivencia (Reid et al. 2003, Reed et al. 2008, Bouwhuis et al. 2010). Sin embargo, se sabe muy poco de estos efectos a largo plazo, y la mayoría de estudios que abordan este tema se han centrado en el análisis de la supervivencia de los hospedadores al año siguiente de ser parasitados. La mayoría de ellos sugieren que el parasitismo no tiene un efecto en la supervivencia de los hospedadores (Brooker y Brooker 1996, Payne y Payne 1998, Hoover 2003, Hauber 2006, Samaš et al. 2019) o en su reproducción futura (Hauber 2006), aunque también se ha encontrado, en menor número, un efecto negativo (Hoover y Reetz 2006, Mark y Rubenstein 2013, Koleček et al. 2015). Una carencia que presentan estos estudios es que no tienen en cuenta que la inversión energética asociada al esfuerzo reproductivo y los costes de la reproducción pueden tener efectos acumulativos a más largo plazo que no sean aparentes en la supervivencia (o en otros rasgos) de un año para otro (Boonekamp et al. 2014b, 2020), por lo que para detectarlos se hace imprescindible utilizar una aproximación longitudinal a nivel individual.

Hasta la fecha son pocos los estudios que han profundizado realmente en las consecuencias del parasitismo a lo largo de la vida de los hospedadores por lo que, por lo general se tiene un visión muy sesgada de los verdaderos costes del parasitismo. La mayoría de los estudios, como hemos visto, se centran en efectos a corto-medio plazo, a pesar de que algunos hospedadores pueden llegar a ser relativamente longevos (Krüger 2007).

Costes del parasitismo a largo plazo: esperanza de vida de los hospedadores y éxito reproductivo a lo largo de la vida

La magnitud de los costes del parasitismo a lo largo de la vida de los hospedadores es lo que realmente van a determinar la intensidad de la presión selectiva que los parásitos ejercen sobre el desarrollo de defensas en los hospedadores y lo que nos permitirán entender la evolución de adaptaciones y contra-adaptaciones, consecuencia de la dinámica coevolutiva derivada de su interacción ecológica. Este tipo de trabajos conllevan la necesidad de estudiar rasgos de historia vital de los hospedadores lo que implica monitorizar a los individuos a lo largo de su vida y

determinar cómo de frecuentemente son parasitados, por lo que son muy difíciles de llevar a cabo, y probablemente por ello muy escasos.

Uno de los rasgos de las historias vitales de los hospedadores que ha recibido muy poca atención en el estudio de estas interacciones es la esperanza de vida, un rasgo comúnmente asociado al éxito reproductivo (Vedder y Bouwhuis 2018). La esperanza de vida y/o la mortalidad, está determinada por factores extrínsecos, como la depredación, condiciones ambientales adversas o enfermedades infecciosas, y por factores intrínsecos, es decir, el declive fisiológico asociado a la edad y en definitiva, con el envejecimiento (Carnes y Olshansky 1997). La mortalidad debida a factores extrínsecos es lo que se conoce como mortalidad basal, y la mortalidad asociada al envejecimiento es lo que se conoce como senescencia actuarial (McDonald et al. 1996). El parasitismo de cría podría afectar estos dos componentes de la mortalidad de los hospedadores modificando así la su esperanza de vida.

Por un lado, como ya se ha mencionado anteriormente, el parasitismo puede modificar el tamaño de nidada, las tasas de aprovisionamiento de los nidos y, en definitiva, el esfuerzo reproductivo de los hospedadores. Estos cambios pueden modificar la mortalidad basal de los hospedadores si modifican, por ejemplo, el riesgo de depredación de las nidadas parasitadas y de los adultos al defenderlas frente a los depredadores: los depredadores utilizan las tasas de visitas a los nidos como una pista para encontrarlos y los nidos con más actividad (más visitas) tienen mayor probabilidad de ser depredados (Martin et al. 2000). Por lo tanto, cuando el parasitismo aumenta el tamaño de nidada y las tasas de visitas a los nidos, puede aumentar también su riesgo de depredación (por ejemplo, en especies en que los pollos parásitos y de hospedadores son criados juntos (Dearborn 1999, Burhans et al. 2002, Hannon et al. 2009)); mientras que en los nidos en los que el parasitismo reduce el tamaño de nidada y las tasas de visitas al nido (como en algunas especies en que los parásitos son criados solos), el riesgo de depredación podría verse reducido.

Por otro lado, el parasitismo también podría modificar la mortalidad basal de los hospedadores si el parasitismo no ocurre al azar. En diferentes sistemas se ha observado que no todos los individuos de una misma población tienen la misma probabilidad de ser parasitados (Hauber et al. 2004, Hoover et al. 2006, Molina-Morales et al. 2013) o que los parásitos podrían escoger a sus hospedadores en base a, por ejemplo, su calidad parental (Soler et al. 1995a) o su edad (Smith et al.

1984, Brooker y Brooker 1996). Si alguna de las características seleccionadas por los parásitos está correlacionada con la mortalidad basal de los hospedadores, éstos podrían promover cambios en su esperanza de vida.

Además, los cambios en el esfuerzo reproductivo de los hospedadores debidos al parasitismo pueden provocar cambios en sus tasas de senescencia actuarial, modificando así también su esperanza de vida. Recientemente, Boonekamp y colaboradores (2014, 2020) han demostrado experimentalmente que un aumento en el esfuerzo reproductivo aumenta la senescencia actuarial de las grajillas (*Coloeus monedula*). Si esto ocurriese de manera general, se podría esperar que los cambios en el esfuerzo reproductivo inducidos por el parasitismo promovieran cambios en las tasas de senescencia actuarial de los hospedadores, y la dirección de esos cambios dependería de si criar nidadas parasitadas supusiera un coste extra o un coste menor en comparación con criar nidadas no parasitadas. Pero además, como se ha sugerido anteriormente, el efecto de estos cambios podría ser acumulativo (Boonekamp et al. 2014b, 2020), por lo que sería necesario analizarlo a lo largo de la vida de los hospedadores.

La modificación de la longevidad de los hospedadores debida al parasitismo también podría tener un efecto sobre su éxito reproductivo a lo largo de la vida: si, por ejemplo, el parasitismo aumentara la esperanza de vida de los hospedadores, éstos podrían tener más oportunidades de escapar del parasitismo. Esto sería particularmente relevante en hospedadores en los que los mecanismos de defensa (como el rechazo de huevos parásitos) se desarrollen con la edad (Rothstein 1978, Hauber et al. 2004b, Molina-Morales et al. 2014, Martínez et al. 2020). Sin embargo hasta la fecha, sólo un estudio ha evaluado los costes del parasitismo en el éxito reproductivo a lo largo de la vida de un hospedador, encontrando que las hembras de maluro espléndido (*Malurus splendedens*) parasitadas por el cuclillo de Horsfield (*Chrysococcyx basalis*) tienen el mismo éxito reproductivo a lo largo de su vida que las hembras nunca parasitadas (Brooker y Brooker 1996). En este sistema los costes del parasitismo son pequeños, lo que explicaría que estos hospedadores no presenten defensas frente al parasitismo (Brooker y Brooker 1996, 1998). La evaluación de los costes globales del parasitismo, en términos de pérdida de éxito reproductivo a lo largo de la vida de los hospedadores, es lo que realmente nos va a ayudar a entender la evolución de las defensas en los hospedadores.

Telómeros: posible mecanismo que une el esfuerzo reproductivo, el envejecimiento y la esperanza de vida

En los últimos años se ha propuesto un mecanismo celular que podría ser el responsable del envejecimiento en diferentes especies animales: los telómeros y su dinámica (Young 2018). Los telómeros son secuencias de ADN repetitivo muy conservadas que se encuentran en los extremos de los cromosomas eucariotas y cuya función es, junto con diferentes nucleoproteínas, proteger y dar estabilidad a los cromosomas (Blackburn 1991). Sin embargo, durante el proceso de replicación celular la ADN polimerasa no puede replicar el extremo de la cadena retardada de ADN, por lo que con cada ciclo celular los telómeros se acortan ("problema del final de la replicación"). Cuando los telómeros se acortan hasta llegar a una longitud crítica, la célula entra en un estado de senescencia replicativa o bien se dispara su respuesta apoptótica y muere (Hemann et al. 2001, Zou et al. 2004). Cuando esto ocurre en un número elevado de células, con el paso del tiempo la funcionalidad de los tejidos puede verse afectada, influyendo así en la actividad del organismo entero (Campisi 2005, Rodier et al. 2005). Por ello, los telómeros pueden suponer un nexo de unión entre la senescencia celular, la integridad somática, el envejecimiento de los organismo y su esperanza de vida (Haussmann y Marchetto 2010). Recientemente un meta-análisis ha mostrado que en diferentes especies de vertebrados la longitud telomérica está negativamente correlacionada con la mortalidad (Wilbourn et al. 2018) y diferentes estudios han mostrado en diferentes especies de aves una relación entre la longitud telomérica y la esperanza de vida, tanto en los primeros días de la vida de los individuos (Bize et al. 2009, Heidinger et al. 2012, Watson et al. 2015) como en individuos adultos (Foote et al. 2011, Barrett et al. 2013, Bichet et al. 2020), lo que sugiere que la longitud telomérica podría jugar un papel en la supervivencia de los organismos y su estudio nos podría ayudar a entender la variabilidad en esperanzas de vida que encontramos en la naturaleza.

La tasa a la que los telómeros se acortan o dinámica telomérica, también puede verse afectada por diferentes factores relacionados con el estrés y situaciones que lo favorezcan que pueden causar un aumento en las tasas de acortamiento telomérico (Chatelain et al. 2020). De esta forma, la dinámica telomérica podría suponer también un nexo de unión entre las situaciones a las que

se enfrenta un organismo y su esperanza de vida (Monaghan y Haussmann 2006). De hecho, en algunas especies de aves es la dinámica telomérica, la tasa de acortamiento, y no la longitud de los telómeros la que predice la esperanza de vida de los individuos (Bize et al. 2009, Salomons et al. 2009, Boonekamp et al. 2014a).

Además, la tasa de acortamiento telomérico también podría actuar como un marcador de los costes de la reproducción (Sudyka 2019). La reproducción es una etapa crítica en la vida de un organismo que está asociada a una mayor actividad metabólica (Jimeno et al. 2020) y una reducción de la inversión en el mantenimiento del organismo (como la inmunidad (Knowles et al. 2009) o la protección antioxidante (Wiersma et al. 2004)). Esta reducción de inversión en mantenimiento, habitualmente conlleva un aumento del estrés oxidativo y de daños en el ADN (Noguera 2017), que se podrían reflejar en la dinámica telomérica (Fig.1). De hecho, diferentes estudios muestran un relación entre el esfuerzo reproductivo y la dinámica telomérica desde una aproximación experimental (Reichert et al. 2014, Sudyka et al. 2014) y correlacional (Bauch et al. 2013, Sudyka et al. 2019, Bichet et al. 2020), por lo que la dinámica telomérica podría reflejar como los costes de la reproducción afectan a la supervivencia o la esperanza de vida de los individuos.

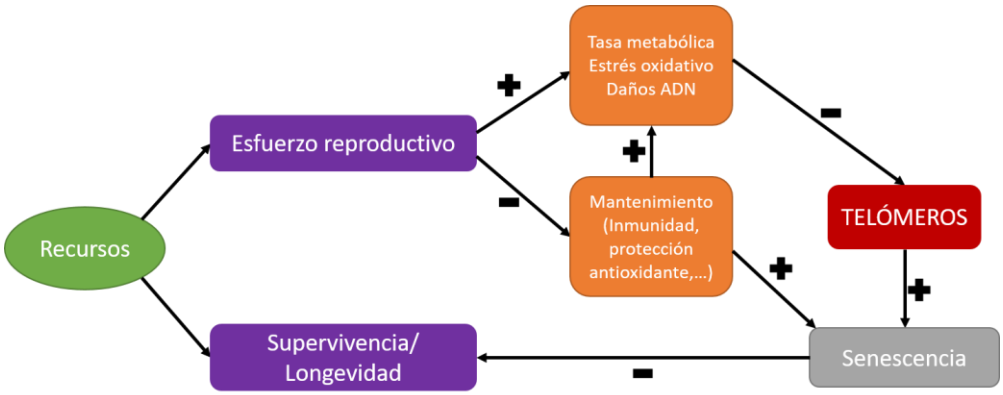


Figura 1: Esquema ilustrativo de la relación entre el esfuerzo reproductivo y la supervivencia mediada por sus efectos sobre diferentes mecanismos moleculares y a su vez el efecto de éstos sobre los telómeros. "+" corresponde a una relación positiva y "-" a una negativa.

A pesar de todo lo que ha avanzado y se ha extendido el estudio de la biología de los telómeros, existen todavía una gran variedad de métodos disponibles para la medida de su longitud, para la toma de muestras, y sobre todo para la preservación y extracción de ADN (Nussey et al. 2014). Pero además, existe un vacío en lo concerniente a los posibles efectos a largo plazo de la conservación de las muestras. Las regiones teloméricas son más sensibles al estrés oxidativo y se reparan menos frecuentemente que otras partes del genoma (Rhee et al. 2011, Coluzzi et al. 2014), lo que podría causar que también fueran especialmente sensibles durante la preservación de las muestras. Este hecho tiene una especial relevancia en estudios a largo plazo en los que las muestras pudieron haber sido tomadas mucho tiempo antes de ser analizadas. Sin embargo, hasta la fecha ningún estudio ha investigado los posibles efectos a largo plazo en la medida de la longitud telomérica de ningún medio de conservación de muestras, lo que debería ser un paso imprescindible antes de trabajar con muestras que han estado preservadas durante un periodo largo de tiempo.

Motivación de esta tesis

Esta tesis se centra en el estudio de las interacciones en el sistema parásito de cría-hospedador formado por la urraca (*Pica pica*) y su parásito, el críalo europeo (*Clamator glandarius*). La mayoría de los estudios llevados a cabo en este tipo de sistemas se centran en la investigación de diferentes aspectos de las interacciones en un plazo de tiempo corto (en pocas temporadas de cría), por lo que se hace imposible determinar los verdaderos costes que el parasitismo supone para los hospedadores. Para llevar a cabo este tipo de trabajo es necesario usar una aproximación longitudinal, con individuos marcados que hayan sido muestreados durante toda su vida.

Esta tesis continúa con el estudio longitudinal que se está llevando a cabo en los últimos años en una población de urraca en el Sur de la Península (Molina-Morales et al. 2012, 2013, 2014, Avilés et al. 2014, Martínez et al. 2020) centrándose en determinar los costes del parasitismo a largo plazo y su efecto sobre la longevidad y el éxito reproductivo de los hospedadores. Este estudio se complementa con una aproximación experimental a corto plazo para estimar los efectos del esfuerzo reproductivo y del parasitismo sobre la dinámica telomérica

de las urracas, asumiendo que esta dinámica podría ser el mecanismo molecular que determinara la esperanza de vida en esta especie.

Esta tesis pretende, combinando una aproximación molecular con una comportamental y evolutiva, proporcionar una mejor comprensión de los mecanismos que explican la dinámica evolutiva en el sistema críalo europeo-urraca.

Referencias

Antonov, A., Stokke, B. G., Moksnes, A. y Røskaft, E. 2007. Factors influencing the risk of common cuckoo *Cuculus canorus* parasitism on marsh warblers *Acrocephalus palustris*. - J. Avian Biol. 38: 390–393.

Avilés, J. M., Rutila, J. y Møller, A. P. 2005. Should the redstart *Phoenicurus phoenicurus* accept or reject cuckoo *Cuculus canorus* eggs? - Behav. Ecol. Sociobiol. 58: 608–617.

Avilés, J. M., Molina-Morales, M. y Martínez, J. G. 2014. Climatic effects and phenological mismatch in cuckoo-host interactions: A role for host phenotypic plasticity in laying date? - Oikos 123: 993–1002.

Bán, M., Moskát, C., Barta, Z. y Hauber, M. E. 2013. Simultaneous viewing of own and parasitic eggs is not required for egg rejection by a cuckoo host. - Behav. Ecol. 24: 1014–1021.

Barrett, E. L. B. B., Burke, T. A., Hammers, M., Komdeur, J. y Richardson, D. S. 2013. Telomere length and dynamics predict mortality in a wild longitudinal study. - Mol. Ecol. 22: 249–259.

Bauch, C., Becker, P. H. y Verhulst, S. 2013. Telomere length reflects phenotypic quality and costs of reproduction in a long-lived seabird. - Proc. R. Soc. B Biol. Sci. 280: 20122540.

Bichet, C., Bouwhuis, S., Bauch, C., Verhulst, S., Becker, P. H. y Vedder, O. 2020. Telomere length is repeatable, shortens with age and reproductive success, and predicts remaining lifespan in a long-lived seabird. - Mol. Ecol. 29: 429–441.

Bize, P., Criscuolo, F., Metcalfe, N. B., Nasir, L. y Monaghan, P. 2009. Telomere dynamics rather than age predict life expectancy in the wild. - Proc. R. Soc. B Biol. Sci. 276: 1679–1683.

Blackburn, E. H. 1991. Structure and function of telomeres. - Nature 350: 569–573.

Blažek, R., Polačik, M., Smith, C., Honza, M., Meyer, A. y Reichard, M. 2018. Success of cuckoo catfish brood parasitism reflects coevolutionary history and individual experience of their cichlid hosts. - Sci. Adv. 4: eaar4380.

Boonekamp, J. J., Mulder, G. A., Salomons, H. M., Dijkstra, C. y Verhulst, S. 2014a. Nestling telomere shortening, but not telomere length, reflects developmental stress and predicts survival in wild birds. - Proc. R. Soc. B Biol. Sci. 281: 20133287.

Boonekamp, J. J., Salomons, H. M., Bouwhuis, S., Dijkstra, C. y Verhulst, S. 2014b. Reproductive effort accelerates actuarial senescence in wild birds: An experimental study. - Ecol. Lett. 17: 599–605.

Boonekamp, J. J., Bauch, C. y Verhulst, S. 2020. Experimentally increased brood size accelerates actuarial senescence and increases subsequent reproductive effort in a wild bird population. - J. Anim. Ecol. 89: 1395–1407.

Boulton, A. M. y Polis, G. A. 2002. Brood parasitism among spiders: Interactions between salticids and *Diguetia mojavea*. - Ecology 83: 282–287.

Bouwhuis, S., Charmantier, A., Verhulst, S. y Sheldon, B. C. 2010. Individual variation in rates of senescence: Natal origin effects and disposable soma in a wild bird population. - J. Anim. Ecol. 79: 1251–1261.

Brooke, M. D. L. y Davies, N. B. 1988. Egg mimicry by cuckoos *Cuculus canorus* in relation to discrimination by hosts. - Nature 335: 630–632.

Brooker, M. y Brooker, L. 1996. Acceptance by the splendid fairy-wren of parasitism by Horsfield's bronze-cuckoo: further evidence for evolutionary equilibrium in brood parasitism. - Behav. Ecol. 7: 395–407.

Burhans, D. E., Dearborn, D., Thompson, F. R. y Faaborg, J. 2002. Factors Affecting Predation at Songbird Nests in Old Fields. - J. Wildl. Manage. 66: 240.

Campisi, J. 2005. Senescent cells, tumor suppression, and organismal aging: Good citizens, bad neighbors. - Cell 120: 513–522.

Campobello, D. y Sealy, S. G. 2010. Enemy Recognition of Reed Warblers (*Acrocephalus scirpaceus*): Threats and Reproductive Value Act Independently in Nest Defence Modulation. - Ethology 116: 498–508.

Carnes, B. A. y Olshansky, S. J. 1997. A biologically motivated partitioning of mortality. - Exp. Gerontol. 32: 615–631.

Chatelain, M., Drobniak, S. M. y Szulkin, M. 2020. The association between stressors and telomeres in non-human vertebrates: a meta-analysis. - Ecol. Lett. 23: 381–398.

Coluzzi, E., Colamartino, M., Cozzi, R., Leone, S., Meneghini, C., O'Callaghan, N. y Sgura, A. 2014. Oxidative Stress Induces Persistent Telomeric DNA Damage Responsible for Nuclear Morphology Change in Mammalian Cells. - PLoS One 9: e110963.

Davies, N. B. 2000. Cuckoos, Cowbirds and Other Cheats. - T & A D Poyser.

Davies, N. b. 2011. Cuckoo adaptations: trickery and tuning. - J. Zool. 284: 1–14.

Davies, N. B. y Brooke, M. de L. 1988. Cuckoos versus reed warblers: Adaptations and counteradaptations. - Anim. Behav. 36: 262–284.

Davies, N. B. y Welbergen, J. A. 2008. Cuckoo-hawk mimicry? An experimental test. - Proc. R. Soc. B Biol. Sci. 275: 1817–1822.

Davies, N. B., Butchart, S. H. M., Burke, T. A., Chaline, N. y Stewart, I. R. K. 2003. Reed warblers guard against cuckoos and cuckoldry. - Anim. Behav. 65: 285–295.

De Mársico, M. C., Gantchoff, M. G. y Reboreda, J. C. 2012. Host-parasite coevolution beyond the nestling stage? Mimicry of host fledglings by the specialist screaming cowbird. - Proc. R. Soc. B Biol. Sci. 279: 3401–3408.

Dearborn, D. C. 1999. Brown-Headed Cowbird Nestling Vocalizations and Risk of Nest Predation. - Auk 116: 448–457.

Dearborn, D. C., Anders, A. D., Thompson, F. R. y Faaborg, J. 1998. Effects of Cowbird Parasitism on Parental Provisioning and Nestling Food Acquisition and Growth. - Condor 100: 326–334.

Expósito-Granados, M., Parejo, D., Martínez, J. G., Precioso, M., Molina-Morales, M. y Avilés, J. M. 2017. Host nest site choice depends on risk of cuckoo parasitism in magpie hosts. - Behav. Ecol. 28: 1492–1497.

Fiorini, V. D., Tuero, D. T. y Reboreda, J. C. 2009. Host behaviour and nest-site characteristics affect the likelihood of brood parasitism by shiny cowbirds on chalk-browed mockingbirds. - Behaviour 146: 1387–1403.

Foote, C. G., Daunt, F., González-Solís, J., Nasir, L., Phillips, R. A. y Monaghan, P. 2011. Individual state and survival prospects: Age, sex, and telomere length in a long-lived seabird. - Behav. Ecol. 22: 156–161.

Gloag, R., Fiorini, V. D., Reboreda, J. C. y Kacelnik, A. 2013. The wages of violence: Mobbing by mockingbirds as a frontline defence against brood-parasitic cowbirds. - Anim. Behav. 86: 1023–1029.

Gluckman, T. L. y Mundy, N. I. 2013. Cuckoos in raptors' clothing: Barred plumage illuminates a fundamental principle of Batesian mimicry. - Anim. Behav. 86: 1165–1181.

González-Megías, A. y Sánchez-Piñero, F. 2003. Effects of brood parasitism on host reproductive success: Evidence from larval interactions among dung beetles. - Oecologia 134: 195–202.

Grayson, P., Glassey, B. y Forbes, S. 2013. Does Brood Parasitism Induce Paternal Care in a Polygynous Host? - Ethology 119: 489–495.

Grim, T. 2011. Ejecting chick cheats: A changing paradigm? - Front. Zool. 8: 1–6.

Hannon, S. J., Wilson, S. and McCallum, C. A. 2009. Does cowbird parasitism increase predation risk to American redstart nests? - Oikos 118: 1035–1043.

Hauber, M. E. 2006. A future cost of misdirected parental care for brood parasitic young? - Folia Zool. 55: 367–374.

Hauber, M. E. y Kilner, R. M. 2007. Coevolution, communication, and host chick mimicry in parasitic finches: Who mimics whom? - Behav. Ecol. Sociobiol. 61: 497–503.

Hauber, M. E., Yeh, P. J. y Roberts, J. O. L. 2004a. Patterns and coevolutionary consequences of repeated brood parasitism. - Proc. R. Soc. London B Biol. Sci. 271: S317–S320.

Hauber, M. E., Yeh, P. J. y Roberts, J. O. L. 2004b. Patterns and coevolutionary consequences of repeated brood parasitism. - Proc. R. Soc. B Biol. Sci. 271: 317–320.

Haussmann, M. F. y Marchetto, N. M. 2010. Telomeres: Linking stress and survival, ecology and evolution. - Curr. Zool. 56: 714–727.

Heidinger, B. J., Blount, J. D., Boner, W., Griffiths, K., Metcalfe, N. B. y Monaghan, P. 2012. Telomere length in early life predicts lifespan. - PNAS 109: 1743–1748.

Hemann, M. T., Strong, M. A., Hao, L. Y. y Greider, C. W. 2001. The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability. - Cell 107: 67–77.

Honza, M., Taborsky, B., Taborsky, M., Teuschl, Y., Vogl, W., Moksnes, A. y Røskaft, E. 2002. Behaviour of female common cuckoos, *Cuculus canorus*, in the vicinity of host nests before and during egg laying: A radiotelemetry study. - Anim. Behav. 64: 861–868.

Hoover, J. P. 2003. Multiple effects of brood parasitism reduce the reproductive success of prothonotary warblers, *Protonotaria citrea*. - Anim. Behav. 65: 923–934.

Hoover, J. P. y Reetz, M. J. 2006. Brood parasitism increases provisioning rate, and reduces offspring recruitment and adult return rates, in a cowbird host. - Oecologia 149: 165–173.

Hoover, J. P., Yasukawa, K. y Hauber, M. E. 2006. Spatially and temporally structured avian brood parasitism affects the fitness benefits of hosts' rejection strategies. - Anim. Behav. 72: 881–890.

Hosoi, S. A. y Rothstein, S. I. 2000. Nest desertion and cowbird parasitism: Evidence for evolved responses and evolutionary lag. - Anim. Behav. 59: 823–840.

Huang, M. H. y Dornhaus, A. 2008. A meta-analysis of ant social parasitism: Host characteristics of different parasitism types and a test of Emery's rule. - Ecol. Entomol. 33: 589–596.

Igic, B., Braganza, K., Hyland, M. M., Silyn-Roberts, H., Cassey, P., Grim, T., Rutila, J., Csaba, M. y Hauber, M. E. 2011. Alternative mechanisms of increased eggshell hardness of avian brood parasites relative to host species. - J. R. Soc. Interface 8: 1654–1664.

Jimeno, B., Prichard, M. R., Landry, D., Wolf, C., Larkin, B., Cheviron, Z. y Breuner, C. 2020. Metabolic rates predict baseline corticosterone and reproductive output in a free-living passerine. - Integr. Org. Biol. 2: abaa030.

Kilner, R. M. y Langmore, N. E. 2011. Cuckoos versus hosts in insects and birds: Adaptations, counter-adaptations and outcomes. - Biol. Rev. 86: 836–852.

Knowles, S. C. L., Nakagawa, S. y Sheldon, B. C. 2009. Elevated reproductive effort increases blood parasitaemia and decreases immune function in birds: A meta-regression approach. - Funct. Ecol. 23: 405–415.

Krüger, O. 2011. Brood parasitism selects for no defence in a cuckoo host. - Proc. R. Soc. B Biol. Sci. 278: 2777–2783.

Krüger, O. 2007. Cuckoos, cowbirds and hosts: Adaptations, trade-offs and constraints. - Philos. Trans. R. Soc. B Biol. Sci. 362: 1873–1886.

Krüger, O. y Davies, N. B. 2002. The evolution of cuckoo parasitism: A comparative analysis. - Proc. R. Soc. B Biol. Sci. 269: 375–381.

Krüger, O. y Davies, N. B. 2004. The evolution of egg size in the brood parasitic cuckoos. - Behav. Ecol. 15: 210–218.

Krüger, O., Davies, N. B. y Sorenson, M. D. 2007. The evolution of sexual dimorphism in parasitic cuckoos: Sexual selection or coevolution? - Proc. R. Soc. B Biol. Sci. 274: 1553–1560.

Langmore, N. E. N. E., Hunt, S. y Kilner, R. M. 2003. Escalation of a coevolutionary arms race through host rejection of brood parasitic young. - Nature 422: 157–160.

Lhomme, P. y Hines, H. M. 2019. Ecology and evolution of cuckoo bumble bees. - Ann. Entomol. Soc. Am. 112: 122–140.

López, A. V., Fiorini, V. D., Ellison, K. y Peer, B. D. 2018. Thick eggshells of brood parasitic cowbirds protect their eggs and damage host eggs during laying. - Behav. Ecol. 29: 965–973.

Lotem, A. y Rothstein, S. I. 1995. Cuckoo-host coevolution: from snapshots of an arms race to the documentation of microevolution. - Trends Ecol. Evol. 10: 436–437.

Lotem, A., Nakamura, H. y Zahavi, A. 1992. Rejection of cuckoo eggs in relation to host age: A possible evolutionary equilibrium. - Behav. Ecol. 3: 128–132.

Mann, C. F. 2017. A Taxonomic Review of Obligate and Facultative Interspecific Avian Brood Parasitism. - In: Soler, M. (ed), Avian Brood Parasitism-Behaviour, Ecology, Evolution and Coevolution. Springer International Publishing, pp. 61–92.

Marchetti, K. 2000. Egg rejection in a passerine bird: size does matter. - Anim. Behav. 59: 877–883.

Martin, T. E., Scott, J. y Menge, C. 2000. Nest predation increases with parental activity: Separating nest site and parental activity effects. - Proc. R. Soc. B Biol. Sci. 267: 2287–2293.

Martínez, J. G., Soler, M. y Soler, J. J. 1996. The Effect of Magpie Breeding Density and Synchrony on Brood Parasitism by Great Spotted Cuckoos. - Condor 98: 272–278.

Martínez, J. G., Molina-Morales, M., Precioso, M. y Avilés, J. M. 2020. Age-related brood parasitism and egg rejection in magpie hosts. - Am. Nat. 195: 876–885.

Massoni, V. and Reboreda, J. C. 2001. Number of Close Spatial and Temporal Neighbors Decreases the Probability of Nest Failure and Shiny Cowbird Parasitism in Colonial Yellow-Winged Blackbirds. - Condor 103: 521–529.

Massoni, V. y Reboreda, J. C. 2002. A Neglected Cost of Brood Parasitism: Egg Punctures by Shiny Cowbirds During Inspection of Potential Host Nests. - Condor 104: 407–412.

McDonald, D. B., Fitzpatrick, J. W. y Woolfenden, G. E. 1996. Actuarial Senescence and Demographic Heterogeneity in the Florida Scrub Jay. - Ecology 77: 2373–2381.

Molina-Morales, M., Martínez, J. G. y Avilés, J. M. 2012. Factors affecting natal and breeding magpie dispersal in a population parasitized by the great spotted cuckoo. - Anim. Behav. 83: 671–680.

Molina-Morales, M., Gabriel Martínez, J., Martín-Gálvez, D., A. Dawson, D., Rodríguez-Ruiz, J., Burke, T., y Avilés, J. M. 2013. Evidence of long-term structured cuckoo parasitism on individual magpie hosts. - J. Anim. Ecol. 82: 389–398.

Molina-Morales, M., Martínez, J. G., Martín-Gálvez, D., Dawson, D. A., Burke, T. y Avilés, J. M. 2014. Cuckoo hosts shift from accepting to rejecting parasitic eggs across their lifetime. - Evolution 68: 3020–3029.

Monaghan, P. y Haussmann, M. F. 2006. Do telomere dynamics link lifestyle and lifespan? - Trends Ecol. Evol. 21: 47–53.

Moskát, C. y Honza, M. 2000. Effect of nest and nest site characteristics on the risk of cuckoo Cuculus canorus parasitism in the great reed warbler Acrocephalus arundinaceus. - Ecography 23: 335–341.

Moskát, C., Bán, M., Székely, T., Komdeur, J., Lucassen, R. W. G., Van Boheemen, L. A. y Hauber, M. E. 2010. Discordancy or template-based recognition? Dissecting the cognitive basis of the rejection of foreign eggs in hosts of avian brood parasites. - J. Exp. Biol. 213: 1976–1983.

Muñoz, A. R., Altamirano, M., Takasu, F. y Nakamura, H. 2007. Nest Light Environment and the Potential Risk of Common Cuckoo (*Cuculus Canorus*) Parasitism. - Auk 124: 619–627.

Noguera, J. C. 2017. Interacting effects of early dietary conditions and reproductive effort on the oxidative costs of reproduction. - PeerJ 5: e3094.

Nussey, D. H., Baird, D., Barrett, E., Boner, W., Fairlie, J., Gemmell, N., Hartmann, N., Horn, T., Haussmann, M., Olsson, M., Turbill, C., Verhulst, S., Zahn, S. y Monaghan, P. 2014. Measuring telomere length and telomere dynamics in evolutionary biology and ecology. - Methods Ecol. Evol. 5: 299–310.

Payne, R. B. 2005. The Cuckoos. - Oxford University Press.

Payne, R. B. y Payne, L. L. 1998. Brood parasitism by cowbirds: Risks and effects on reproductive success and survival in indigo buntings. - Behav. Ecol. 9: 64–73.

Požgayová, M., Procházka, P. y Honza, M. 2009. Adjustment of incubation according to the threat posed: A further signal of enemy recognition in the Blackcap *Sylvia atricapilla*? - J. Ornithol. 150: 569–576.

Požgayová, M., Beňo, R., Procházka, P., Jelínek, V., Abraham, M. M. y Honza, M. 2015. Lazy males and hardworking females? Sexual conflict over parental care in a brood parasite host and its consequences for chick growth. - Behav. Ecol. Sociobiol. 69: 1053–1061.

Reed, T. E., Kruuk, L. E. B., Wanless, S., Frederiksen, M., Cunningham, E. J. A. y Harris, M. P. 2008. Reproductive Senescence in a Long- Lived Seabird: Rates of Decline in Late- Life Performance Are Associated with Varying Costs of Early Reproduction. - Am. Nat. 171: E89–E101.

Reichert, S., Bize, P., Arrivé, M., Zahn, S., Massemin, S. y Criscuolo, F. 2014. Experimental increase in telomere length leads to faster feather regeneration. - Exp. Gerontol. 52: 36–38.

Reid, J. M., Bignal, E. M., Bignal, S., McCracken, D. I. y Monaghan, P. 2003. Age-specific reproductive performance in red-billed choughs *Pyrrhocorax pyrrhocorax*: Patterns and processes in a natural population. - J. Anim. Ecol. 72: 765–776.

Rhee, D. B., Ghosh, A., Lu, J., Bohr, V. A. y Liu, Y. 2011. Factors that influence telomeric oxidative base damage and repair by DNA glycosylase OGG1. - DNA Repair 10: 34–44.

Rodier, F., Kim, S. H., Nijjar, T., Yaswen, P. y Campisi, J. 2005. Cancer and aging: The importance of telomeres in genome maintenance. - Int. J. Biochem. Cell Biol. 37: 977–990.

Rohwer, S. y Spaw, C. D. 1988. Evolutionary lag versus bill-size constraints: a comparative study of the acceptance of cowbird eggs by old hosts. - Evol. Ecol. 2: 27–36.

Røskaft, E., Moksnes, A., Stokke, B. G., Bicík, V. y Moskát, C. 2002. Aggression to dummy cuckoos by potential European cuckoo hosts. - Behaviour 139: 613–628.

Rothstein, S. I. 1975. Mechanisms of avian egg-recognition: Do birds know their own eggs? - Anim. Behav. 23: 268–278.

Rothstein, S. I. 1978. Mechanisms of avian egg-recognition: Additional evidence for learned components. - Anim. Behav. 26: 671–677.

Rothstein, S. I. 1990. A Model System for Coevolution: Avian Brood Parasitism. - Annu. Rev. Ecol. Syst. 21: 481–508.

Salomons, H. M., Mulder, G. A., Van De Zande, L., Haussmann, M. F., Linskens, M. H. K. K. y Verhulst, S. 2009. Telomere shortening and survival in free-living corvids. - Proc. R. Soc. B Biol. Sci. 276: 3157–3165.

Samaš, P., Rutila, J., Honza, M., Kysučan, M. y Grim, T. 2018. Rearing a virulent common cuckoo is not extra costly for its only cavity-nesting host. - Proc. R. Soc. B Biol. Sci. 285: 20181710.

Samaš, P., Grim, T., Jelínek, V., Abraham, M. M., Šulc, M. y Honza, M. 2019. No immediate or future extra costs of raising a virulent brood parasite chick. - Behav. Ecol. 30: 1020–1029.

Smith, J. N. M., Arcese, P. y McLean, I. G. 1984. Age, experience, and enemy recognition by wild song sparrows. - Behav. Ecol. Sociobiol. 14: 101–106.

Soler, M. 2014. Long-term coevolution between avian brood parasites and their hosts. - Biol. Rev. 89: 688–704.

Soler, M. y Soler, J. J. 1991. Growth and Development of Great Spotted Cuckoos and Their Magpie Host. - Condor 93: 49–54.

Soler, J. J., Soler, M., Møller, A. P. y Martínez, J. G. 1995a. Does the great spotted cuckoo choose magpie hosts according to their parenting ability? - Behav. Ecol. Sociobiol. 36: 201–206.

Soler, M., Martínez, J. G., Soler, J. J. y Møller, A. P. 1995b. Preferential allocation of food by magpies *Pica pica* to great spotted cuckoo *Clamator glandarius* chicks. - Behav. Ecol. Sociobiol. 37: 7–13.

Soler, M., Soler, J. J. y Martínez, J. G. 1997. Great spotted cuckoos improve their reproductive success by damaging magpie host eggs. - Anim. Behav. 54: 1227–1233.

Soler, J. J., Martínez, J. G., Soler, M. y Moller, A. P. 1999. Host sexual selection and cuckoo parasitism: an analysis of nest size in sympatric and allopatric magpie *Pica pica* populations parasitized by the great spotted cuckoo *Clamator glandarius*. - Proc. R. Soc. London B Biol. Sci. 266: 1765–1771.

Soler, M., Rodríguez-Navarro, A. B., Pérez-Contreras, T., García-Ruiz, J. M. y Soler, J. J. 2019. Great spotted cuckoo eggshell microstructure characteristics can make eggs stronger. - J. Avian Biol. 50: jav.02252.

Sorenson, M. D. y Payne, R. B. 2002. Molecular Genetic Perspectives on Avian Brood Parasitism. - Integr. Comp. Biol. 42: 388–400.

Sorenson, M. D. y Payne, R. B. 2005. A molecular genetic analysis of cuckoo phylogeny. - In: Payne, R. B. (ed), The cuckoos. Oxford University Press, pp. 68–94.

Spottiswoode, C. N. y Koorevaar, J. 2012. A stab in the dark: Chick killing by brood parasitic honeyguides. - Biol. Lett. 8: 241–244.

Stevens, M., Troscianko, J. y Spottiswoode, C. N. 2013. Repeated targeting of the same hosts by a brood parasite compromises host egg rejection. - Nat. Commun. 4: 1–6.

Stokke, B. G., Moksnes, A., Røskaft, E., Rudolfsen, G. y Honza, M. 1999. Rejection of artificial cuckoo (*Cuculus canorus*) eggs in relation to variation in egg appearance among reed warblers (*Acrocephalus scirpaceus*). - Proc. R. Soc. B Biol. Sci. 266: 1483–1488.

Stokke, B. G., Moksnes, A. y Røskaft, E. 2002. Obligate brood parasites as selective agents for evolution of egg appearance in passerine birds. - Evolution 56: 199–205.

Stokke, B. G., Moksnes, A. y Røskaft, E. 2005. The enigma of imperfect adaptations in hosts of avian brood parasites. - Ornithol. Sci. 4: 17–29.

Stokke, B. G., Takasu, F., Moksnes, A. y Røskaft, E. 2007. The importance of clutch characteristics and learning for antiparasite adaptations in hosts of avian brood parasites. - Evolution 61: 2212–2228.

Sudyka, J. 2019. Does Reproduction Shorten Telomeres? Towards Integrating Individual Quality with Life-History Strategies in Telomere Biology. - BioEssays 41: 1900095.

Sudyka, J., Arct, A., Drobniak, S., Dubiec, A., Gustafsson, L. y Cichoń, M. 2014. Experimentally increased reproductive effort alters telomere length in the blue tit (*Cyanistes caeruleus*). - J. Evol. Biol. 27: 2258–2264.

Sudyka, J., Arct, A., Drobniak, S. M., Gustafsson, L. y Cichon, M. 2019. Birds with high lifetime reproductive success experience increased telomere loss. - Biol. Lett. 15: 9–12.

Taborsky, M. 2001. The Evolution of Bourgeois, Parasitic, and Cooperative Reproductive Behaviors in Fishes. - J. Hered. 92: 100–110.

Tanaka, K. D. y Ueda, K. 2005. Horsfield's Hawk-Cuckoo Nestlings Simulate Multiple Gapes for Begging. - Science 308: 653.

Vedder, O. y Bouwhuis, S. 2018. Heterogeneity in individual quality in birds: overall patterns and insights from a study on common terns. - Oikos 127: 719–727.

Watson, H., Bolton, M. y Monaghan, P. 2015. Variation in early-life telomere dynamics in a long-lived bird: Links to environmental conditions and survival. - J. Exp. Biol. 218: 668–674.

Welbergen, J. A. y Davies, N. B. 2009. Strategic Variation in Mobbing as a Front Line of Defense against Brood Parasitism. - Curr. Biol. 19: 235–240.

Welbergen, J. A. y Davies, N. B. 2011. A parasite in wolf's clothing: hawk mimicry reduces mobbing of cuckoos by hosts. - Behav. Ecol. 22: 574–579.

Wiersma, P., Selman, C., Speakman, J. R. y Verhulst, S. 2004. Birds sacrifice oxidative protection for reproduction. - Proc. R. Soc. B Biol. Sci. 271: 360–363.

Wilbourn, R. V., Moatt, J. P., Froy, H., Walling, C. A., Nussey, D. H. y Boonekamp, J. J. 2018. The relationship between telomere length and mortality risk in non-model vertebrate systems: A meta-analysis. - Philos. Trans. R. Soc. B Biol. Sci. 373: 20160447.

Yom-Tov, Y. y Geffen, E. 2017. Conspecific Brood Parasitism Among Birds: The Effects of Phylogeny, Mode of Reproduction and Geographic Distribution. - In: Soler, M.

(ed), Avian Brood Parasitism-Behaviour, Ecology, Evolution and Coevolution. Springer International Publishing, pp. 95–103.

Young, A. J. 2018. The role of telomeres in the mechanisms and evolution of life-history trade-offs and ageing. - Philos. Trans. R. Soc. B Biol. Sci. 373: 20160452.

Zou, Y., Sfeir, A., Gryaznov, S. M., Shay, J. W. y Wright, W. E. 2004. Does a sentinel or a subset of short telomeres determine replicative senescence? - Mol. Biol. Cell 15: 3709–3718.

Objetivos

Objetivos

Los objetivos que se plantean en esta tesis son:

1. Estimar el esfuerzo reproductivo en nidos parasitados y no parasitados y determinar si el parasitismo afecta a la supervivencia de un año para otro de los adultos. Además explorar si hay diferencias en el esfuerzo reproductivo de machos y hembras, y si el parasitismo tiene un efecto sobre el papel de los sexos en los cuidados parentales y sobre otros rasgos de la historia vital de los individuos, como su fenología (Capítulo 1).

2. Investigar mediante una aproximación experimental el efecto del parasitismo sobre la dinámica telomérica de las urracas, y comprobar si dicho efecto es debido al parasitismo *per se* o a que las nidadas parasitadas contienen un menor número de pollos que las nidadas no parasitadas. Además, se exploran los factores que podrían afectar la longitud telomérica de las urracas adultas (Capítulo 2).

3. Determinar si el método que se usa para preservar las muestras de sangre tiene un efecto a largo plazo sobre la estima de la longitud telomérica. Específicamente a) determinar si el número de años que las muestras de sangre han estado preservadas en etanol tiene un efecto sobre la longitud telomérica; b) modelizar el efecto del tiempo de preservación (de 1 a 12 años) sobre las diferencias en las medidas teloméricas obtenidas a partir de la misma muestra de sangre extraída en dos años consecutivos; c) corregir el efecto del tiempo de preservación a partir del modelo anterior; y d) validar este método comparando los valores de longitud telomérica corregidos, con los de muestras extraídas y analizadas poco tiempo después de haber sido tomadas (Capítulo 3).

4. Investigar el efecto del parasitismo sobre la supervivencia y el éxito reproductivo a lo largo de la vida de los hospedadores, explorando si la longitud telomérica al principio de la vida está relacionada con la esperanza de vida de los hospedadores y si el efecto del parasitismo sobre la supervivencia de los hospedadores difiere en edades tempranas comparado con edades tardías. Específicamente se pretende determinar, a) el efecto del parasitismo sobre las trayectorias de mortalidad dependientes de la edad, comparándolas con las de

individuos que no han sido nunca parasitados, b) si el éxito reproductivo a lo largo de la vida difiere entre individuos que han sido parasitados alguna vez e individuos que no han sido nunca parasitados; c) si la longitud telomérica de los volantones de urraca está correlacionada con sus trayectorias de mortalidad; y d) explorar si los costes de la reproducción y del parasitismo sobre la mortalidad de los hospedadores son diferentes en edades tempranas y tardías (Capítulo 4).

Metodología general

Metodología general

Sistema de estudio: críalo europeo-urraca

La urraca

La urraca (*Pica pica*) es un ave paseriforme de la Familia Corvidae propia de la región Paleártica. Es una ave de tamaño medio (43-50 cm, (Birkhead 1991)) y de vida relativamente larga, ya que algunos individuos pueden llegar a vivir 15 años (Birkhead 1991). Las urracas son aves sedentarias, territoriales y principalmente monógamas (Birkhead 1991), y sólo presentan un leve dimorfismo sexual en el tamaño, siendo los machos algo más grandes que las hembras (Birkhead 1991). Las urracas construyen nidos abovedados que pueden variar mucho en tamaño(Álvarez y Arias de Reyna 1974a). Comienzan la puesta alrededor de principios de abril, y su tamaño de puesta suele ser de entre 4 y 9 huevos (Arias de Reyna et al. 1984). El periodo de incubación es de 21 días aproximadamente y solamente incuban las hembras, que comienzan a incubar habitualmente tras poner el cuarto huevo. Por ello, la eclosión de los huevos es asincrónica, primero eclosionan los primeros huevos y el resto van eclosionando gradualmente, lo que conlleva que haya una jerarquía de tamaño en la nidada (Birkhead 1991, Martínez 2011). Los volantones abandonan el nido cuando tienen aproximadamente 27 días y los padres continúan alimentándolos durante varias semanas (Birkhead 1991, Martínez 2011).

El críalo

El críalo europeo (*Clamator glandarius*) es una especie de ave cuculiforme de la familia Cuculidae. Es un ave migratoria de tamaño medio (38-40cm) que pasa el invierno en África y vuelve al Sur de Europa alrededor de Febrero-Marzo para reproducirse (Ibáñez-Álamo et al. 2019). Es un parásito de cría obligado, y su principal hospedador en Europa es la urraca, aunque puede parasitar a otras especies de córvidos (Soler 1990), en particular la corneja (Baglione et al. 2017) y una misma hembra es capaz de parasitar a ambas especies hospedadoras (Martínez et al. 1998, Baglione et al. 2017). Las hembras de críalo son capaces de poner más de 15 huevos en una temporada de cría, y a veces pueden poner más de un huevo en un mismo nido, o incluso varias hembras pueden parasitar un mismo nido, dependiendo de la disponibilidad de éstos (Martínez et al. 1998, Martínez et al.

1998). A menudo, las hembras de críalo dañan uno o varios huevos de urraca cuando ponen sus huevos (Soler et al. 1997).

Interacciones entre el críalo y la urraca

Las urracas son capaces de reconocer a los críalos como una amenaza y los persiguen y expulsan cuando se encuentran cerca del nido (Soler et al. 1999a, Avilés et al. 2014). Se ha descrito que los machos de críalo realizan una estrategia de distracción de los hospedadores para permitir que las hembras pongan los huevos (Álvarez y Arias de Reyna 1974b), sin embargo, es habitual que las hembras de críalo los pongan cuando la hembra de urraca está en el nido y que por ello algunos huevos de urraca resulten dañados (Soler et al. 2014a).

Además, se ha sugerido que los críalos podrían elegir a los hospedadores que van a parasitar utilizando como signo de calidad parental el tamaño de los nidos de urraca (Soler et al. 2001, De Neve y Soler 2002), parasitando nidos de mayor tamaño para aumentar su éxito reproductivo (Soler et al. 1995b). Sin embargo, la relación entre el tamaño del nido y la probabilidad de parasitismo puede variar dentro de la temporada de cría (Molina-Morales et al. 2013) y dependiendo de la tasa de parasitismo y la disponibilidad de nidos para parasitar (Molina-Morales et al. 2016).

Una vez que un nido ha sido parasitado, el principal mecanismo de defensa que presenta el hospedador es el reconocimiento y rechazo de los huevos parásitos (Soler y Møller 1990). Esta adaptación varía entre distintas poblaciones dependiendo del tiempo que críalos y urracas han vivido en simpatría (Soler y Møller 1990) y de las distancias genéticas y geográficas entre poblaciones (Soler et al. 1999b). Esta adaptación tiene una base genética (Martín-Gálvez et al. 2006), que se ha sugerido que produciría un polimorfismo en las poblaciones, caracterizadas por individuos de dos fenotipos, aceptadores y rechazadores. Sin embargo, se ha mostrado que el comportamiento de rechazo varía con la edad (Molina-Morales et al. 2014, Martínez et al. 2020), lo que sugiere que, incluso existiendo un polimorfismo genético para que se exprese el comportamiento es necesario un periodo de aprendizaje (Rothstein 1975, Lotem et al. 1995).

Los críalos no han desarrollado mimetismo de sus huevos con respecto a los de urraca (Soler et al. 2003), lo que favorecería que las urracas no los reconocieran aumentando su éxito reproductivo, pero se ha descrito en algunos

individuos un comportamiento "mafioso", que consiste en destruir las puestas de nidos donde se han rechazado sus huevos (Soler et al. 1995d), obligando a los hospedadores a realizar una puesta de reposición, en la que, además, los huevos de críalo son aceptados con mayor probabilidad (Soler et al. 1999c).

Un vez que el críalo parasita exitosamente un nido de urraca, el periodo de incubación del huevo de críalo es de unos 14 días, por lo que eclosionan antes que los pollos de urraca y las hembras de urraca dejan de incubar y comienzan a alimentar al pollos de críalo (Soler y Soler 1991). Si los huevos de urraca llegan a eclosionar, el pollo de críalo ya tiene cierto tamaño, posee papilas en la cavidad bucal que lo hacen más llamativo y eficiente al pedir comida (Soler et al. 1995c) y monopoliza los cuidados parentales, y las urracas recién eclosionadas a menudo mueren de inanición (Soler 1990, Soler et al. 1996); por ello los críalos son habitualmente criados solos en el nido o junto a otros pollos de críalo (Martínez et al. 1998). Los pollos de críalo abandonan el nido cuando tienen unos 17-20 días, a menudo forman grupos con otros pollos de críalo (Soler et al. 1995a), y son alimentados por sus hospedadores y por otras parejas que también han sido parasitadas durante un periodo de tiempo variable (Soler et al. 1995a, 2014b).

Población de estudio

Este estudio se ha llevado a cabo durante los años 2005-2019 en una población de urracas situada en La Calahorra, provincia de Granada (37º10´N, 3º03´W). El área de estudio está compuesta por pequeños campos de cereal rodeados de almendros, que son los árboles donde las urracas preferentemente construyen sus nidos en la zona (Fig. 1). Existen alrededor de unas 100 parejas reproductoras en la población. El porcentaje de nidos parasitados por los críalos en la población es bastante variable, y puede ir desde un 19.4% a un 65.6% de los nidos dependiendo del año, siendo de media 32% (DS = 14).

El parasitismo en nuestra población está estructurado de manera que algunos individuos escapan sistemáticamente de ser parasitados, mientras otros son parasitados con mayor o menor frecuencia a lo largo de su vida (Molina-Morales et al. 2013). Además, la estructura de edad de la población juega un papel fundamental en la dinámica de las interacciones entre críalos y urracas, de manera que individuos más jóvenes tienen mayor probabilidad de ser parasitados porque son más numerosos, pero además, los individuos más viejos tienen más

probabilidades de escapar del parasitismo mediante el rechazo de huevos (Martínez et al. 2020)

Figura 1: Área de estudio (Fuente: https://igsierranevada.blogspot.com)

El porcentaje de rechazo de huevos modelo miméticos a los del críalo ha oscilado alrededor del 30% en los 15 años que se ha estudiado esta población.

Trabajo de campo

Captura y marcaje de individuos

Durante todo el periodo de estudio se han capturado urracas adultas en las proximidades de los nidos mediante diferentes tipos de trampas que contenían una urraca viva como señuelo (Fig. 2). Cada individuo capturado fue anillado con una combinación de anillas de colores única y se tomaron diferentes medidas morfométricas como son el peso, que se tomó con una pesola (precisión 2 gr), la longitud del tarso con un calibre (precisión 0.1 mm) y la longitud del ala y de la

cola con una regla con tope (precisión 1mm). También se midió con el calibre la cantidad de negro del extremo de la primera pluma primaria de los individuos capturados; en las urracas la mancha negra del extremo de esta pluma es mayor en individuos de un año de edad (que todavía no han mudado su primer plumaje) que en individuos de dos o más años, de manera que si la cantidad de negro de la primera primaria del individuo capturado era mayor de 14 mm pudimos determinar que tenía un año de edad (Birkhead 1991, Martínez et al. 2020). Además, de cada individuo capturado se tomó una muestra de sangre (100 microlitros aproximadamente) que se introdujo en un tubo de microcentrífuga con 1ml de etanol absoluto, y de cada muestra se extrajo posteriormente ADN para poder sexar a los individuos molecularmente y en determinados casos, medir la longitud relativa de sus telómeros.

Figura 2: Trampa de captura con una urraca viva como señuelo (Fuente: Alfredo Sánchez Tójar).

Además, en cada nido en el que volaron pollos de urraca, se marcó a cada pollo con una combinación de anillas única cuando tenían entre 15 y 20 días aproximadamente (Fig. 3), se tomó su peso y una muestra de sangre de la misma forma que hemos descrito anteriormente. Algunos de estos pollos se incorporaron después a la población como adultos reproductores.

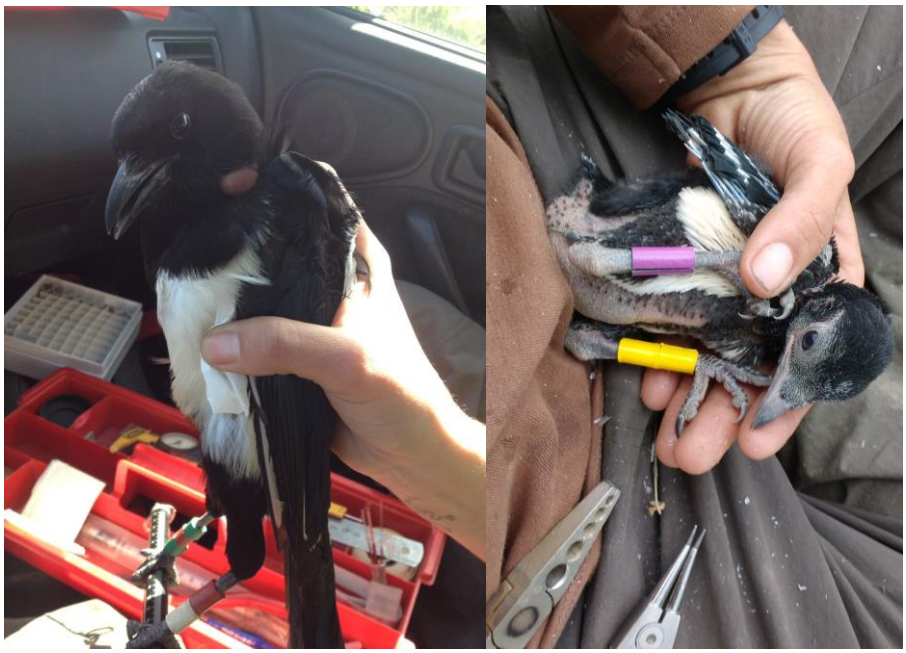


Figura 3: Urraca adulta capturada (izquierda). Volantón de urraca recién anillado (derecha).

Monitoreo de las parejas reproductoras

Las urracas comienzan a construir sus nidos a principios de marzo. A partir de este momento se localizan los nidos y posicionan mediante sus coordenadas GPS. Cada nido fue observado con un telescopio desde unos 50-100 metros durante su fase de construcción para identificar a los adultos. Una vez que el nido estaba terminado se visitaba cada 4—5 días para determinar su fecha de puesta, y una vez que estaba terminada la puesta se visitaba el nido para determinar el tamaño del nido, el tamaño de puesta, su estatus de parasitismo, su fecha de eclosión y su éxito reproductivo (número de pollos que vuelan del nido y de qué especie).

Experimento de manipulación del esfuerzo reproductivo

Durante las temporadas de cría 2016-2018 se llevó a cabo un experimento en el que se manipuló el esfuerzo reproductivo y el estatus de parasitismo de determinados individuos durante dos temporadas de cría consecutivas. La finalidad de este experimento fue determinar el efecto del esfuerzo reproductivo y el parasitismo sobre la dinámica telomérica de urracas adultas. Para ello se capturaron adultos al principio de la temporada de cría (2016 o 2017), se les extrajo una muestra de sangre y se les asignó uno de los siguientes tratamientos: un pollo de críalo, un pollo de urraca o varios pollos de urraca (entre 3 y 7 pollos de urraca), y se manipuló su nidada de acuerdo al tratamiento que se le había asignado durante dos temporadas de cría seguidas, de forma que algunos individuos criaron dos años consecutivos un pollo de críalo, otros un pollo de urraca y otros una pollada de entre 3 y 7 pollos de urraca (Fig. 4). Después de la segunda temporada de cría, se volvió a capturar a los individuos y se tomó una segunda muestra de sangre. De las dos muestras de sangre de cada individuo se obtuvo ADN y dos medidas relativas de su longitud telomérica y se pudo estimar una tasa de acortamiento telomérico y analizar si los tratamientos de nidada tenían un efecto diferencial sobre esa tasa (metodología más detallada en Capítulo 2).

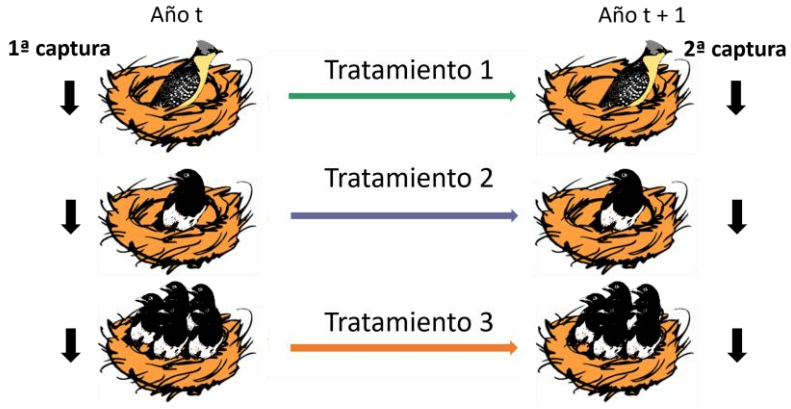


Figura 4: Esquema ilustrativo del diseño del experimento de manipulación de nidada.

Trabajo de laboratorio

Todas las muestras de sangre fueron llevadas al Laboratorio de Ecología Molecular del Departamento de Ciencias Animales y de Plantas de la Universidad de Sheffield en el Reino Unido para su análisis.

Extracción de ADN y determinación del sexo

Los eritrocitos de las aves presentan núcleo, por lo que a partir de muestras de sangre se puede extraer ADN fácilmente. De cada muestra de sangre obtenida en el campo y preservada en etanol absoluto se extrajo ADN mediante el método de precipitación con acetato de amonio (Richardson et al. 2001). Cada muestra de ADN fue diluida con agua purificada y preservada a -20 ºC para su posterior análisis. Lo primero que se hizo con las muestras de ADN fue determinar el sexo de cada individuo mediante reacción en cadena de la polimerasa (PCR) utilizando los cebadores específicos de sexo en aves P2/P8 (Griffiths et al. 1998) y Z43B (Dawson et al. 2016) en reacciones separadas. El producto de las amplificaciones fue analizado un secuenciador 3730 DNA Analyzer (Applied Biosystems) y el programa ABI Genemapper Software versión 3.7.

Medida relativa de la longitud telomérica

Uno de los objetivos de esta tesis es realizar una estima de la longitud telomérica de determinados individuos con diferentes fines (ver capítulos 2, 3 y 4). Existen diferentes métodos para estimar la longitud de los telómeros (Nakagawa et al. 2004. Nussey et al. 2014) y en este estudio se ha utilizado la PCR cuantitativa o a tiempo real (Cawthon 2002). Esta técnica permite estimar las cantidad relativa de secuencia telomérica (T) presente en una muestra de ADN en función de la cantidad de una secuencia no telomérica de referencia no variable en número de copias (S), permitiendo obtener un ratio entre las dos cantidades (T/S). En una PCR cuantitativa (qPCR) la cantidad de ADN amplificada es estimada en tiempo real en cada ciclo de amplificación: esto se consigue añadiendo colorantes fluorescentes que se unen al ADN de doble cadena que se va amplificando y estableciendo un punto de detección de fluorescencia en cada ciclo de amplificación. En un ciclo determinado (denominado Cq), la fluorescencia que ha ido aumentando de forma exponencial cruza un umbral fijo, y es proporcional a la cantidad de ADN molde presente en la muestra. En el caso de la medida relativa

telomérica se amplifican dos secuencias, la telomérica y la de referencia, y se pueden amplificar en reacciones separadas (Cawthon 2002), o en una reacción multiplex y monocromática (Cawthon 2009), que es el método que se utiliza en este estudio. Para ello se usan los cebadores específicos teloméricos telc y telg diseñados por Cawthon (2009), y el gen de la enzima Gliceraldehído-3-fosfato deshidrogenasa (GAPDH) como secuencia de referencia. Existen cebadores descritos para amplificar el gen que codifica esta enzima en aves (Criscuolo et al. 2009) y han sido utilizados con urracas en reacciones en singleplex (Soler et al. 2015). Sin embargo, la secuencia para este gen en urracas difiere bastante de la de estos cebadores. Los cebadores podían formar dímeros con los cebadores telc y telg en reacciones multiplex (ambos hechos podrían comprometer la eficiencia de la amplificación) y además el producto obtenido fue excesivamente largo (lo que podría provocar que la secuencia telomérica, al ser más corta, se amplificara con preferencia y las amplificaciones no fueran comparables), por lo que se diseñaron un par de cebadores para amplificar una parte del gen Gliceraldehído-3-fosfato deshidrogenasa (GAPDH) a partir de la sequencia de Genbank de urraca EF052752.

Telc y telg amplifican un producto de longitud fija, lo que hace que tengan una temperatura de fusión fija, y esta característica es la que va a permitir que la amplificación de la secuencia telomérica y de GAPDH se puedan llevar a cabo en la misma reacción. Además, a los cebadores de GAPDH se les añade una cola de Guanina-Citosina para aumentar su temperatura de fusión, y retrasar su amplificación con respecto a la de la secuencia telomérica dentro del mismo ciclo de amplificación. Al principio de cada ciclo hay una etapa a alta temperatura en la que todo el ADN se desnaturaliza, tras lo cual la temperatura baja hasta alcanzar la temperatura de alineamiento de la secuencia telomérica y ésta se amplifica; en ese momento en que el único ADN de cadena doble es la secuencia telomérica recién amplificada se mide la fluorescencia de la muestra. A continuación la temperatura aumenta de manera que la secuencia telomérica se desnaturaliza y alcanza la temperatura de alineamiento de GAPDH, que empieza a amplificarse y es el único ADN de cadena doble en la muestra, momento en el que se mide otra vez la fluorescencia de la muestra (Fig. 5). De esta forma, en un mismo ciclo se va a medir la fluorescencia de la muestra en dos momentos que van a corresponder con cada una de las secuencias (Fig. 5).

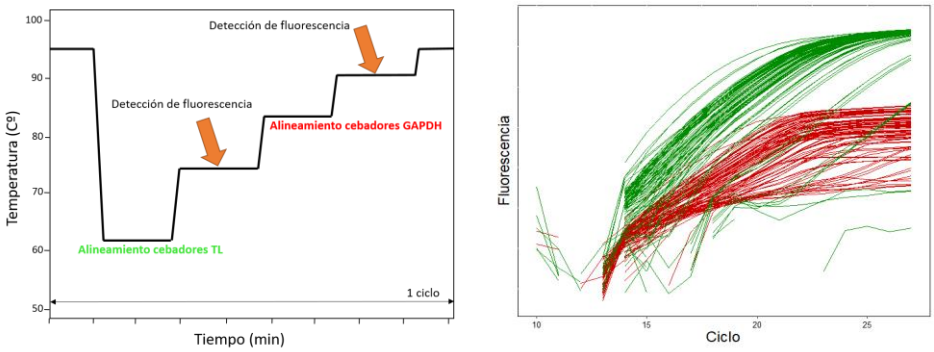


Figura 5: Esquema ilustrativo del funcionamiento del la PCR multiplex monocromática para la medida de la longitud telomérica en un ciclo de amplificación (izquierda). Plot de amplificación (derecha) de la secuencia telomérica (verde) y GAPDH (rojo); cada línea verde está asociada a una roja y corresponden a la misma muestra.

La cantidad de ADN amplificado tras cada ensayo de cada muestra se extrapola comparando los puntos en que la florescencia atraviesa el umbral Cq con los puntos en que la florescencia atraviesa los umbrales Cq de una curva patrón construida a partir de diluciones seriadas de concentración conocida de una muestra de referencia, para la secuencia telomérica y para GAPDH independientemente. Estas curvas se pueden linearizar usando el logaritmo de las concentraciones de la muestra de referencia (Fig. 6) y a partir de ella se calcula la eficiencia de la amplificación de cada secuencia de la siguiente forma (Bustin et al. 2009):

$$E = (10^{(-1/\text{pendiente})} - 1) \times 100$$

Usando la pendiente de la recta patrón de la secuencia telomérica y de GAPDH, respectivamente. A partir de esta recta patrón se calcula la cantidad de secuencia telomérica (T) y la cantidad de secuencia de GAPDH (S) amplificada en cada muestra según las ecuaciones (Ruijter et al. 2009):

$$T = 10^{\wedge}((\log(Nt_{TL}/E_{TL}^{Cq}) - \text{intercepto}_{\text{patrónTL}})/\text{pendiente}_{\text{patrónTL}})$$

$$S = 10^{\wedge}((\log(Nt_{GAPDH}/E_{GAPDH}^{Cq}) - \text{intercepto}_{\text{patrónGAPDH}})/\text{pendiente}_{\text{patrónGAPDH}})$$

Donde Nt es el umbral, Cq es el ciclo en el que la fluorescencia atraviesa el umbral (para la amplificación de la secuencia telomérica y GAPDH por separado), y el

intercepto y la pendiente de las rectas patrón (de la secuencia telomérica y GAPDH por separado). A partir de T y S obtenemos el ratio T/S, que debe ser proporcional a la longitud telomérica media.

La secuencia de los cebadores que usamos fue la siguiente:

Telc- TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA
Telg- ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT
GAPDH-Magpie-F-5'-CGGCGGCGGGCGGCGCGGGCTGGGCGGAGCC-AAAGTGGCTCCAATCCCACT-3'
GAPDH-Magpie-R-5'-GCCCGGCCCGCCGCGCCCGTCCCGCCG-CTGAACTCCCATCCACCCT-3'

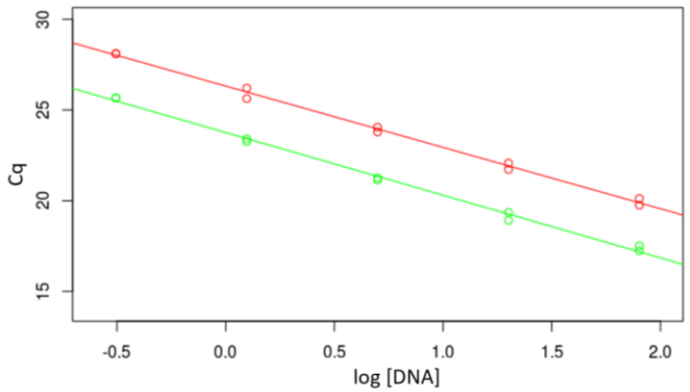


Figura 6: Rectas patrón construidas a partir de cinco diluciones seriadas de la misma muestra de referencia (verde: secuencia telomérica; rojo: GAPDH).

Se optimizó la técnica para estos cebadores probando diferentes concentraciones de los mismos (que fueron desde 600 a 1200nM), y finalmente se utilizó una concentración de 900nM para cada uno y una concentración de ADN por reacción de 25 nanogramos/microlitro. El volumen de la reacción fue 20 microlitros, de los cuales 10 microlitros eran SYBR Select Master Mix (Applied Biosystems). Todas las muestras fueron analizadas por duplicado en placas ópticas de 96 pocillos MicroAmp y selladas con film adhesivo óptico MicroAmp (Applied Biosystems). Cada placa incluyó dos controles negativos (NTC) y diluciones seriadas de ADN de la misma muestra de referencia (concentraciones 80, 20, 5, 1.25, 0.3125 nanogramos/microlitro) por duplicado para generar la recta patrón. Todos los

ensayos se llevaron a cabo en un instrumento de qPCR QuantStudio 12K Flex (ThermoFisher SCIENTIFIC) con el siguiente perfil térmico: etapa inicial: 2 min a 50ºC, 2 min a 95ºC; Etapa 1 (2 ciclos): 15 s a 94ºC, 10 s a 49ºC; Etapa 2 (40 ciclos): 15 s a 94ºC, 10 s a 62ºC, 15 s a 74ºC (con detección de fluorescencia para la amplificación telomérica), 10 s a 84ºC, 15 s a 86ºC (con detección de fluorescencia para la amplificación de GAPDH); Etapa de curva de fusión (con detección de fluorescencia): 15 s a 95ºC, 1 min a 60ºC, 15s a 95ªC. Ésta última etapa consiste en la desnaturalización paulatina de todo el ADN de la muestra con detección de fluorescencia, y a partir de estos datos podemos obtener una curva de disociación de ADN frente a la temperatura (curva de fusión), con la que podremos verificar la existencia de los dos productos de la amplificación (Fig. 7) y la especificidad de los cebadores (Morinha et al. 2020).

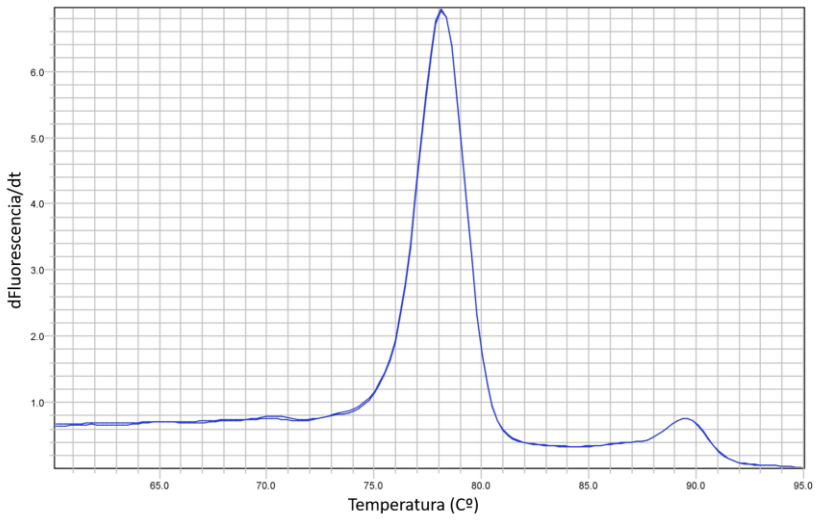


Figura 7: Curva de fusión de una muestra analizada por duplicado tras finalizar un ensayo.

El número de ensayos de cada estudio, así como las eficiencias de amplificación, el ajuste a la recta patrón y la variabilidad intra- e inter-ensayo están especificados en cada capítulo (Capítulos 2, 3 y 4).

Referencias

Álvarez, F. y Arias de Reyna, L. 1974a. Reproducción de la urraca, *Pica pica*, en Doñana. - Doñana, Acta Vertebr. 1: 77–96.

Álvarez, F. y Arias de Reyna, L. 1974b. Mecanismos de parasitación por *Clamator glandarius* y defensa por *Pica pica*. - Doñana Acta Vert. 1: 43–66.

Arias de Reyna, L. Recuerda, P., Corvillo, M. y Cruz, A. 1984. Reproducción de la urraca (*Pica pica*) en Sierra Morena (Andalucía). - Doñana, Acta Vertebr. 11: 79–92.

Avilés, J. M., Bootello, E. M., Molina-Morales, M. y Martínez, J. G. 2014. The multidimensionality of behavioural defences against brood parasites: Evidence for a behavioural syndrome in magpies? - Behav. Ecol. Sociobiol. 68: 1287–1298.

Baglione, V., Bolopo, D., Canestrari, D., Martínez, J. G., Roldan, M., Vila, M. y Soler, M. 2017. Spatiotemporal variation of host use in a brood parasite: The role of the environment. - Behav. Ecol. 28: 49–58.

Birkhead, T. R. 1991. The Magpies: The Ecology and Behaviour of Black-Billed and Yellow-Billed Magpies. - T & A D Poyser.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J. y Wittwer, C. T. 2009. The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. - Clin. Chem. 55: 611–622.

Cawthon, R. M. 2002. Telomere measurement by quantitative PCR. - Nucleic Acids Res. 30: 1–6.

Cawthon, R. M. 2009. Telomere length measurement by a novel monochrome multiplex quantitative PCR method. - Nucleic Acids Res. 37: e21–e21.

Criscuolo, F., Bize, P., Nasir, L., Metcalfe, N. B., Foote, C. G., Griffiths, K., Gault, E. A. y Monaghan, P. 2009. Real-time quantitative PCR assay for measurement of avian telomeres. - J. Avian Biol. 40: 342–347.

Dawson, D. A., dos Remedios, N. y Horsburgh, G. J. 2016. A new marker based on the avian spindlin gene that is able to sex most birds, including species problematic to sex with CHD markers. - Zoo Biol. 35: 533–545.

De Neve, L. y Soler, J. J. 2002. Nest-building activity and laying date influence female reproductive investment in magpies: An experimental study. - Anim. Behav. 63: 975–980.

Griffiths, R., Double, M. C., Orr, K., Dawson, D. A. y Robert, J. G. 1998. A DNA test to sex most birds. - Mol. Ecol. 7: 1071–1075.

Ibáñez-Álamo, J. D., Rühmann, J., Pérez-Contreras, T. y Soler, M. 2019. Migration behavior and performance of the great spotted cuckoo (*Clamator glandarius*). - PLoS One 14: 1–20.

Lotem, A., Nakamura, H. y Zahavi, A. 1995. Constraints on egg discrimination and cuckoo-host co-evolution. - Anim. Behav. 49: 1185–1209.

Martín-Gálvez, D., Soler, J. J., Martínez, J. G., Krupa, A. P., Richard, M., Soler, M., Møller, A. P. y Burke, T. 2006. A quantitative trait locus for recognition of foreign eggs in the host of a brood parasite. - J. Evol. Biol. 19: 543–550.

Martinez, J. G., Burke, T., Dawson, D., Soler, J. J., Soler, M. y Møller, A. P. 1998. Microsatellite typing reveals mating patterns in the brood parasitic great spotted cuckoo (*Clamator glandarius*). - Mol. Ecol. 7: 289–297.

Martínez, J. G. 2011. Urraca – *Pica pica.* - Encicl. Virtual los Vertebr. Españoles

Martínez, J. G., Soler, J. J., Soler, M. y Burke, T. 1998. Spatial patterns of egg laying and multiple parasitism in a brood parasite: A non-territorial system in the great spotted cuckoo (*Clamator glandarius*). - Oecologia 117: 286–294.

Martínez, J. G., Molina-Morales, M., Precioso, M. y Avilés, J. M. 2020. Age-related brood parasitism and egg rejection in magpie hosts. - Am. Nat. 195: 876–885.

Molina-Morales, M., Gabriel Martínez, J., Martín-Gálvez, D., A. Dawson, D., Rodríguez-Ruiz, J., Burke, T. y Avilés, J. M. 2013. Evidence of long-term structured cuckoo parasitism on individual magpie hosts. - J. Anim. Ecol. 82: 389–398.

Molina-Morales, M., Martínez, J. G., Martín-Gálvez, D., Dawson, D. A., Burke, T. y Avilés, J. M. 2014. Cuckoo hosts shift from accepting to rejecting parasitic eggs across their lifetime. - Evolution 68: 3020–3029.

Molina-Morales, M., Martínez, J. G. y Avilés, J. M. 2016. Criteria for host selection in a brood parasite vary depending on parasitism rate. - Behav. Ecol. 27: 1441–1448.

Morinha, F., Magalhães, P. y Blanco, G. 2020. Different qPCR master mixes influence telomere primer binding within and between bird species. - J. Avian Biol. 51: 1–9.

Nakagawa, S., Gemmell, N. J. y Burke, T. 2004. Measuring vertebrate telomeres: Applications and limitations. - Mol. Ecol. 13: 2523–2533.

Nussey, D. H., Baird, D., Barrett, E., Boner, W., Fairlie, J., Gemmell, N., Hartmann, N., Horn, T., Haussmann, M., Olsson, M., Turbill, C., Verhulst, S., Zahn, S. y Monaghan, P. 2014. Measuring telomere length and telomere dynamics in evolutionary biology and ecology. - Methods Ecol. Evol. 5: 299–310.

Richardson, D. S., Jury, F. L., Blaakmeer, K., Komdeur, J. y Burke, T. 2001. Parentage assignment and extra-group paternity in a cooperative breeder: the Seychelles warbler (*Acrocephalus sechellensis*). - Mol. Ecol. 10: 2263–2273.

Rothstein, S. I. 1975. Mechanisms of avian egg-recognition: Do birds know their own eggs? - Anim. Behav. 23: 268–278.

Ruijter, J. M., Ramakers, C., Hoogaars, W. M. H. H., Karlen, Y., Bakker, O., van den Hoff, M. J. B. B. y Moorman, A. F. M. M. 2009. Amplification efficiency: Linking baseline and bias in the analysis of quantitative PCR data. - Nucleic Acids Res. 37: e45–e45.

Soler, M. 1990. Relationships between the Great Spotted Cuckoo *Clamator glandarius* and Its Corvid Hosts in a Recently Colonized Area. - Ornis Scand. 21: 212–223.

Soler, M. y Møller, A. P. 1990. Duration of sympatry and coevolution between the great spotted cuckoo and its magpie host. - Nature 343: 748–750.

Soler, M. y Soler, J. J. 1991. Growth and Development of Great Spotted Cuckoos and Their Magpie Host. - Condor 93: 49–54.

Soler, M., Palomino, J. J., Martinez, J. G. y Soler, J. J. 1995a. Communal parental care by monogamous magpie hosts of fledgling great spotted cuckoos. - Condor 97: 804–810.

Soler, J. J., Soler, M., Møller, A. P. y Martínez, J. G. 1995b. Does the great spotted cuckoo choose magpie hosts according to their parenting ability? - Behav. Ecol. Sociobiol. 36: 201–206.

Soler, M., Martínez, J. G., Soler, J. J. y Møller, A. P. 1995c. Preferential allocation of food by magpies Pica pica to great spotted cuckoo Clamator glandarius chicks. - Behav. Ecol. Sociobiol. 37: 7–13.

Soler, M., Soler, J. J., Martinez, J. G. y Moller, A. P. 1995d. Magpie host manipulation by great spotted cuckoos: Evidence for an avian mafia? - Evolution 49: 770–775.

Soler, M., Martînez, J. G. y Soler, J. J. 1996. Effects of brood parasitism by the great spotted cuckoo on the breeding success of the magpie host: an experimental study. - Ardeola 43: 87–96.

Soler, M., Soler, J. J. y Martínez, J. G. 1997. Great spotted cuckoos improve their reproductive success by damaging magpie host eggs. - Anim. Behav. 54: 1227–1233.

Soler, J. J., Soler, M., Pérez-Contreras, T., Aragón, S. y Møller, A. P. 1999a. Antagonistic antiparasite defenses: nest defense and egg rejection in the magpie host of the great spotted cuckoo. - Behav. Ecol. 10: 707–713.

Soler, J. J., Martinez, J. G., Soler, M. y Møller, A. P. 1999b. Genetic and geographic variation in rejection behavior of cuckoo eggs by European magpie populations: An experimental test of rejecter-gene flow. - Evolution 53: 947–956.

Soler, J. J., Sorci, G., Soler, M. y Møller, A. P. 1999c. Change in host rejection behavior mediated by the predatory behavior of its brood parasite. - Behav. Ecol. 10: 275–280.

Soler, J. J., Neve, L. de, Soler, M., y Martínez, J. G. 2001. Nest size affects clutch size and the start of incubation in magpies: An experimental study. - Behav. Ecol. 12: 301–307.

Soler, J. J., Aviles, J. M., Soler, M. y Møller, A. P. 2003. Evolution of host egg mimicry in a brood parasite, the great spotted cuckoo. - Biol. J. Linn. Soc. 79: 551–563.

Soler, M., Pérez-Contreras, T. y de Neve, L. 2014a. Great Spotted Cuckoos Frequently Lay Their Eggs While Their Magpie Host is Incubating. - Ethology 120: 965–972.

Soler, M., Pérez-Contreras, T., Ibáñez-Álamo, J. D., Roncalli, G., Macías-Sánchez, E. y de Neve, L. 2014b. Great Spotted Cuckoo Fledglings Often Receive Feedings from Other Magpie Adults than Their Foster Parents: Which Magpies Accept to Feed Foreign Cuckoo Fledglings? - PLoS One 9: e107412.

Soler, J. J., Ruiz Castellano, C., Martínez-de la Puente, J., Tomás, G., Ruiz-Rodríguez, M. y Figuerola, J. 2015. Telomere dynamics in parasitic great spotted cuckoos and their magpie hosts. - J. Evol. Biol. 28: 1610–1617.

Capítulo 1

Brood parasitism, provisioning rates and breeding phenology of male and female magpie hosts

Este capítulo reproduce íntegramente el artículo:

Precioso, M., Molina-Morales, M., Sánchez-Tójar, A., Avilés, J. M. and Martínez, J. G. 2020. Brood parasitism, provisioning rates and breeding phenology of male and female magpie hosts. - J. Avian Biol.: jav.02522.

Brood parasitism, provisioning rates and breeding phenology of male and female magpie hosts

Marta Precioso[1], Mercedes Molina-Morales[1], Alfredo Sánchez-Tójar[2], Jesús M. Avilés[3] and Juan G. Martínez[1]

[1]Departamento de Zoología, Facultad de Ciencias, Universidad de Granada, Spain
[2]Department of Evolutionary Biology, Bielefeld University, Germany
[3]Departamento de Ecología Funcional y Evolutiva, Estación Experimental de Zonas Áridas (EEZA-CSIC), Almería, Spain.

Abstract

Parental care is a costly behaviour that raises the prospects of offspring survival. In species with biparental care these costs are shared by both parents, although there may be a conflict regarding the relative investment of each sex. Avian brood parasites leave all the costs of rearing offspring to their hosts. The magnitude of these costs and their consequences on the relative role of both sexes in parental care and future reproduction remain mostly unknown. Here, we investigate whether provisioning rate of nestlings by magpie hosts (*Pica pica*) differs between broods parasitized by the great spotted cuckoo (*Clamator glandarius*) and non-parasitized broods, and whether the relative contribution of each sex to provisioning is affected by parasitism. Furthermore, we explore the effect of parasitism on magpie's future reproduction. We found that provisioning rate was similar in parasitized and non-parasitized broods, and that the relative contribution of males and females was also similar, irrespectively of the parasitism status. However, rearing parasitic offspring seems to have a negative long-term effect on magpie's breeding phenology in the following breeding season. Our results suggest that, although brood parasitism by great spotted cuckoos does not seem to influence the relative contribution of both sexes to parental care, it may entail long-term extra costs in terms of breeding delay for magpies.

Introduction

Parental care comprises any investment made by parents that increases the prospects of survival of their progeny (Clutton-Brock 1991). Because parental investment has associated costs in terms of fecundity and/or survival for the caring parents (Trivers 1972, Stearns 1992) different species have evolved distinct strategies to optimize the trade-off between benefits and costs of parental care (Clutton-Brock 1991). In species with biparental care, such as most bird species, the offspring survival relies on the effort of both parents (e.g. Schroeder et al. 2013); however, the relative investment of each parent might differ. How much effort to invest can be influenced by many factors, such as adult sex-specific longevity(Bonduriansky et al. 2008), food availability (e.g. Eldegard and Sonerud 2009), extra-pair paternity (e.g. Møller and Cuervo 2000), and brood size (e.g. Komdeur et al. 2002). Furthermore, each member of the pair can adjust its parental effort to that of its mate by either matching it (Johnstone and Hinde 2006) or exploiting it (Houston et al. 2005, Harrison et al. 2009), although there may be a continuum of possibilities between matching and exploiting a mate's effort (Johnstone and Hinde 2006).

Avian brood parasites lay their eggs in the nests of hosts, and hosts assume all the parental duties of rearing the parasitic offspring (Davies 2000, Payne 2005). Brood parasitism entails short-term costs for the hosts, being the reduction in current reproductive success the main one. Some brood parasites (e.g. *Cuculidae*) reduce their host's breeding success to zero through, for example, the eviction of host eggs performed by parasitic cuckoo nestlings; whereas in other species (e.g. *Molothrus sp.*), the negative effect of brood parasitism can be less drastic, and parasitic nestlings are regularly raised together with host nestlings (Rothstein 1990). In any case, brood parasitism imposes a strong selective pressure on hosts that has driven the evolution of defences against brood parasites, which, as a consequence, has favoured the evolution of counter-adaptations in brood parasites which facilitate host exploitation, leading in some instances to coevolutionary arms races (Rothstein 1990, Soler 2014).

Brood parasitism might also influence host parental care (Krüger 2007). For instance, studies on brood parasite–host systems where parasitic and host nestlings do not share the nest have either failed to find statistically significant differences in provisioning rate between parasitized and non-parasitized nests (Kilner et al. 1999, Mark and Rubenstein 2013, Samaš et al. 2019), or found lower provisioning rates in

parasitized nests (Soler et al. 1995b, Požgayová et al. 2015, Samaš et al. 2018). In contrast, studies on brood parasite–host systems in which parasitic and host nestlings share the nest (e.g. some species of *Molothrus* and their hosts) have shown that provisioning rate tends to be higher in parasitized than non-parasitized nests (Dearborn et al. 1998, Hoover and Reetz 2006, Grayson et al. 2013, but see Canestrari et al. 2014 for a non-cowbird brood parasite where provisioning rates are lower in parasitized nests). Furthermore, brood parasitism could also affect the relative parental contribution of males and females, for example, by affecting brood size (Komdeur et al. 2002), a possibility seldom explored. For example, Požgayová et al. (2015) found that males work less in great reed warbler (*Acrocephalus arundinaceus*) nests parasitized by the common cuckoo (*Cuculus canorus*) than in non-parasitized nests, even in monogamous pairs, which constitute an important part of the population. Contrastingly, red-winged blackbird (*Agelaius phoeniceus*) nests parasitized by the brown-headed cowbird (*Molothrus ater*) were more likely to receive care from the male than non-parasitized nests (Grayson et al 2013). Hence the potential effect of brood parasitism on the relative role of each sex might be species-specific, and overall, the potential effect of brood parasitism on parental care remains poorly understood.

Beyond detrimental effects on the parasitized breeding attempt, brood parasitism may also have long-term consequences and impact adult host survival and future reproduction. Life-history theory predicts that current reproductive effort compromises future reproductive output by increasing adult mortality and/or decreasing the capacity to invest in future reproduction (Stearns 1989, Wedell et al. 2006). So far the long-term consequences of raising brood parasites are not well understood and most studies have focused on host breeding dispersal, survival and return rates in response to previous brood parasitism. These studies have found that brood parasitism either has no clear effect on host survival and return rates (Brooker and Brooker 1996, Payne and Payne 1998, Hoover 2003a, Hauber 2006, Samaš et al. 2019), or that it can affect them negatively (Hoover and Reetz 2006, Mark and Rubenstein 2013, Koleček et al. 2015). By contrast, parasitism does not seem to affect breeding dispersal distances between years (Hoover 2003a, b, Sedgwick 2004, Hoover and Reetz 2006, Koleček et al. 2015) or may even reduce dispersal distances of some male hosts (Molina-Morales et al. 2012). However, brood parasitism, through the costs of parental care, may potentially mediate long-term changes in life-history traits such as clutch size or laying date, a possibility seldom considered. Up

to date, only two studies have evaluated this scenario. Hauber (2006) found no statistically significant effect of parasitism on clutch size or laying date in second breeding attempts in eastern phoebes (*Sayornis phoebe*), even though parasitic cowbird nestlings were capable of increasing host provisioning rates (Hauber and Montenegro 2002). In contrast, parasitism by the Central America striped cuckoo (*Tapera naevia*) negatively affects rufous-and-white wren (*Thryophilus rufalbus*) hosts' re-nesting phenology and the probability of breeding in the subsequent season (Mark and Rubenstein 2013). Altogether, these mixed results imply that the long-term consequences of brood parasitism on adult host survival or future reproduction remain poorly understood.

In this study we explore both short- and long-term effects of brood parasitism in magpies (*Pica pica*), a common host of the great spotted cuckoo (*Clamator glandarius*). Great spotted cuckoo parasitism often reduces magpie's current breeding success to zero, and in most parasitized nests cuckoo nestlings are reared alone or together with other cuckoo nestlings (i.e. multiparasitism; Martínez et al. 1998). Soler et al. (1995a) have previously reported that the total amount of food delivered in non-parasitized magpie nests is higher than that delivered in both natural and experimentally parasitized nests, suggesting that magpies could be working less when rearing parasitic cuckoos. Moreover, Buitron (1988) found that male' provisioning rates were significantly higher than those of females, but this study was carried out in a non-parasitized magpie population. Changes in host parental workload and in the relative role of each sex in response to parasitism, as above explained, may have long-term life-history effects worth investigating.

To address these issues here we first quantify provisioning rate (as a proxy to parental workload) in parasitized nests and compared it to that in non-parasitized nests. We predict i) that provisioning rates should be smaller in parasitized nests due to the smaller number of nestlings in the nests. We explore the relative role of males and females in provisioning in a context of parasitism. As a consequence of the lower brood size in parasitized nests (see Methods), ii) we expect that males should reduce their provisioning rates as it has been found in other hosts (Požgayová et al. 2015). Finally, to identify possible long-term consequences of raising parasitic nestlings for hosts, we investigate whether parasitism status and magpie provisioning rates in a breeding attempt can influence its presence in the breeding population and its breeding phenology in the subsequent breeding season. We predict iii) a negative effect of increased provisioning rates on the probability of host presence and egg

laying dates in the following season. As we predict lower provisioning rates in parasitized nests and the time to fledge for cuckoo nestlings is shorter than that for magpies (Soler and Soler 1991), we expect that parasitized magpies would have higher probability of surviving to the next breeding season and would advance their breeding phenology.

Methods

Study area and system

This study was conducted in La Calahorra (37°10'N, 3°03'W, Granada, Southern Spain) during the breeding seasons (March–July) of 2008–2012 and 2016–2017. The study area is characterized by cereal fields patched with groves of almond trees (*Prunus dulcis*) in which magpies preferentially build their nests. Magpies are territorial, sedentary and socially monogamous (Birkhead 1991, Molina-Morales et al. 2012) and pair bonds have been reported to be long-lasting (Birkhead 1991). In our study area, the percentage of nests parasitized by great spotted cuckoos (hereafter cuckoos) ranges from 15.9% to 65.6%, depending on the year (15.9% in 2007, 25.4% in 2008, 65.6% in 2009, 50.7% in 2010, 55.8% in 2011, 35.6% in 2012, 24.5% in 2016 and 24.4% in 2017; see Molina-Morales et al. 2013, Martínez et al. 2020). Cuckoo eggs hatch earlier than magpie eggs (Soler et al. 1997), and so, by the time magpie eggs hatch, cuckoo nestlings are already 4–5 days old (Soler and Soler 1991) and monopolize feeds, leading in most cases to the starvation of magpie nestlings.

Individual marking and sex assignment

This magpie population is under long-term monitoring since 2005 (Molina-Morales et al. 2013, Avilés et al. 2014, Molina-Morales et al. 2014, Martínez et al. 2020) and a large number of individuals have been ringed either shortly before fledgling (15–18 days old) or captured and marked as adults with a unique combination of colour rings. At the time of ringing, a blood sample was collected from each individual by puncturing the brachial vein with a sterile needle. Since magpies are only slightly dimorphic in size, individual sex was assigned by extracting DNA from each blood sample and using the sex-specific primers P2/P8 (Griffiths et al. 1998) and Z43B (Dawson et al. 2016).

Nest and pair monitoring

Magpie nests were monitored from the beginning of March each year, when the earliest pairs start building their nests, to the beginning of July, when the last fledglings leave the nest. Nests were found by careful examination of all trees in the study area and GPS positioned. To identify the individuals of each nest, we observed the nests during the nest building stage using a telescope. Observations were performed from a hide or a car, 70 to 100 meters away from the nest. Nests were visited every four to five days to record laying date, parasitism status, clutch size, hatching date, number of eggs hatched whenever possible, and number of chicks fledged (estimated as the number of chicks in the nest 15–18 days after hatching).

Laying and hatching dates were estimated as the number of days after the first of April. All dates were expressed as deviations from the annual average laying date of each year (mean-centered by year) to account for inter-annual variability in phenology. As nest size has been suggested to be a signal of parental ability in magpies (Soler et al. 1995a, De Neve et al. 2004b), after the clutch was completed, the nests were measured. Nests were assumed to be spheroidal and their volume was estimated from their height and width, which were measured using a measuring tape (precision within 1cm), and calculated as $4/3(\pi \times a \times b^2)/1000$ (in litres), where a is the largest radius of the nest and b is the smallest radius of the nest (Soler et al. 1995a).

Provisioning rate

We estimated provisioning rate (Schroeder et al. 2013, Bowers et al. 2014) of males and females only in nests where at least one of the caring parents was ringed, so that we were able to distinguish between male and female. Unringed individuals involved in provisioning observations could have changed their partner during the study period, and thus there is a possibility that we observed the same unringed individual twice in two different years, although we think that it is unlikely because magpie pair bonds are long-lasting (Birkhead 1991)

Provisioning observations were carried out between 7 a.m. and 3 p.m., when nestlings were 8–18 days old (cuckoo nestlings ages ranged from 8 to 18 days, mean age = 11.9, median = 11, SD = 3.3; magpie nestlings ages ranged from 9 to 16 days, mean age = 11.9, median = 11.5, SD = 2.2), during the breeding seasons of 2008–2011 and 2016. We observed nests that allowed a clear identification of adults using

a telescope from a hide or a car at a distance of 70 to 100 meters. We registered the number of visits performed by each individual during 90 to 180 minutes from the first visit of an adult to the nest. This duration has been suggested to be adequate to quantify parental effort (Lendvai et al. 2015). Individual provisioning rate was estimated as the number of visits performed per hour. The number of parasitized nests observed was 17, containing a mean of 1.88 nestlings (median = 2, SD = 0.86, ranging from 1 to 4 cuckoo nestlings), while the number of non-parasitized nests observed was 22, containing a mean of 4.54 nestlings (median = 4.5, SD = 1.59, ranging from 1 to 7 magpie nestlings). These mean brood sizes are similar to the mean brood sizes observed at the population level (parasitized nests: 1.65 (0.76) nestlings, median =1, n = 100; non-parasitized nests: 4.33 (1.57) nestlings, median =4, n = 164; data from years 2008–2014). A small percentage of nests in the population contains mixed broods (3%, 8 out of 272 nests, data from years 2008–2014), and due to the scarcity of this type of broods we did not perform observations on them and they were not included in average calculations of brood sizes. Mean brood size significantly differed between parasitized and non-parasitized nests in both cases (our dataset: Mann-Whitney test, U = 33.5, p-value <0.001; population: Mann-Whitney test, U = 1214, p-value <0.001). Most parasitized nests in the population contain 1 or 2 cuckoo nestlings (83% of the parasitized nests), while most non-parasitized nests contain 4 or more magpie nestlings (74% of the non-parasitized nests). Similarly, of the nests observed in this study, 83% of parasitized nests contained 1 or 2 cuckoo nestlings and 82% of non-parasitized nests contained 4 or more magpie nestlings.

Statistical analyses

All statistical analyses were performed in R version 3.6.1 (R Core Team 2019), and all mixed models were constructed using the lme4 package (version 1.1.21, Bates et al. 2015). All continuous variables were z-standardized and categorical variables were mean-centered (Schielzeth 2010). Non-significant interactions were excluded from final models by backward stepwise selection, although results of full models (including those interactions) are shown in Supplementary material, Appendix D.

Provisioning behaviour

Aiming to analyse whether provisioning rates differed between individuals rearing parasitized or non-parasitized broods, we constructed a generalized linear mixed

model (hereafter GLMM) with a Poisson distribution (log link function and log observation duration as an offset) in which the individual number of visits was the dependent variable, and parasitism status (non-parasitized = 0; parasitized =1) and parental sex (male = 0; female = 1) were included as fixed effects. We also incorporated brood size and brood age as continuous fixed effects to the model because they may affect provisioning rates. Nest volume and laying date were also included as continuous fixed effects because they have been suggested as proxies of parental quality in magpies (Soler et al. 1995a, De Neve et al. 2004a, b). Moreover, we included two two-way interactions: one between parental sex and parasitism status to ascertain whether provisioning rate differed between sexes depending on the species being raised, and another one between parasitism status and brood size to separate the effect of both variables. Pair identity and year were firstly included as random effects but the model could not handle two random effects (it did not converge), and so pair id was finally included as a random effect and year was included as a categorical fixed effect (levels: 2008, 2009, 2010, 2011 and 2016).

Since brood size was smaller in parasitized nests (see section "Provisioning behaviour"), there was a strong correlation between parasitism status and brood size (correlation ratio $\eta = 0.72$). Collinearity between two fixed effects increases the standard error of the coefficients estimated for all the other fixed effects in the model, and can reduce the statistical significance of influential predictors (Dormann et al. 2013). We tested the possible effect of collinearity by performing an additional LMM with the same structure as described above, but in which we eliminated one of the correlated fixed effects (as suggested in (Forstmeier and Schielzeth 2011): since brood size was a strong predictor in the model (see Results, Table 1), we did not drop it, so parasitism status was dropped instead, and estimates from this additional model were compared with that of the former model. A variance inflation factor (VIF) was also calculated for both fixed effects, and never exceeded a threshold of five (Sheather 2009). As there were no important differences in the estimates of both models (Table 1 and Supplementary material Appendix A, Table A1), VIF were low, and parasitism status was one of the factors we were most interested in, we decided to keep both fixed effects in the final model. Moreover, these two predictors are not redundant, and the model may lose biological sense without one or the other, besides the fact that this model will be used to draw predictions in the system in which this study was conducted, where collinearity between these two fixed effects should remain constant (Dormann et al. 2013).

Additionally, we tested whether individual provisioning rates differed between non-parasitized nests that contained four or more magpie nestlings (n = 18 nests) and parasitized nests that contain one or two cuckoo nestlings (n = 14 nests), which are the most common situations in our population (see section Provisioning rate). We then constructed a GLMM (Poisson distribution, log link function and log observation duration as an offset) in which the individual number of visits to the nests was the dependent variable and parasitism status, brood age, nest volume, laying date, parental sex and a two-way interaction between parasitism status and sex were included as fixed effects and pair identity was included as a random effect. Year was not included in this model to avoid model over-fitting and because it had little effect on provisioning rates (see Table 1).

Parents' presence and breeding phenology in the following season

We tested whether different variables related to magpies' current reproduction (such as provisioning rate or laying date) could affect adults' presence (the equivalent to return rate) in the population and/or their breeding phenology in the following season. Thus, we firstly constructed a GLMM (binomial distribution, logit link function) in which adults' presence in the breeding population in the subsequent breeding season (year t +1; yes = 1, no = 0) was the dependent variable and the following variables related to the previous breeding season (year t) were included as fixed effects: parasitism status (non-parasitized = 0, parasitized = 1), brood size (continuous variable), provisioning rate (continuous variable), laying date (continuous variable), sex (male = 0, female = 1) and a two-way interaction between parasitism status and brood size. Pair identity was included as a random effect. Year was included as a fixed effect, however, the model could not appropriately handle it, since the estimates for pair id were zero but different from zero if year was excluded from the model; so we decided to finally exclude year, also because it seemed to have little effect on provisioning rates (Table 1). This model included 49 marked individuals (n = 39 nests) involved in provisioning observations of which 26 (n = 24 nests) were observed breeding in the following season.

Secondly, we constructed a linear model (LM) in which laying date in the subsequent breeding season (year t + 1) was the dependent variable and parasitism status (non-parasitized = 0, parasitized = 1), provisioning rate (continuous variable) and laying date (continuous variable) in the previous breeding season (year t) were

included as fixed effects. We also included a two-way interaction between parasitism status and laying date and another one between parasitism status and provisioning rate. This model included 23 of those 26 individuals found breeding in the following season. One individual was excluded because its breeding attempt corresponded to a replacement clutch. This data set included two pairs so one member of each pair was randomly excluded to avoid pseudoreplication and we ran the same model excluding the other member of the pair (Supplementary material, Appendix E, Table A9) and results remained qualitatively similar. Year and sex seemed to have little effect on provisioning rates (Table 1) and laying dates already accounted for the variability between years (they were mean-centered by year), so year and sex were not included in the model in order to avoid model over-fitting. We were unable to analyse other reproductive variables (i.e. clutch size) since a large proportion of those 23 individuals (43%) were parasitized in the following breeding season. Additionally, in the same data set, we tested whether the difference in laying date between two consecutive years (i.e., laying date in t+1 minus laying date in t) differed according to parasitism status in year t. We then constructed a LM fitted by generalized least squares (GLS; R package nlme version 3.1-144 (Pinheiro et al. 2020)) with a variance structure that allowed different variance per parasitism status level (parasitism status showed residual heterogeneity), in which the difference in laying dates between both breeding seasons (year t and year t + 1) was the dependent variable and parasitism status (non-parasitized = 0, parasitized = 1), provisioning rate (continuous variable) and a two-way interaction between them were included as fixed effects. The same model was run excluding the other member of each pair as above (Supplementary material, Appendix E, Table A10).

Results

Provisioning behaviour

Both sexes provisioned nestlings at similar rates regardless of the type of chick reared in the nest (interaction sex and parasitism status; female provisioning rates on non-parasitized nests: mean = 2.91, median = 3.17, SD = 1.43, parasitized nests: mean = 2.83, median = 2.5, SD = 1.56; male provisioning rates on: non-parasitized nests: mean = 3.74, median = 3.67, SD = 1.70, parasitized nests: mean = 2.82, median = 2.5, SD = 1.37; Supplementary material, Appendix D, Table A4). In addition, there were no clear differences in provisioning rates between individuals that reared

cuckoo nestlings (mean = 2.83, median = 2.5, SD = 1.45) and those that reared magpie nestlings (mean = 3.32, median = 3.33, SD = 1.61; Table 1; Fig. 1), not even when controlling for brood size (interaction between brood size and parasitism status; Supplementary material, Appendix D, Table A4). Provisioning rates increased with brood size and brood age (Table 1). The associations of nest volume and laying date with provisioning rates were not statistically significant (Table 1).

Table 1: Factors affecting provisioning rate in magpies (N = 78 individuals, 39 nests).

Fixed effects		β	Lower CI	Upper CI	Z	*p*-value
Intercept		**1.08**	**0.98**	**1.18**	**20.64**	**<0.001**
Parasitism status		0.15	-0.17	0.48	0.92	0.356
Brood size		**0.19**	**0.03**	**0.34**	**2.34**	**0.019**
Brood age		**0.14**	**0.02**	**0.26**	**2.40**	**0.016**
Nest volume		-0.10	-0.22	-0.01	-1.80	0.072
Laying date		0.03	-0.08	0.15	0.57	0.565
Sex		-0.13	-0.32	0.06	-1.35	0.177
Year:	2009	-0.11	-0.54	0.32	-0.50	0.617
	2010	0.07	-0.25	0.39	0.51	0.681
	2011	0.15	-0.29	0.58	0.66	0.507
	2016	0.21	-0.17	0.59	1.09	0.273
Random effect		σ			LRT	*p*-value
Pair identity		0.06			0.06	0.800

Results of a GLMM (Poisson distribution, log link function) testing the effect of parasitism status, brood size, brood age, nest volume, laying date, sex, year and pair identity on provisioning rates in magpies. 95% CI were calculated by the Wald approximation; parameter estimates were calculated by the Gauss-Hermite approximation to the log-likelihood with 25 quadrature points; *p*-values for fixed effects were calculated by a Wald Z test; *p*-value for the random effect was calculated by a likelihood ratio test. Marginal $R^2 = 0.20$; Conditional $R^2 = 0.21$ (calculated following Nakagawa and Schielzeth (2013); MuMIn package, version 1.43.15, Bartoń (2019)). Significant estimates are highlighted in bold.

However, individual provisioning rates significantly differed between parasitized and non-parasitized nests when parasitized broods contained one or two cuckoo nestlings (mean = 2.57, median = 2.29, SD = 1.38) and non-parasitized

broods four or more magpie nestlings (mean = 3.57, median = 3.50, SD = 1.59; Supplementary material, Appendix B, Table A2; Fig. 2).

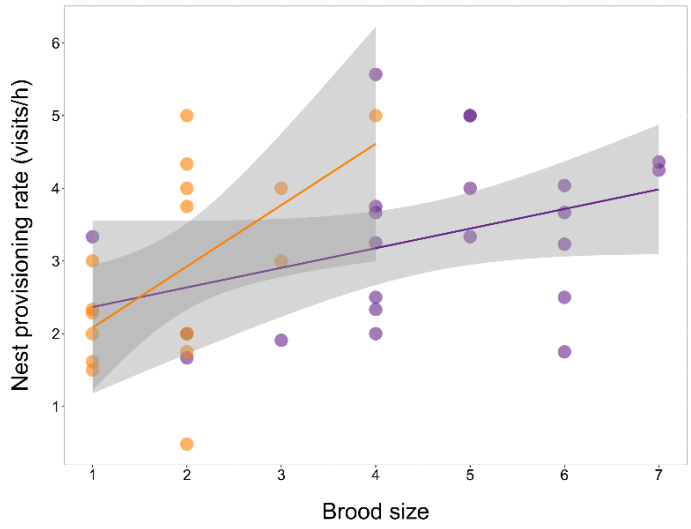


Figure 1: Nest provisioning rates (feeding visits/h) do not significantly differ regarding the nest parasitism status. Each point represents the mean provisioning rate per nest (n = 39 nests). Solid lines represent regression lines and shaded areas represent the 95% confidence interval for non-parasitized (purple circles, n = 22 nests) and parasitized nest (orange circles, n = 17 nests).

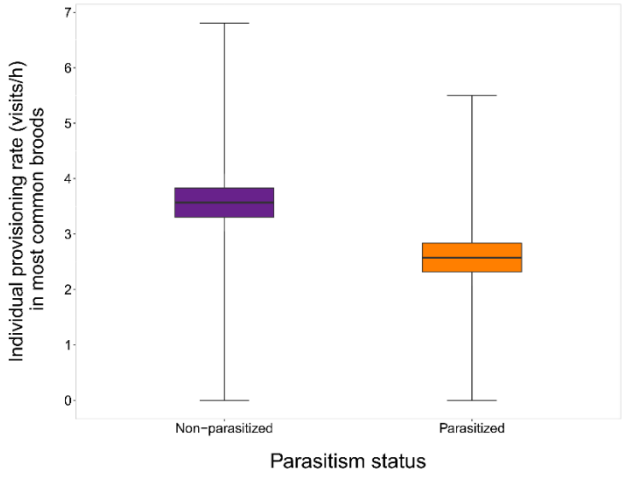


Figure 2: Magpie provisioning rates (feeding visits/h) are higher in non-parasitized nests that contain 4 or more magpie nestlings (purple box, mean = 3.57, SE = 0.27, n = 36 observations, 18 nests) compared to parasitized nests that contain 1 or 2 cuckoo nestlings (orange box, mean = 2.29, SE = 0.26, n = 28 observations, 14 nests). Boxes represent the mean and standard error of magpie provisioning rates. Error bars represent 95% confidence interval.

Parents' presence and breeding phenology in the following season

The presence/absence of adults in a given breeding season was not affected by parasitism status, brood size, provisioning rate or laying date in the previous breeding season and there were no clear differences between males and females in their probability of being present at the study site in the following season (Table 2).

Table 2: Factors affecting adults' presence in the following breeding season (t + 1) (n = 49 individuals, 39 nests).

Fixed effects	β	Lower CI	Upper CI	df	LRT	*p*-value
Intercept	0.16	-0.53	0.85	1	0.23	0.63
Parasitism status in t	0.49	-1.59	2.57	1	0.21	0.64
Brood size in t	0.88	-0.36	2.12	1	2.56	0.11
Provisioning rate in t	0.1	-0.61	0.81	1	0.08	0.77
Laying date in t	-0.58	-1.42	0.26	1	2.49	0.11
Sex	-0.64	-2.14	0.85	1	0.87	0.35
Random effects	σ				LRT	*p*-value
Pair identity	0.53				0.01	0.92

Results of a GLMM (Binomial distribution, logit link function) testing the effect of parasitism status, brood size, provisioning rate, laying date, sex and pair identity on the presence/absence of the adult magpies in the subsequent breeding season. 95% CI were calculated by the Wald approximation; parameter estimates were calculated by the Gauss-Hermite approximation to the log-likelihood with 25 quadrature points; *p*-values for fixed and random effects were calculated by a likelihood ratio test. Marginal R^2 = 0.22; Conditional R^2: = 0.28 (calculated following Nakagawa and Schielzeth (2013); MuMIn package, version 1.43.15, Bartoń (2019)).

On the other hand, laying date in a given season was positively associated with the previous season's laying date, but it was not related to provisioning rates in that previous breeding season and individuals that reared cuckoo nestlings one season bred later in the following season than individuals that reared magpie nestlings (Supplementary material, Appendix C, Table A3). Furthermore, differences in laying dates between two consecutive breeding seasons were not related to provisioning rates in the previous breeding season either, but were larger for parasitized magpies (mean = 2.33, SD = 3.08) compared to non-parasitized ones (mean = -2.29, SD = 6.73; Table 3; Fig 3).

Table 3: Factors affecting the difference in laying dates between two consecutive breeding seasons in magpies (n = 23 individuals).

	β	Lower CI	Upper CI	df	F	*p*-value
Intercept	0	-0.39	0.39	1,20	3.36	0.081
Parasitism status in t	**0.71**	**-0.02**	**1.44**	**1,20**	**4.81**	**0.040**
Provisioning rate in t	-0.09	-0.42	0.24	1,20	0.27	0.606

Results of a LM fitted by GLS testing the effect of parasitism status and provisioning rate in year t on the differences in laying dates between year t and year t + 1 (i.e., laying date in t+1 minus laying date in t). R^2 = 0.15 (piecewiseSEM package, version 2.1.0, Lefcheck (2016)). Significant estimates are highlighted in bold.

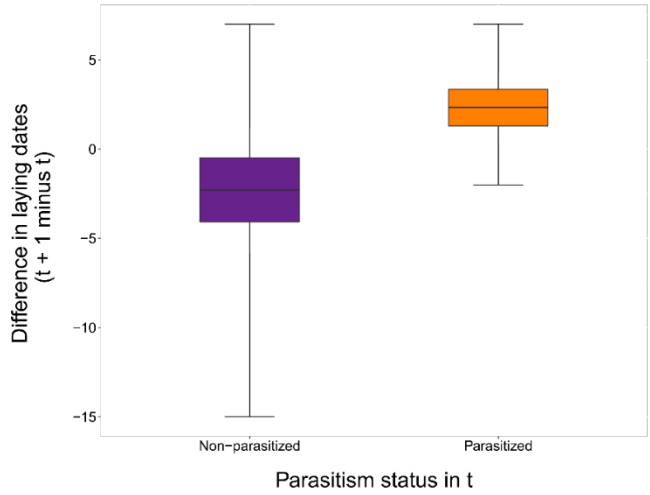


Figure 3: Magpies parasitized in year t delay their breeding in year t + 1 compared to non-parasitized magpies (n = 23 individuals). Boxes represent the mean and standard error of the difference in laying dates between two consecutive breeding seasons (t + 1 minus t) for non-parasitized (purple boxes, mean = -2.29, SE = 1.80) and parasitized magpies (orange boxes, mean = 2.33, SE =1.03). Error bars represent 95% confidence interval. Negative values correspond to an advance in laying dates and positive values correspond to a delay.

Discussion

In this study we explored parental investment of male and female magpie hosts in naturally parasitized and non-parasitized nests, and tested whether parental investment and raising parasitic cuckoos affects future reproductive performance. Our results indicate that magpie provisioning rates were higher in nests with older nestlings and larger broods, which is consistent with Buitron (1988) and Moreno-

Rueda et al. (2007), and with previous studies in passerines. Furthermore, our results show that, contrary to our prediction, there are no significant differences in the provisioning rates between parasitized and non-parasitized nests in general, and parasitism does not affect the relative contribution of sexes. Also, parasitism does not affect the presence/absence of individuals in the following year but, surprisingly, individuals that have been parasitized in a particular year breed later and delay their laying dates compared to non-parasitized individuals in the following year.

.*Nest provisioning rate and parasitism*

Our analyses showed that magpie's provisioning rate did not statistically differ between parasitized and non-parasitized nests (Fig. 1), even though we expected to find a lower provisioning rate in parasitized nests since they contain fewer nestlings. Indeed, our analyses confirmed that, brood size is one of the main predictors of provisioning rate. Besides, Soler et al. (1995b) showed that broods parasitized with a single great spotted cuckoo nestling received less food than non-parasitized broods (mean brood size: 4.1 (SD = 0.3)) in the same area. The absence of clear differences in our first analysis (Table 1) could be due to the fact that our dataset included both non-parasitized nests with a small brood size (i.e. one to three magpie nestlings, 18.2 % of the non-parasitized nests) and parasitized ones with a large brood size (i.e. three or four cuckoo nestlings, 17.6% of the parasitized nests), which occur naturally, but at low frequency in the population (26 % and 17%, respectively). Indeed, when we analysed the subset of observations in nests with the most common brood size (non-parasitized nests containing 4 or more nestlings and parasitized nests containing 1 or 2 nestlings; Supplementary material, Appendix B, Table A2), individual provisioning rates were significantly smaller in parasitized nests (Fig. 2). This suggests that for most parasitized individuals (83% of the parasitized nests in the population contain one or two cuckoo nestlings) rearing a parasitized brood would suppose a smaller nest provisioning effort than rearing a non-parasitized brood (most frequently with 4 or more magpies chicks) although the net gain in terms of fitness would be zero.

In any case, our results are in accordance with the general pattern observed in brood parasite–host systems, suggesting that the consequences of parasitism on host provisioning rates seem to depend upon whether parasitic chicks are reared along with nest mates, increasing then brood size, or not. In the case of brown-headed cowbirds (*Molothrus ater*) whose chicks commonly share the nest with host

chicks, hosts respond to parasitism by increasing their provisioning rates (for example, indigo bunting *Passerina cyanea* (Dearborn et al. 1998), eastern phoebes *Sayornis phoebe* (Hauber and Montenegro 2002), prothonotary warbler *Protonotaria citrea* (Hoover and Reetz 2006) or red-winged blackbirds *Agelaius phoeniceus*(Grayson et al. 2013)). In contrast, in the case of parasites whose nestlings are reared alone in host nests, several studies have found that host provisioning rates were either similar between parasitized and non-parasitized broods (Brooke and Davies 1989, Kilner et al. 1999, Mark and Rubenstein 2013, Samaš et al. 2019, this study), or were lower in parasitized nests. For example, common cuckoo nestlings reared by rufous bush robins (*Cercotrichas galactotes*) received less food than a normal host brood (Martín-Gálvez et al. 2005), or were fed less frequently when they are reared by common redstarts (*Phoenicurus phoenicurus*, Samaš et al. 2018).

Most of these studies are correlational, and as such, could be affected if parasitism was not random. Some parasites choose their hosts according to their characteristics. For example, Brooke and Brooke (1996) found that young or inexperienced splendid fairy-wren females (*Malurus splendens*) were more likely parasitized by the Horsfield's bronze cuckoo (*Chrysococcyx basalis*). Only one of the studies above mentioned has discarded the effect of the host selection by parasites by testing whether provisioning rates differed between experimentally and naturally parasitized nests, finding no clear differences between them (Grayson et al. 2013). In this study we analysed data from naturally parasitized and non-parasitized nests, so our results may be influenced by host choice made by parasites.

Contrary to our prediction and previous work on this species (Buitron 1988), we did not find significant differences between male and female provisioning rates, neither in non-parasitized nor in parasitized nests. Some authors have recently suggested that in bird species with biparental care where partners tend to stay together in long-term pair bonds, changes in the reproductive value of broods may induce a matching response of both sexes in parental care (see for example Mariette et al. 2015). Magpies meet some of these conditions as they are long-lived and have long-term pair bonds (Birkhead 1991). Altogether, our results suggest that cooperation between the sexes prevails in the species and parasitism does not substantially affect the relative contribution of males and females to parental care.

Parental effort and adults' presence and breeding phenology in the following season

Despite the fact that our sample size is modest, our analysis suggests that individual provisioning rates in a given year were unrelated with host's presence/absence at the breeding site or laying date in the following breeding season.

Extra costs imposed by brood parasitism on future reproduction have been reported in some hosts. For example, male and female rufous-and-white wrens that had reared a parasitic Central America striped cuckoo were less likely to breed in the following breeding season (Mark and Rubenstein 2013). In other systems, the effect of parasitism seems to be sex-dependent, hinging on which sex provides more care: prothonotary warbler males (Hoover and Reetz 2006) and great reed warbler females (Koleček et al. 2015) had reduced return rates when they had been previously parasitized by brown-headed cowbird and by common cuckoo, respectively. On the other hand, Samaš et al. (2019) reported that parasitism by common cuckoo does not seem to affect great reed warbler return rates. Our study system differs from those of hosts mentioned above, as magpies are sedentary and stay all year around in the area where they breed. Moreover, parasitism only affects interannual breeding dispersal of magpie males, reducing their dispersal distances (Molina-Morales et al. 2012); therefore the lack of differences in the presence/absence between the previously parasitized and non-parasitized individuals would suggest no costs of cuckoo parasitism in terms of adult magpie survival (Table 2).

Our results, however, have shown that, when accounting for the potential effects of previous year's provisioning rate and laying date, the laying date of individuals in the subsequent season was affected by their parasitism status in the previous year (Supplementary material, Appendix C, Table A3). Specifically, non-parasitized magpies advanced their laying dates in the subsequent breeding season, while parasitized individuals delayed them (Table 3; Fig. 3). This is important since the individuals that breed earlier in the breeding season have higher fledgling success (Birkhead 1991, Soler et al. 1995a). Moreover, delaying reproduction may entail high costs for magpies because the risk of parasitism increases throughout the season in our magpie population (Molina-Morales et al. 2016). Thus, the delay in laying date in the subsequent season of magpies suffering parasitism would be a consequence of an extra cost of parasitism that seems to be independent of the nest provisioning rates. This effect of parasitism on future reproduction might be related to other stages of the parental care period that we have not considered in this study, such as

the post-fledgling stage: Soler et al. (1995c, 2014) found that great spotted cuckoo fledglings are frequently fed by magpies other than their foster parents that had also reared cuckoo chicks that season. Feeding a greater number of cuckoo fledglings than those reared in the nest and maybe, other aspects of the post-fledgling caring period may raise the costs of parental care even further and have cumulative carry over effects that may not affect magpies' apparent survival but their breeding phenology in the future. However, this latter result should be taken with caution due to the small sample size. Further studies should try to evaluate the possible fitness costs of delayed reproduction in magpies due to parasitism.

Conclusions

In summary, magpies' workload (estimated as provisioning rate) in parasitized and non-parasitized nests was similar overall, but smaller in parasitized nests when comparing the most common brood size of parasitized nests (1 or 2 cuckoo nestlings) versus the most common brood size of non-parasitized nests (4 or more magpie nestlings). Brood parasitism did not seem to modify the relative contribution of host males and females to nestling provisioning. Moreover, rearing parasitic broods did not influence hosts' apparent survival (neither for males nor females) but seemed to negatively affect their breeding phenology in the subsequent season by delaying breeding and possibly increasing the likelihood of being parasitized. This suggests the possibility of extra post-fledging costs of parasitism for magpies that would be worth investigating in the future. Our results stress the need of evaluating the costs of parasitism at all breeding stages as well as its effect on different hosts' life history traits. This study provides valuable information about the short- and long-term costs of parasitism in magpies, but further research about the long-term costs of parasitism should be done to draw stronger conclusions about the role of parental care on great spotted cuckoo and magpie interactions.

Acknowledgments

We thank Vicente Serrano for his assistance in the field. We also would like to thank Milica Požgayová, Judith Morales and an anonymous reviewer for their valuable comments, which have significantly improved the manuscript.

Funding

This study was supported by the Junta de Andalucía (Project P06-RNM-01862) and the Spanish Ministry of Science through a project (CGL2014-55362-P), a grant (BES-2015-075675) and a Juan de la Cierva –Incorporación Fellowship (IJC2018-036411-I). AST was partially funded by the German Research Foundation (DFG) as part of the SFB TRR 212 (NC^3) – Project numbers 316099922 and 396782608.

References

Avilés, J. M., Molina-Morales, M. and Martínez, J. G. 2014. Climatic effects and phenological mismatch in cuckoo-host interactions: A role for host phenotypic plasticity in laying date? - Oikos 123: 993–1002.

Bartoń, K. 2019. MuMIn: Multi-Model Inference R package ver. 1.43.15. <https://CRAN.R-project.org/package=MuMIn>.

Bates, D., Mächler, M., Bolker, B. and Walker, S. 2015. Fitting Linear Mixed-Effects Models Using lme4. - J. Stat. Softw. 67: 1-48.

Birkhead, T. R. 1991. The Magpies: The Ecology and Behaviour of Black-Billed and Yellow-Billed Magpies. - T & A D Poyser.

Bonduriansky, R., Maklakov, A., Zajitschek, F. and Brooks, R. 2008. Sexual selection, sexual conflict and the evolution of ageing and life span. - Funct. Ecol. 22: 443–453.

Bowers, E. K., Nietz, D., Thompson, C. F. and Sakaluk, S. K. 2014. Parental provisioning in house wrens: Effects of varying brood size and consequences for offspring. - Behav. Ecol. 25: 1485–1493.

Brooke, M. L. and Davies, N. B. 1989. Provisioning of nestling Cuckoos *Cuculus canorus* by Reed Warbler *Acrocephalus scirpaceus* hosts. - Ibis 131: 250–256.

Brooker, M. and Brooker, L. 1996. Acceptance by the splendid fairy-wren of parasitism by Horsfield's bronze-cuckoo: further evidence for evolutionary equilibrium in brood parasitism. - Behav. Ecol. 7: 395–407.

Buitron, D. 1988. Female and Male Specialization in Parental Care and Its Consequences in Black-Billed Magpies. - Condor 90: 29–39.

Canestrari, D., Bolopo, D., Turlings, T. C. J., Roder, G., Marcos, J. M. and Baglione, V. 2014. From Parasitism to Mutualism: Unexpected Interactions Between a Cuckoo and Its Host. - Science 343: 1350–1352.

Clutton-Brock, T. H. 1991. The Evolution of Parental Care. - Princeton University Press.

Davies, N. B. 2000. Cuckoos, Cowbirds and Other Cheats. - T & A D Poyser.

Dawson, D. A., dos Remedios, N. and Horsburgh, G. J. 2016. A new marker based on the avian spindlin gene that is able to sex most birds, including species problematic to sex with CHD markers. - Zoo Biol. 35: 533–545.

De Neve, L., Soler, J. J., Pérez-Contreras, T. and Soler, M. 2004a. Genetic, environmental and maternal effects on magpie nestling-fitness traits under different nutritional conditions: A new experimental approach. - Evol. Ecol. Res. 6: 415–431.

De Neve, L., Soler, J. J., Soler, M. and Pérez-Contreras, T. 2004b. Nest size predicts the effect of food supplementation to magpie nestlings on their immunocompetence: An experimental test of nest size indicating parental ability. - Behav. Ecol. 15: 1031–1036.

Dearborn, D. C., Anders, A. D., Thompson, F. R. and Faaborg, J. 1998. Effects of Cowbird Parasitism on Parental Provisioning and Nestling Food Acquisition and Growth. - Condor 100: 326–334.

Dormann, C. F., Elith, J., Bacher, S., Buchmann, C., Carl, G., Carré, G., Marquéz, J. R. G. G., Gruber, B., Lafourcade, B., Leitão, P. J., Münkemüller, T., Mcclean, C., Osborne, P. E., Reineking, B., Schröder, B., Skidmore, A. K., Zurell, D. and Lautenbach, S. 2013. Collinearity: A review of methods to deal with it and a simulation study evaluating their performance. - Ecography 36: 27–46.

Eldegard, K. and Sonerud, G. A. 2009. Female offspring desertion and male-only care increase with natural and experimental increase in food abundance. - Proc. R. Soc. B Biol. Sci. 276: 1713–1721.

Forstmeier, W. and Schielzeth, H. 2011. Cryptic multiple hypotheses testing in linear models: overestimated effect sizes and the winner's curse. - Behav. Ecol. Sociobiol. 65: 47–55.

Grayson, P., Glassey, B. and Forbes, S. 2013. Does Brood Parasitism Induce Paternal Care in a Polygynous Host? - Ethology 119: 489–495.

Griffiths, R., Double, M. C., Orr, K., Dawson, D. A. and Robert, J. G. 1998. A DNA test to sex most birds. - Mol. Ecol. 7: 1071–1075.

Harrison, F., Barta, Z., Cuthill, I. and Székely, T. 2009. How is sexual conflict over parental care resolved? A meta-analysis. - J. Evol. Biol. 22: 1800–1812.

Hauber, M. E. 2006. A future cost of misdirected parental care for brood parasitic young? - Folia Zool. 55: 367–374.

Hauber, M. E. and Montenegro, K. 2002. What are the costs of raising a brood parasite? Comparing host parental care at parasitized and non-parasitized broods. - Etologia 10–11: 1–9.

Hoover, J. P. 2003a. Decision rules for site fidelity in a migratory bird, the Prothonotary Warbler. - Ecology 84: 416–430.

Hoover, J. P. 2003b. Multiple effects of brood parasitism reduce the reproductive success of prothonotary warblers, *Protonotaria citrea*. - Anim. Behav. 65: 923–934.

Hoover, J. P. and Reetz, M. J. 2006. Brood parasitism increases provisioning rate, and reduces offspring recruitment and adult return rates, in a cowbird host. - Oecologia 149: 165–173.

Houston, A. I., Székely, T. and McNamara, J. M. 2005. Conflict between parents over care. - Trends Ecol. Evol. 20: 33–38.

Johnstone, R. A. and Hinde, C. A. 2006. Negotiation over offspring care—how should parents respond to each other's efforts? - Behav. Ecol. 17: 818–827.

Kilner, R. M., Noble, D. G. and Davies, N. B. 1999. Signals of need in parent–offspring communication and their exploitation by the common cuckoo. - Nature 397: 667–672.

Koleček, J., Jelínek, V., Požgayová, M., Trnka, A., Baslerová, P., Honza, M. and Procházka, P. 2015. Breeding success and brood parasitism affect return rate and dispersal distances in the great reed warbler. - Behav. Ecol. Sociobiol. 69: 1845–1853.

Komdeur, J., Wiersma, P. and Magrath, M. 2002. Paternal care and male mate–attraction effort in the European starling is adjusted to clutch size. - Proc. R. Soc. London B Biol. Sci. 269: 1253–1261.

Krüger, O. 2007. Cuckoos, cowbirds and hosts: Adaptations, trade-offs and constraints. - Philos. Trans. R. Soc. B Biol. Sci. 362: 1873–1886.

Lefcheck, J. 2016. piecewiseSEM: Piecewise structural equation modeling. R package version 1.2.1. - Methods Ecol. Evol. 7: 573–579.

Lendvai, Á. Z., Akçay, Ç., Ouyang, J. Q., Dakin, R., Domalik, A. D., St John, P. S., Stanback, M., Moore, I. T. and Bonier, F. 2015. Analysis of the Optimal Duration of Behavioral Observations Based on an Automated Continuous Monitoring System in Tree Swallows (*Tachycineta bicolor*): Is One Hour Good Enough? (C Moskát, Ed.). - PLoS One 10: e0141194.

Mariette, M. M., Griffith, S. C., Adkins-Regan, A. E. E. and Kalisz, E. S. 2015. The adaptive significance of provisioning and foraging coordination between breeding partners. - Am. Nat. 185: 270–280.

Mark, M. M. and Rubenstein, D. R. 2013. Physiological costs and carry-over effects of avian interspecific brood parasitism influence reproductive tradeoffs. - Horm. Behav. 63: 717–722.

Martín-Gálvez, D., Soler, M., Soler, J. J., Martín-Vivaldi, M. and Palomino, J. J. 2005. Food acquisition by common cuckoo chicks in rufous bush robin nests and the advantage of eviction behaviour. - Anim. Behav. 70: 1313–1321.

Martínez, J. G., Soler, J. J., Soler, M. and Burke, T. 1998. Spatial patterns of egg laying and multiple parasitism in a brood parasite: A non-territorial system in the great spotted cuckoo (*Clamator glandarius*). - Oecologia 117: 286–294.

Martínez, J. G., Molina-Morales, M., Precioso, M. and Avilés, J. M. 2020. Age-related brood parasitism and egg rejection in magpie hosts. - Am. Nat. 195: 876–885.

Molina-Morales, M., Martínez, J. G. and Avilés, J. M. 2012. Factors affecting natal and breeding magpie dispersal in a population parasitized by the great spotted cuckoo. - Anim. Behav. 83: 671–680.

Molina-Morales, M., Gabriel Martínez, J., Martín-Gálvez, D., A. Dawson, D., Rodríguez-Ruiz, J., Burke, T. and Avilés, J. M. 2013. Evidence of long-term structured cuckoo parasitism on individual magpie hosts. - J. Anim. Ecol. 82: 389–398.

Molina-Morales, M., Martínez, J. G., Martín-Gálvez, D., Dawson, D. A., Burke, T. and Avilés, J. M. 2014. Cuckoo hosts shift from accepting to rejecting parasitic eggs across their lifetime. - Evolution 68: 3020–3029.

Molina-Morales, M., Martínez, J. G. and Avilés, J. M. 2016. Criteria for host selection in a brood parasite vary depending on parasitism rate. - Behav. Ecol. 27: 1441–1448.

Møller, A. P. and Cuervo, J. J. 2000. The evolution of paternity and paternal care in birds. - Behav. Ecol. 11: 472–485.

Moreno-Rueda, G., Soler, M., Soler, J. J., Martínez, J. G. and Pérez-contreras, T. 2007. Rules of food allocation between nestlings of the black-billed magpie *Pica pica*, a species showing brood reduction. - Ardeola 54: 15–25.

Nakagawa, S. and Schielzeth, H. 2013. A general and simple method for obtaining R2 from generalized linear mixed-effects models. - Methods Ecol. Evol. 4: 133–142.

Payne, R. B. 2005. The Cuckoos - Robert B. Payne - Oxford University Press. - Oxford University Press.

Payne, R. B. and Payne, L. L. 1998. Brood parasitism by cowbirds: Risks and effects on reproductive success and survival in indigo buntings. - Behav. Ecol. 9: 64–73.

Pinheiro, J., Bates, D., Debroy, S. and Sarkar, D. 2020. nlme: Linear and Nonlinear Mixed Effects Models ver. 3.1-144 < https://CRAN.R-project.org/package=nlme>.

Požgayová, M., Beňo, R., Procházka, P., Jelínek, V., Abraham, M. M. and Honza, M. 2015. Lazy males and hardworking females? Sexual conflict over parental care in a brood parasite host and its consequences for chick growth. - Behav. Ecol. Sociobiol. 69: 1053–1061.

R Core Team 2019. R: A language and environment for statistical computing. R Foundation for Statistical Computing.<https://www.r-project.org/>.

Rothstein, S. I. 1990. A Model System for Coevolution: Avian Brood Parasitism. - Annu. Rev. Ecol. Syst. 21: 481–508.

Samaš, P., Rutila, J., Honza, M., Kysučan, M. and Grim, T. 2018. Rearing a virulent common cuckoo is not extra costly for its only cavity-nesting host. - Proc. R. Soc. B Biol. Sci. 285: 20181710.

Samaš, P., Grim, T., Jelínek, V., Abraham, M. M., Šulc, M. and Honza, M. 2019. No immediate or future extra costs of raising a virulent brood parasite chick. - Behav. Ecol. 30: 1020–1029.

Schielzeth, H. 2010. Simple means to improve the interpretability of regression coefficients. - Methods Ecol. Evol. 1: 103–113.

Schroeder, J., Cleasby, I., Dugdale, H. L., Nakagawa, S. and Burke, T. 2013. Social and genetic benefits of parental investment suggest sex differences in selection pressures. - J. Avian Biol. 44: 133–140.

Sedgwick, J. A. 2004. Site fidelity, territory fidelity, and natal philopatry in willow flycatchers (*Empidonax traillii*). - Auk 121: 1103–1121.

Sheather, S. J. 2009. A Modern Approach to Regression with R . - Springer.

Soler, M. 2014. Long-term coevolution between avian brood parasites and their hosts. - Biol. Rev. 89: 688–704.

Soler, M. and Soler, J. J. 1991. Growth and Development of Great Spotted Cuckoos and Their Magpie Host. - Condor 93: 49–54.

Soler, J. J., Soler, M., Møller, A. P. and Martínez, J. G. 1995a. Does the great spotted cuckoo choose magpie hosts according to their parenting ability? - Behav. Ecol. Sociobiol. 36: 201–206.

Soler, M., Martínez, J. G., Soler, J. J. and Møller, A. P. 1995b. Preferential allocation of food by magpies *Pica pica* to great spotted cuckoo *Clamator glandarius* chicks. - Behav. Ecol. Sociobiol. 37: 7–13.

Soler, M., Palomino, J. J., Martinez, J. G. and Soler, J. J. 1995c. Communal Parental Care by Monogamous Magpie Hosts of Fledgling Great Spotted Cuckoos. - Condor 97: 804–810.

Soler, M., Soler, J. J. and Martínez, J. G. 1997. Great spotted cuckoos improve their reproductive success by damaging magpie host eggs. - Anim. Behav. 54: 1227–1233.

Soler, M., Pérez-Contreras, T., Ibáñez-Álamo, J. D., Roncalli, G., Macías-Sánchez, E. and de Neve, L. 2014. Great Spotted Cuckoo Fledglings Often Receive Feedings from Other Magpie Adults than Their Foster Parents: Which Magpies Accept to Feed Foreign Cuckoo Fledglings? - PLoS One 9: e107412.

Stearns, S. C. 1989. Trade-Offs in Life-History Evolution. - Funct. Ecol. 3: 259.

Stearns, S. C. 1992. The Evolution of Life Histories. - Oxford University Press.

Trivers, R. L. 1972. Parental investment and sexual selection. - In: Campbell, B. (ed), Sexual Selection and the Descent of Man: The Darwinian Pivot. Ed. Aldine, pp. 136–179.

Wedell, N., Kvarnemo, C., Lessells, C. (K). M. and Tregenza, T. 2006. Sexual conflict and life histories. - Anim. Behav. 71: 999–1011.

Supplementary material

Appendix:

Evaluation of the possible effect of collinearity between parasitism status and brood size (A);
Model analysing magpies' provisioning rates in a subset of nests with the most common brood sizes (B);
Model analysing magpies' breeding phenology in the following breeding season (C);
Results of full models including non-significant interactions (D);
Models explaining magpies' breeding phenology in the subsequent breeding season including the other member of the pair (E, see Methods).

A) Evaluation of the possible effect of collinearity between parasitism status and brood size

Table A1: Factors affecting provisioning rate in magpies. Model excluding parasitism status.

Fixed effects		β	Lower CI	Upper CI	Z	*p*-value
Intercept		**1.08**	**0.98**	**1.18**	**20.46**	**<0.001**
Brood size		**0.13**	**0.02**	**0.24**	**2.41**	**0.016**
Brood age		**0.14**	**0.02**	**0.26**	**2.26**	**0.023**
Nest volume		-0.10	-0.22	0.01	-1.73	0.083
Laying date		0.03	-0.08	0.15	0.55	0.585
Sex		-0.13	-0.31	0.06	-1.35	0.177
Year:	2009	-0.04	-0.45	0.37	-0.20	0.839
	2010	0.12	-0.18	0.42	0.77	0.440
	2011	0.23	-0.17	0.64	1.13	0.259
	2016	0.25	-0.12	0.62	1.30	0.192
Random effect		σ			LRT	*p*-value
Pair identity		0.09			0.15	0.70

Results of a GLMM (Poisson distribution, log link function) testing the effect of brood size, brood age, nest volume, laying date, sex, year and pair identity on provisioning visits in magpies. Significant estimates are highlighted in bold. 95% CI were calculated by the Wald approximation; parameter estimates were calculated by the Gauss-Hermite approximation to the log-likelihood with 25 quadrature points; *p*-values for fixed effects were calculated by a Wald Z test; *p*-value for year and the random effect was calculated by a likelihood ratio test. Marginal $R^2 = 0.19$; Conditional $R^2 = 0.21$ (calculated following Nakagawa and Schielzeth (2017); MuMIn package, version1.43.15, Bartoń (2019)).

B) Model analysing magpies' provisioning rates in a subset of nests with the most common brood sizes

Table A2: Factors affecting magpies' provisioning rates in a subset of nests with the most common brood sizes in parasitized nests (i.e., 1 or 2 cuckoo nestlings) and non-parasitized ones (i.e., 4 or more magpie nestlings) (N = 64 individuals, 32 nests).

Fixed effects	B	Lower CI	Upper CI	Z	*p*-value
Intercept	**1.09**	**0.96**	**1.22**	**16.97**	**<0.001**
Parasitism status	**-0.30**	**-0.56**	**-0.04**	**-2.29**	**0.022**
Brood age	0.06	-0.07	0.19	0.89	0.375
Nest volume	-0.10	-0.24	0.03	-1.53	0.127
Laying date	0.05	-0.08	0.18	0.74	0.457
Sex	-0.17	-0.38	0.04	-1.60	0.110
Random effect	Σ			LRT	*p*-value
Pair identity	0.18			1.56	0.211

Results of a GLMM (Poisson distribution, log link function) testing the effect of parasitism status, brood age, nest volume, laying date, sex and pair identity on individual provisioning rates. Significant estimates are highlighted in bold. 95% CI were calculated by the Wald approximation; parameter estimates were calculated by the Gauss-Hermite approximation to the log-likelihood with 25 quadrature points; *p*-values were calculated by a Wald Z test; *p*-value for random effect was calculated by a likelihood ratio test. Marginal R^2 = 0.14; Conditional R^2 = 0.23 (calculated following Nakagawa and Schielzeth (2013); MuMIn package, version 1.43.15, Bartoń (2019)).

C) Model analysing magpies' breeding phenology in the following breeding season

Table A3: Factors affecting magpies breeding phenology in the following breeding season (n = 23 individuals).

	β	Lower CI	Upper CI	df	F	*p*-value
Intercept	0	-0.32	0.32	1,19	0	1.000
Parasitism status in t	**0.72**	**0.01**	**1.44**	**1,19**	**4.48**	**0.048**
Provisioning rate in t	0.21	-0.16	0.58	1,19	1.38	0.255
Laying date in t	**0.62**	**0.27**	**0.97**	1,19	**13.44**	**0.002**

Results of a LM testing the effect of parasitism status, provisioning rates and laying date in year t on magpies' laying date in t + 1. Significant estimates are highlighted in bold. Multiple R^2 = 0.52; Adjusted R^2 = 0.44.

D) Results of full models including non-significant interactions

Table A4: Factors affecting provisioning rate in magpies (N = 78 individuals, 39 nests).

Fixed effects		β	Lower CI	Upper CI	Z	*p*-value
Intercept		**1.14**	**0.97**	**1.31**	**12.86**	**<0.001**
Parasitism status		0.23	-0.13	0.58	1.26	0.207
Brood size		**0.23**	**0.05**	**0.41**	**2.48**	**0.013**
Brood age		**0.13**	**0.02**	**0.25**	**2.23**	**0.026**
Nest volume		-0.10	-0.22	0.01	-1.76	0.079
Laying date		0.02	-0.10	0.14	0.36	0.715
Sex		-0.12	-0.31	0.07	-1.28	0.200
Year:	2009	-0.08	-0.51	0.35	-0.37	0.713
	2010	0.09	-0.23	0.41	0.54	0.589
	2011	0.11	-0.34	0.55	0.48	0.633
	2016	0.22	-0.15	0.60	1.17	0.242
Sex × Parasitism status		0.28	-0.10	0.66	1.42	0.154
Brood size × Parasitism status		0.19	-0.23	0.61	0.87	0.384
Random effect		σ			LRT	*p*-value
Pair identity		0.06			0.03	0.86

Results of a GLMM (Poisson distribution, log link function) testing the effect of parasitism status, brood size, brood age, nest volume, laying date, sex, year, sex in interaction with parasitism status, brood size in interaction with parasitism status and pair identity on provisioning rates in magpies . Significant estimates are highlighted in bold. 95% CI were calculated by the Wald approximation; parameter estimates were calculated by the Gauss-Hermite approximation to the log-likelihood with 25 quadrature points; *p*-values for fixed effects were calculated by a Wald Z test; *p*-value for the random effect was calculated by a likelihood ratio test. Marginal R^2: 0.21; Conditional R^2: 0.22 (calculated following Nakagawa and Schielzeth (2013); MuMIn package, version 1.43.15, Bartoń (2019)).

Table A5: Factors affecting magpies' provisioning rates in a subset of nests with the most common brood sizes in parasitized nests (i.e., 1 or 2 cuckoo nestlings) and non-parasitized ones (i.e., 4 or more magpie nestlings) (N = 64 individuals, 32 nests).

Fixed effects	β	Lower CI	Upper CI	Z	*p*-value
Intercept	**1.09**	**0.96**	**1.21**	**16.97**	**<0.001**
Parasitism status	**-0.30**	**-0.55**	**-0.04**	**-2.26**	**0.024**
Brood age	0.06	-0.07	0.19	0.89	0.375
Nest volume	-0.10	-0.24	0.03	-1.53	0.127
Laying date	0.05	-0.08	0.18	0.74	0.457
Sex	-0.16	-0.38	0.05	-1.51	0.131
Sex × Parasitism status	0.09	-0.34	0.53	0.43	0.670
Random effect	σ			LRT	*p*-value
Pair identity	0.18			1.56	0.211

Results of a GLMM (Poisson distribution, log link function) testing the effect of parasitism status, brood size, brood age, nest volume, laying date, sex, year, the interaction between sex and parasitism status and pair identity on individual provisioning rates in a subset of nests which contain 1 or 2 cuckoo nestlings and 4 or more magpie nestlings. Significant estimates are highlighted in bold. 95% CI were calculated by the Wald approximation; parameter estimates were calculated by the Gauss-Hermite approximation to the log-likelihood with 25 quadrature points; *p*-values were calculated by a Wald Z test; *p*-value for random effect was calculated by a likelihood ratio test. Marginal R^2: 0.14; Conditional R^2: 0.23 (calculated following Nakagawa and Schielzeth (2013); MuMIn package, version1.43.15, Bartoń (2019)).

Table A6: Factors affecting adults' presence in the following breeding season (t + 1) (n = 49 individuals, 39 nests).

Fixed effects	β	Lower CI	Upper CI	df	LRT	*p*-value
Intercept	0.48	-0.79	1.76	1	0.65	0.42
Parasitism status in t	0.86	-1.56	3.29	2	0.63	0.73
Brood size in t	1.11	-0.41	2.63	2	2.98	0.23
Provisioning rate in t	0.08	-0.63	0.79	1	0.05	0.82
Laying date in t	-0.68	-1.60	0.24	1	2.91	0.09
Sex	-0.59	-2.08	0.89	1	0.72	0.39
Brood size in t × Parasitism status in t	0.91	-1.93	3.75	1	0.42	0.52
Random effects	σ				LR	*p*-value
Pair identity	0.54				0.01	0.91

Results of a GLMM (Binomial distribution, logit link function) testing the effect of parasitism status, brood size, provisioning rate, laying date, sex, brood size in interaction with parasitism status and pair identity on the presence/absence of adult magpies in the subsequent breeding season. 95% CI were calculated by the Wald approximation; parameter estimates were calculated by the Gauss-Hermite approximation to the log-likelihood with 25 quadrature points; *p*-values for fixed and random effects were calculated by a likelihood ratio test. Marginal R^2: 0.23; Conditional R^2: 0.28 (calculated following Nakagawa and Schielzeth (2013); MuMIn package, version1.43.15, Bartoń (2019)).

Table A7: Factors affecting magpies' breeding phenology in the following breeding season (n = 23 individuals).

	β	Lower CI	Upper CI	df	F	*p*-value
Intercept	0.06	-0.27	0.40	1,17	0.16	0.690
Parasitism status in t	**0.70**	**0.01**	**1.40**	**1,17**	**4.47**	**0.049**
Provisioning rate in t	0.14	-0.23	0.52	1,17	0.65	0.431
Laying date in t	**0.52**	**0.16**	**0.89**	1,17	**9.04**	**0.008**
Parasitism status in t × Provisioning rate in t	0.10	-0.71	0.92	1,17	0.07	0.790
Parasitism status in t × Laying date in t	0.59	-0.14	1.31	1,17	2.93	0.105

Results of a LM testing the effect of parasitism status, provisioning rates, laying date and the interaction between brood parasitism and laying date in year t on magpies' laying date in t + 1 (N = 23). Significant estimates are highlighted in bold. Multiple R^2: 0.61; Adjusted R^2: 0.50.

Table A8: Factors affecting difference in laying dates between two consecutive breeding seasons in magpies (n = 23 individuals).

	β	Lower CI	Upper CI	df	F	*p*-value
Intercept	-0.03	-0.45	0.39	1,19	3.32	0.084
Parasitism status in t	**0.73**	**-0.02**	**1.48**	**1,19**	**4.61**	**0.045**
Provisioning rate in t	-0.02	-0.46	0.41	1,19	0.27	0.610
Parasitism status in t × Provisioning rate in t	-0.19	-0.96	0.59	1,19	0.22	0.640

Results of a LM fitted by GLS testing the effect of parasitism status and provisioning rate in year t on the differences in laying dates between year t and year t + 1. Significant estimates are highlighted in bold. $R^2 = 0.16$ (piecewiseSEM package, version 2.1.0, Lefcheck (2016)).

E) Models explaining magpies' breeding phenology in the subsequent breeding season including the other member of the pair.

Table A9: Factors affecting magpies' breeding phenology in the following breeding season. Model including the member of the pair excluded in Supplementary material, Appendix 1C, Table A3 (n = 23 individuals).

	β	Lower CI	Upper CI	df	F	*p*-value
Intercept	0.06	-0.27	0.41	1,17	0.18	0.675
Parasitism status in t	**0.93**	**0.24**	**1.64**	1,17	**7.95**	**0.012**
Provisioning rate in t	0.22	-0.15	0.60	1,17	1.59	0.224
Laying date in t	**0.42**	**0.06**	**0.78**	1,17	**5.93**	**0.026**
Parasitism status in t × Provisioning rate in t	0.32	-0.47	1.11	1,17	0.73	0.403
Parasitism status in t × Laying date in t	0.41	-0.32	1.14	1,17	1.43	0.249

Results of a LM testing the effect of parasitism status, provisioning rates, laying date and the interaction between brood parasitism and laying date in year t on magpies laying date in t + 1. Significant estimates are highlighted in bold. Multiple R^2: 0.59; Adjusted R^2: 0.47.

Table A10: Factors affecting the difference in laying dates between two consecutive breeding seasons in magpies. Model including the member of the pair excluded in Table 3 (n = 23 individuals).

	β	Lower CI	Upper CI	df	F	*p*-value
Intercept	0	-0.41	0.42	1,19	0.77	0.392
Parasitism status in t	**0.88**	**0.09**	**1.67**	**1,19**	**4.88**	**0.040**
Provisioning rate in t	0.06	-0.38	0.49	1,19	0.09	0.764
Parasitism status in t × Provisioning rate in t	0.02	-0.79	0.82	1,19	0.00	0.964

Results of a LM fitted by GLS testing the effect of parasitism status and provisioning rate in year t on the differences in laying dates between year t and year t + 1 (i.e., laying date in t+1 minus laying date in t). Significant estimates are highlighted in bold. $R^2 = 0.18$ (piecewiseSEM package, version 2.1.0, Lefcheck (2016)).

Capítulo 2

Breeding effort, brood parasitism and telomere dynamics in magpies (*Pica pica)*

Breeding effort, brood parasitism and telomere dynamics in magpies (*Pica pica)*

Marta Precioso[1], Mercedes Molina-Morales[1], Deborah A. Dawson[2], Terry Burke[2], Juan G. Martínez[1]

[1]*Departamento de Zoología, Universidad de Granada, Granada, Spain*
[2]*Department of Animal and Plant Sciences, University of Sheffield, Sheffield, United Kingdom*

Abstract

Brood parasites exploit their hosts, leaving them with the costs of rearing parasitic chicks. The effect of these costs on hosts' survival and longevity remains unknown in many host species. Telomeres are related to longevity and shorten with cell divisions at a rate that is affected by lifestyle and reproduction. Thus, this rate of shortening is used as a biomarker of the costs of reproduction in terms of survival. Here, we experimentally tested whether the costs in terms of telomere dynamics for magpie hosts (*Pica pica*) of rearing a single parasitic great spotted cuckoo (*Clamator glandarius*) are different from the costs of rearing either single or multiple magpie chicks in a non-parasitized brood. We forced 27 individuals to rear during consecutive breeding seasons one of the following experimental brood treatments: one cuckoo, one magpie or several magpie chicks; two telomere length measurements were taken before and after the experiment to estimate a telomere rate of change (TROC). TROCs differed between treatments depending on initial telomere length: individuals with longer telomeres had significantly smaller rates of change when they reared one cuckoo or one magpie than when they reared several magpies, but there were no differences in TROCs between individuals with shorter telomeres. Results suggest that brood parasitism itself does not impose an extra cost in terms of telomere attrition; however reduced breeding effort implies smaller telomere attrition for individuals with larger telomere lengths. We also investigated factors associated with telomere length in 61 magpies of known age. We found that younger individuals have longer telomeres and that the number of magpie chicks fledged within the breeding season is correlated with telomere length in this species. These results suggest that the costs of parental care in terms of telomere loss may vary with both age and quality in magpie hosts, which could have an effect on their future survival and might have implications in the evolution of host longevity.

Introduction

Avian brood parasites lay their eggs in the nest of their hosts, which assume all the costs of rearing the parasitic offspring (Davies 2000). These costs include the loss of fitness in the parasitized breeding attempt, which can be reduced drastically to zero in some host-parasite systems (Rothstein 1990). However, there may be other costs than those related to the breeding success of the parasitized clutch that may depend on how much parental effort is required to rear parasitic broods compared to rearing host broods, and, more importantly, may have long-term consequences on hosts future reproductive performance (Mark and Rubenstein 2013) or survival (Hoover and Reetz 2006, Koleček et al. 2015). The study of these kinds of costs has received little attention, even though they can affect different host life history traits such as host survival or lifespan. Indeed, we know little about the possible relationship between parasitism and hosts longevity. Hosts' lifespan might be affected by parasitism depending on whether rearing parasitic broods entails extra costs compared to rearing host broods.

Different mechanisms have been proposed to underlie these trade-offs between current and future reproduction, linking breeding effort, ageing and lifespan (Monaghan et al. 2009, Ricklefs 2010). One candidate mechanism that has received substantial attention in the last decade is the shortening of telomeres associated with breeding effort (Reichert et al. 2014, Sudyka 2019). Telomeres are non-coding repetitive DNA sequences that are found in the extremes of eukaryotic chromosomes and maintain the integrity of chromosomes (Blackburn 1991). However, during the replication process, the DNA polymerase cannot replicate the end of the lagging DNA strand ("the end-replication problem"), and thus, telomeres shorten with each cellular cycle (Blackburn 1991). When telomeres shorten to a critical length, the cell enters a state of replicative senescence or dies via apoptosis (Zou et al. 2004, Hemann et al. 2001). This process can result in an accumulation of defects that may affect the normal function of different tissues and may be associated with organismal ageing (Campisi 2005, López-Otín et al. 2013). Indeed, a recent meta-analysis in non-model vertebrates has shown that telomere length is negatively related to mortality risk, especially in birds (Wilbourn et al. 2018). Moreover, the rate at which telomeres shorten is also affected by several factors related to stress (Chatelain et al. 2020), and stressful situations, such as infection or reproduction, can lead to persistent telomere erosion, which suggests that the rate

of erosion may constitute a link between lifestyle, senescence and lifespan (Monaghan and Haussmann 2006). Indeed, most experimental studies suggest a connection between reproduction and telomere loss (reviewed in Sudyka 2019). For these reasons, telomere dynamics may act as a biomarker of the costs of reproduction and may reflect the effects of these costs on individuals' lifespans. If parasitism influences hosts lifespan through differential costs of rearing parasitic offspring, this should be reflected in hosts' telomere dynamics.

Here, we present an experimental test of the effect of breeding effort and parasitism by great spotted cuckoos (*Clamator glandarius*) on magpies' (*Pica pica*) telomere dynamics. Magpies are the main host of great spotted cuckoos (hereafter cuckoos) in Europe; cuckoo parasitism commonly reduces magpie breeding success to zero and cuckoo nestlings are normally reared alone or in the company of few other cuckoo nestlings in the nest (Soler et al. 1996, Martínez et al. 1998). It has been previously shown that the parental effort (estimated as provisioning rates) required for magpies to rear non-parasitized and parasitized broods is significantly smaller for the most common brood sizes in parasitized nests (1-2 cuckoo nestlings) compared to the most common non-parasitized brood sizes (4 or more magpie nestlings; Precioso et al. 2020). Moreover, parasitism does not affect magpies' survival to the subsequent breeding season (Precioso et al. 2020). We aim to test whether brood parasitism itself has a differential effect on magpies' telomere dynamics. Because parasitized broods are normally smaller than non-parasitized ones, we have tried to separate the effect of parasitism from the effect of brood size through an experiment. If brood parasitism brings about extra costs, we predict that rearing a single parasitic cuckoo would have a larger effect on telomere dynamics than rearing a single magpie nestling; however, if brood parasitism does not entail extra costs, we predict that rearing a single parasitic cuckoo would have a similar effect on magpies' telomere dynamics to rearing a single magpie, and this would be smaller than the effect of rearing a non-parasitized and non-manipulated brood (ranging from 3 to 8 chicks in this study) because parasitized broods typically contain a smaller number of chicks (Soler et al. 1996, Precioso et al. 2020).

There is also evidence that telomere shortening depends on the initial telomere length in different species, with longer telomeres tending to shorten more quickly (Salomons et al. 2009, Verhulst et al. 2013, Boonekamp et al. 2014). Moreover, if telomere length is related to other characteristics, such as individual quality/reproductive performance (Le Vaillant et al. 2015, Parolini et al. 2017) or

physiological state (Monaghan 2010), it may also be indicative of how individuals deal with different life-history trade-offs, including the costs of reproduction, and thus might help to explain and understand rates of telomere change associated with differential breeding effort. For this reason, we additionally explored different factors that may be related to magpies' telomere lengths, such as age, sex, body condition and breeding success.

Methods

Study area and system

The study was performed in a wild population of European magpies located in La Calahorra (37°10'N, 3°03'W, Granada, Southern Spain) during the breeding seasons (March to July) of 2016-2018. The study area consists of cereal fields patched with groves of almond trees (*Prunus dulcis*), in which magpies preferentially build their nests. Magpies start building their nests around the beginning of March. Nests are monitored from the moment they are located and GPS positioned, until chicks fledge. Nest are observed with a telescope during the nest building stage to identify the adults, which are ringed (see below), and visited every four-five days to record different reproductive variables (laying date, parasitism status, hatching date and fledging success).

In our study population, parasitism rates are rather variable depending on the year (from 16% to 65%; Molina-Morales et al. 2016). This population of magpies has been under long-term monitoring since 2005 and a large number of magpies in the area have been ringed in the nest as fledglings (at 15-18 days) with a unique combination of coloured rings; some of these individuals have recruited into the population, and thus a percentage of the breeding adults are marked individuals of known age.

Adult capture and blood sampling

Adult magpies were captured in the proximity of their nests with Larsen and corvid traps. These magpies were colour-ringed and different morphometric measures were recorded (weight, wing length and tarsus length). Additionally, a blood sample (approximately 100 microlitres) was taken from each individual by puncturing the brachial vein with a sterile needle, and placed in a microcentrifuge tube containing 1 ml of analytical grade absolute ethanol (Fisher) and stored in a rubber-sealed screw-

topped microfuge tube at room temperature. Sexing of magpies in the field is not possible due to monomorphic plumage and little difference in size between the sexes, and so genomic DNA was extracted from each blood sample and PCR amplified with two sex-specific primer sets P2/P8 (Griffiths et al. 1998) and Z43B (Dawson et al. 2016) to determine sex.

Experimental Design

To test how breeding effort and brood parasitism may affect magpies' telomere dynamics, we carried out an experiment to attempt to separate the effects of brood parasitism itself from the effects of the smaller brood size typical of parasitized nests. We captured adults from 61 nests (a total of 66 individuals), with each matched in a triplet with others with similar hatching dates (although one nest was out of any triplet and we randomly assigned its treatment). Within each triplet each nest was randomly assigned to one of the following treatments: (1) nests with only one great spotted cuckoo chick, (2) nests with only one magpie chick and (3) non-parasitized broods (non-parasitized nests with several magpie chicks; mean brood size (SD): 4.5 (1.7)). The treatments were repeated during two consecutive breeding seasons, and individuals were forced to raise the same type of brood (either treatment 1, 2 or 3) in both years. To create the experimental broods, chicks were cross-fostered when they were 1-2 days old according to the treatment assigned. Any chicks remaining that were not cross-fostered were left in non-experimental nests and not included in this study. After the second breeding season the experimental adults were recaptured when chicks were close to fledging. A blood sample was taken from experimental adults at every capture; in this way we could obtain two measures of relative telomere length (RTL) from each experimental adult, one before breeding and the second one after raising the chicks in the second breeding season; we could then estimate a telomere rate of change (TROC) from these two measures. Most of the adults involved in the experiment were not ringed when they were captured, and we only knew the exact age of 10 of those individuals.

We did not manage however to capture all the experimental adults after their second breeding event; in that case, in the following year we repeated the same treatment for those individuals and they were finally recaptured in the third year when their chicks were between 12 and 18 days old (n = 8). Because of this, the estimates of TROC were corrected for time between both sampling events (see below). Because some individuals could not be recaptured and some nests failed due

to predation, the final samples sizes of individuals captured and sampled before and after the experimental treatments were: Treatment 1 (n = 8; 5 females, 3 males), Treatment 2 (n = 8; 3 females, 5 males) and Treatment 3 (n = 11; 6 females, 5 males).

Factors Affecting Telomere Length

To explore factors related to telomere length in magpies, a total of 75 blood samples from 65 individuals of known age were used in this study (26 females, 39 males). Their ages ranged from 1 to 10 years old (mean age (SD): 3.1 (2.7)). Ten of those individuals were caught twice during the study period. For all of them, age was known; forty-one individuals had been ringed at the nest, and their age was known with accuracy. The remaining 24 individuals were considered to be 1 year old when captured based on their plumage (see Martínez et al. 2020). We recorded body condition of all birds, whether they had successfully bred and the number of fledglings leaving the nest in the year they were captured, along with their TL estimate.

Telomere Length Measurements

All laboratory analyses were carried out at the NERC Biomolecular Analysis Facility at the University of Sheffield (United Kingdom). Blood samples were stored in absolute ethanol at room temperature for 5 months until DNA was extracted. Genomic DNA was extracted from the erythrocytes of whole-blood samples by ammonium acetate precipitation (Richardson et al. 2001) and stored at -20ºC. The DNA concentration of each sample was measured using a NanoDrop 8000 Spectrophotometer (ThermoScientific) and samples diluted with ultrapure water to 25 nanograms/microliter. Telomere length was assessed by real-time PCR (qPCR, Cawthon 2002) in a monochrome multiplex reaction (Cawthon 2009), with some modifications. Relative telomere length was estimated as the ratio (T/S) between the amount of telomeric sequence (T) and the amount of a reference single-copy gene (S) amplified within the same reaction.

We used the TELC-TELG primer set to amplify a 79-bp fixed-length product from the telomere sequence (Cawthon 2009). We also designed a new primer set to amplify part of the *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) gene as the reference gene. For this gene, we designed a pair of primers specific for magpies from the GenBank *Pica pica* Sequence: EF052752. Thirty-one and 27 base pair clamps were added to the 5' ends of the forward and reverse primers,

respectively, consisting of a guanine-cytosine tail, in order to elevate their melting temperature (T_m); this allowed amplification of the telomere and *GAPDH* sequences within the same reaction (see Cawthon 2009 for further explanation). The product size for this pair of primers was 97 base pair, similar to the fixed-length telomere product, ensuring that both amplifications were comparable. The sequences of the new forward and reverse primers for *GAPDH* (including the clamp sequences) were GAPDH-Magpie-F-5'-CGGCGGCGGGCGGCGCGGGCTGGGCGGAGCC-AAAGTGGCTCCAATCCCACT-3' and GAPDH-Magpie-R-5'-GCCCGGCCCGCCGCGCCCGTCCCGCCGCTGAACTCCCATCCACCCT-3', respectively. Several primer concentrations were tested (ranging from 600 to 1200 nM) and the final optimized concentration was 900 nM for both pairs of primers; the DNA concentration was 25 nanograms per reaction. Reaction volume was 20 microliters, including 10 microliters of SYBR Select Master Mix (Applied Biosystems).

All samples were run in duplicate in MicroAmp Fast Optical 96-well plates, loaded manually and sealed with MicroAmp Optical adhesive film (Applied Biosystems). Samples were randomly distributed and run on 22 different plates (along with samples belonging to another study). All qPCR assays were performed in a QuantStudio 12K Flex qPCR instrument (ThermoFisher Scientific) and the thermal cycling profile was: hold stage: 2 min at 50ºC, 2 min at 95ºC; Stage 1: 2 cycles of 15 s at 94ºC, 10 s at 49ºC; Stage 2: 40 cycles of 15 s at 94ºC, 10 s of 62ºC, 15 s at 74ºC with signal acquisition (for the telomere amplification), 10 s at 84ºC, 15 s at 86 ºC with signal acquisition (for the *GAPDH* amplification); melt curve stage: 15 s at 95ºC, 1 min at 60ºC, 15s at 95º. The melting curve stage allowed us to confirm the presence of both amplicons.

Each plate included two no-template controls (NTC) and serial dilutions by duplicate (80, 20, 5, 1.25, 0.3125 nanograms/microliters) of DNA from the same sample to generate a standard curve. This standard curve allows us to calculate the amount of DNA amplified in each reaction from its Cq values (cycle at which amplification crosses a fixed threshold), assess amplification efficiency and account for inter-plate variation. Efficiencies were calculated as $E = (10^{(-1/\text{slope})}-1) \times 100$ for the telomeres and *GAPDH* separately, using the slope of the standard curves for each amplification. T and S were calculated following the method by Ruijter et al. (2009), and then divided to get a ratio (T/S).

Amplification efficiencies were on average (SD) 94.8% (5.5) for telomeres and 92.2% (3.3) for *GAPDH*. The intra-plate standard deviations of Cq values were on average 0.083 (0.017) for telomeres and 0.093 (0.027) for *GAPDH*. Intra-plate and inter-plate T/S ratio standard deviations were on average 0.041 (0.014) and 0.089 (0.025), respectively. R^2 for standard curves were on average 0.99 (0.003) for telomeres and 0.99 (0.003) for GAPDH.

T/S ratios varied between 0.41 and 1.61. All T/S ratios were standardized to account for inter-run variability by dividing the average T/S ratio of duplicates by the average T/S ratio of the 20 nanograms/microliter standard sample of the plate where the sample was run. Samples whose SD between replicates were above 0.05 were discarded from the analysis (12 samples).

Statistical analyses

All statistical analyses were performed in R version 4.0.2 (R Core Team 2018). When possible, linear models were applied –model validation was assessed using graphical tools – and when there was a violation of linear model assumptions, generalized linear (mixed) models were applied instead. All continuous variables in the analyses were z-standardized and all the categorical variables were mean-centered (Schielzeth 2010).

Telomere rate of change of brood treatments

We calculated a telomere rate of change (TROC) from the two relative telomere lengths (RTL) from each individual involved in the experiment: the difference between these two relative measurements was corrected for the regression to the mean effect following Verhulst et al. (2013) and divided by the time period between captures in years, obtaining a corrected TROC (D/time).

To test whether individual corrected TROC was affected by the brood treatments, we constructed a first linear model in which D/time was the dependent variable and treatment was included as a fixed effect (Model 1). Bateson et al. (2019) showed that controlling for baseline telomere length (baseline TL) might inflate the estimates of the difference in telomere shortening between groups when there are systematic differences in baseline TL between those groups. However, there were no significant differences in baseline RTL between our treatments (mean baseline T/S ratio (SD): Treatment 1: 0.86 (0.27), Treatment 2: 0.74 (0.14), Treatment 3: 0.85

(0.33); post-hoc pairwise comparisons: Treat. 1 vs Treat. 2 $p = 0.38$, Treat. 1 vs Treat. 3 $p = 0.93$, Treat. 2 vs Treat. 3 $p = 0.39$). Moreover, by not including baseline RTL in our analysis we might be losing important biological information, as RTL could be related to other individual characteristics (such as quality). For this reason, we constructed a second LM in which the baseline RTL was also included as a fixed factor to control for the possible effect of length-dependent shortening of telomeres (Model 2). Third, we constructed a further LM in which we added a two-way interaction between treatment and baseline RTL as a fixed effect (Model 3) to test whether corrected TROC could vary between treatments, depending on the baseline RTL of individuals.

We performed model selection based on Akaike's Information Criterion corrected for small sample sizes (Burnham et al. 1998), calculated different measures of R^2 (multiple and adjusted) from these three models and performed a post-hoc analysis by estimated marginal means (package emmeans version 1.5.0 (Lenth 2020)) for the best model to test the contrast between treatments.

Additionally, and to avoid model over-fitting given the small sample size, we constructed a set of models based on Model 3 in which we included, alternatively, one of the following variables as a fixed effect to test for their possible effect on TROC: difference in body condition between the two captures (Model 3a), sex (Model 3b) and the interval of time elapsed between captures (Model 3c).

Factors affecting magpies' relative telomere length

To test which variables are related to magpies' telomere length, we analyzed the RTL of 26 female and 39 male magpies of known age (12 samples were discarded from this analysis; see section Telomere length measurements). We constructed a generalized linear mixed model (GLMM, lme4 package version1.1-23, (Bates et al. 2015)) with a gamma error distribution and log link in which RTL (T/S ratio) was the dependent variable, age and body condition (calculated as weight divided by wing length) were included as continuous predictors, and sex (female = 1; male = 0), successful breeding (if the individual bred and fledge at least 1 chick = 1; if the individual had no breeding success = 0) and moment of capture (whether the individual had been captured before = 0, or after = 1 the beginning of breeding) were included as categorical predictors. We also included individual identity as a random factor because 10 individuals had been caught twice during the study period. Moreover, 12 of the individuals formed 6 pairs, but the model could not handle two

random effects (the model did not converge), so one of the members of each pair was randomly excluded. To test whether the exclusion of different individuals could lead to different results, we randomized this exclusion and ran the model 100 times. The final samples size for the model was 69 observations, but three individuals that had been captured twice were involved in pairs and another one was involved in two different pairs in different years, so the number of individuals could be 61 or 62, depending of which observation was randomly excluded (see for example Supplementary Material, Table 5). Additionally, we tested whether RTL was related to the number of fledglings successfully raised in a subset of observations in which broods were non-parasitized and had not been manipulated (27 observations). We then constructed a GLM with a Poisson error distribution and log link in which the number of chicks fledged in the breeding season was the dependent variable and individual T/S ratio and age were included as fixed effects. This analysis included two individuals that had been captured twice during the study period, so we randomly excluded one of the observations from the same individual. The model gave similar results, regardless which observation was excluded.

Results

Telomere Length Rate of Change of Brood Treatments

Model selection (Table S1, Supplementary Material) showed that the best model describing corrected telomere rate of change (D/time) was model 3 (Table 1), which included an interaction between baseline RTL and Treatment. To test the differences among treatments a post-hoc analysis was conducted (Table 2). The analyses showed that there were significant differences between Treatment 3 and the other two treatments in interaction with the individuals' baseline RTL (Table 1), so that individuals with larger telomeres suffered less telomere attrition when rearing only one chick (either magpie or cuckoo), but the effect of the treatment was not significant for individuals with shorter telomeres (Fig. 1). Moreover, individual TROC did not seem to be related to the difference in body condition between captures, sex, or the interval of time elapsed between captures (Table S2, Supplementary Material).

Table 1: Best model explaining magpies' telomere rate of change: Model 3 (n=27 individuals).

	β	Lower CI	Upper CI	d.f.	t-value	*p*-value
Intercept	0.03	-0.22	0.29	21	-0.27	0.79
Treatment 2	-0.26	-0.96	0.44	21	0.77	0.45
Treatment 3	**-1.14**	**-1.73**	**-0.54**	**21**	**3.93**	**<0.001**
RTL1	**0.43**	**0.09**	**0.76**	**21**	**-2.66**	**0.01**
Treatment 2*RTL1	-0.26	-1.30	0.77	21	0.53	0.6
Treatment 3*RTL1	**-1.30**	**-1.88**	**-0.71**	**21**	**4.62**	**<0.001**

Results of a LM evaluating the effect of the three treatments on magpies' corrected telomere rate of change (D/time). RTL1 refers to baseline RTL, that is, RTL of individuals before the experimental treatment. Multiple R^2: 0.69; Adjusted R^2: 0.62. Significant estimates are highlighted in bold.

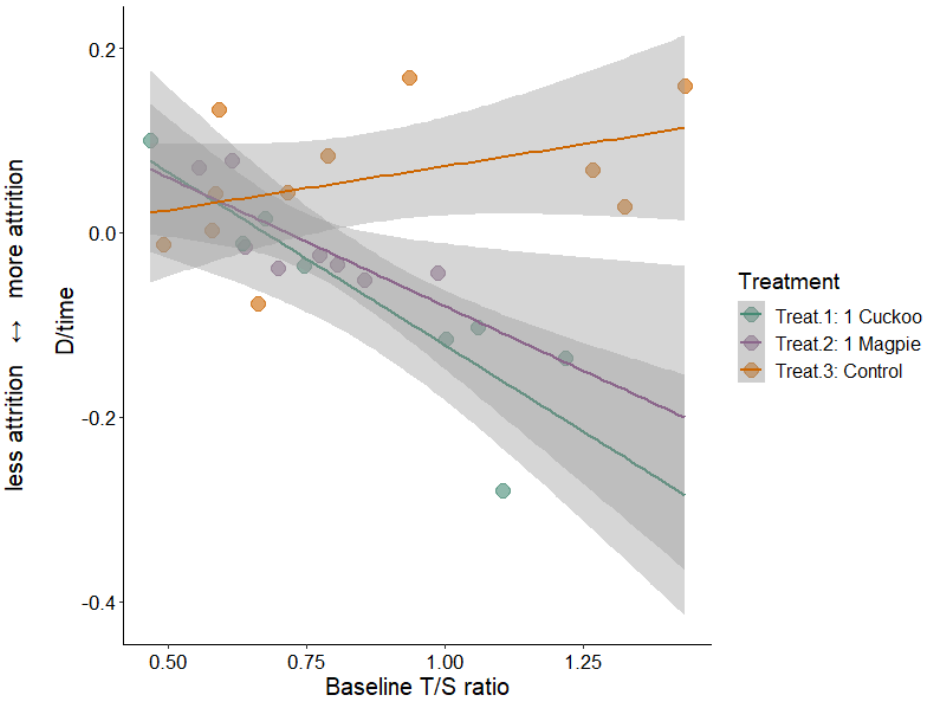


Figure 1: Relationship between individual corrected telomere rate of change (D/time) and brood treatment depending on baseline telomere length (baseline T/S ratio). Solid lines represent regression lines and shades areas represent 95% confidence interval.

Table 2: Post-Hoc pair-wise comparison by estimated marginal means of the interaction: Treatment*RTL1 (Model 3).

	β	SE	d.f.	t-value	*p*-value
Treat.1*RTL1 -Treat.2*RTL1	0.26	0.34	21	0.77	0.45
Treat.1*RTL1 -Treat.3*RTL1	**1.14**	**0.29**	**21**	**3.93**	**<0.001**
Treat.2*RTL1 -Treat.3*RTL1	**0.88**	**0.32**	**21**	**2.77**	**0.01**

Factors Affecting Magpies' Relative Telomere Length

Results of the models exploring different variables that could affect magpies' RTL after the randomized exclusion of one member of each pair led to three different results: in 48% of the models only age was significant (an example of one of these models is shown in Table 3), in 28% of the models age and successful breeding were the only significant variables (an example is shown in Table S3, Supplementary Material) and in 24% of the models age, successful breeding and body condition were significant (an example is shown in Table S4, Supplementary Material). RTL, thus, seems to be clearly related to age in magpies, so that older magpies tend to have shorter telomeres. Successful breeding was significant in 52% of the models, with successful individuals having longer telomeres (Table S3, Supplementary Material), so there is some evidence that the probability of successfully completing breeding was related to telomere length in this species. On the other hand, body condition was significant in only 24% of the models and its estimated coefficient was small in all the models (it ranged from – 0.050 to – 0.048), so even if telomere length was related to body condition in magpies, this relationship seemed to be weak. In no model were there significant differences in RTL between sexes or between individuals that were captured before or after the beginning of breeding.

Furthermore, results of the analysis of the subset of non-manipulated and non-parasitized individuals showed that, in addition to age, the number of chicks fledged in a breeding attempt was related to RTL (Fig. 2, Table 4).

Table 3: Variables affecting relative telomere length (T/S ratio) in magpies (n=69 observations, 61 individuals; 25 females, 36 males).

	β	Lower CI	Upper CI	t-value	*p*-value
Fixed effects					
Intercept	**-0.21**	**-0.34**	**-0.08**	**-3.12**	**<0.01**
Age	**-0.20**	**-0.31**	**-0.09**	**-3.60**	**<0.001**
Sex	-0.15	-0.42	0.11	-1.14	0.25
Body condition	-0.02	-0.06	0.01	-1.23	0.22
Breeding Success	0.08	-0.23	0.39	0.52	0.60
Moment of capture (before/after laying)	-0.01	-0.07	0.05	-0.21	0.83
Random effect	σ	σ^2			
Individual ID	0.34	0.12			
Residual	0.09	0.01			

Results of a GLMM (gamma error distribution, log link function) evaluating the effect of different variables on magpies T/S ratios. 95% CI were calculated by the Wald approximation; parameter estimates were calculated by the Laplace approximation to the maximum likelihood; *p*-values were calculated by a Wald t-test. Marginal R^2: 0.27; Conditional R^2: 0.95 (calculated following Nakagawa et al. (2017); piecewiseSEM package, version 2.1.0, (Lefcheck 2016)). Significant estimates are highlighted in bold.

Table 4: Variables related to magpies' fledgling success (n = 25 individuals; 7 females, 18 males).

	β	Lower CI	Upper CI	z-value	*p*-value
Intercept	**-1.13**	**0.89**	**1.35**	**9.75**	**<0.001**
Age	**0.22**	**0.01**	**0.42**	**2.11**	**0.03**
RTL	**0.26**	**0.02**	**0.49**	**2.15**	**0.03**

Results of a GLM (Poisson error distribution, log link function) evaluating the effect of individual relative telomere length (RTL) and age on the number of chicks fledged. Significant estimates are highlighted in bold. 95% CI were calculated by the Wald approximation; parameter estimates were calculated by iteratively reweighted least squares to the maximum likelihood; *p*-values were calculated by a Wald z-test. Pseudo-R^2: 0.35 (calculated following Nagelkerke (1991); piecewiseSEM package, version 2.1.0, (Lefcheck 2016)).

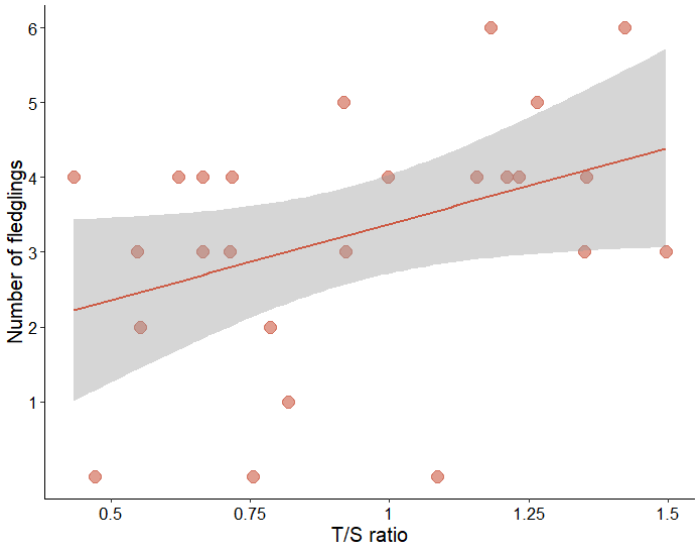


Figure 2: Relationship between the number of chicks fledged and relative telomere lengths (T/S ratio) of individuals whose broods were non-parasitized and non-manipulated (n = 25; 7 females, 18 males). Solid lines represent regression lines and shades areas represent 95% confidence interval.

Discussion

In this study we provide the first experimental test of a potential effect of brood parasitism on host's telomere dynamics, disentangling the effects of parasitism itself from the effects of the small brood size typical of parasitized nests. Our results suggest that it is not parasitism, but brood size (and thus breeding effort), that may have an impact on magpie telomere attrition, depending on initial telomere length; in any case, and because parasitism usually reduces brood size, most parasitized individuals will also experience a relatively small reduction in telomere length. Moreover, we did not find differences in RTL or rates of telomere change between sexes, which is not surprising since both sexes provision the nests at a similar rate (Precioso et al. 2020), and neither RTL nor TROCs were related to body condition.

Breeding effort has been shown to be associated to telomere dynamics (Sudyka 2019). Costly reproductive events have a negative effect on telomere dynamics; for example, an experimental increase in brood size led to a larger telomere attrition (Reichert et al. 2014), and an experimentally induced stress during reproduction was associated with faster telomere shortening (Schultner et al. 2014).

In agreement with this, our study suggests that, for individuals with larger baseline telomere length, a reduction in breeding effort may translate into a smaller rate of telomere shortening, which has also been shown in other species, either by manipulation of brood size (Reichert et al. 2014) or through an improvement in physiological condition during reproduction by medication (Pineda-Pampliega et al. 2020).

Our results are therefore in line with some previous studies of cuckoo hosts that have found that rearing parasitic nestlings does not necessarily imply extra costs, other than the reduction in breeding success due to the parasitism event (Kilner et al. 1999, Samaš et al. 2019), or that raising parasitic offspring may require less breeding effort (Canestrari et al. 2014, Požgayová et al. 2015, Samaš et al. 2018), including previous studies in magpies (Soler et al. 1995a, Precioso et al. 2020).

Although these studies on brood parasite´s hosts did not assess the effect of parasitism on telomere dynamics, some of them did explore the possible effect of parasitism on host survival to the subsequent breeding season, and in most cases found no negative effect of parasitism (Canestrari et al. 2014, Samaš et al. 2019, Precioso et al. 2020). Recently, evidence has accumulating favouring a relationship between lifespan and telomere dynamics in birds (Bize et al. 2009, Salomons et al. 2009, Barrett et al. 2013); if telomere length or dynamics are also related to survival in magpies, then parasitism might have a positive effect on magpies' lifespans by imposing smaller survival costs on parasitized individuals. This effect is, however, yet unclear: Precioso et al. (2020) found that there were no differences in survival to the subsequent breeding season between parasitized and non-parasitized magpies; nonetheless, the effect of differential reproductive effort might not be apparent in the short term, might be cumulative and have long-term consequences on survival (Boonekamp et al. 2014a) and on other life-history traits (Boonekamp et al. 2020).

The effect of reduced brood size on telomere attrition shown in this study is dependent on the individual baseline telomere length (Fig. 1), which suggests that less breeding effort by magpies translates into reduced telomere loss relative to the usual breeding effort only in those individuals with larger telomeres. The relationship between telomere attrition and baseline RTL is not surprising, since the costs of reproduction may vary with both age (Sendecka et al. 2007, Tarwater and Arcese 2017) and individual quality (Wendeln and Becker 1999, Hamel et al. 2010), and our correlational analyses (Tables 3 and 4) suggest that individuals with larger telomeres are likely to be younger individuals, but also suggest that telomere length may be

related to individual quality since non-parasitized magpies with larger telomeres showed higher fledgling success (Fig. 2). The significant effect of treatment on telomere dynamics of individuals with larger telomeres might be explained by younger individuals paying a higher cost of reproduction (Herborn et al. 2016), and/or high-quality individuals adjusting their effort to the brood's requirements and investing more in larger broods.

The lack of a difference in telomere rates of change between treatments when individual baseline RTL is small can be explained in two different ways: if individuals with shorter telomeres are older they might in general be in worse condition and pay larger costs of reproduction, irrespective of their breeding effort; some studies suggest a larger cost of reproduction for older individuals in long-lived species (Herborn et al. 2016). Also, if individuals with shorter telomeres were lower-quality individuals, they might be less capable of adjusting their breeding effort to changes in brood requirements, such as a decrease in brood size, so incurring similar costs to individuals rearing a normal-size brood. Our data show that RTL is a good predictor of reproductive success, because RTL is correlated with the number of fledglings produced by non-parasitized individuals, after controlling for their age (Table 4, Fig. 2), and moreover successful individuals (those that fledged at least one chick) tend to have longer telomeres (Table S2). This suggests that RTL is a good proxy for individual quality in magpies, in line with some previous studies performed in wild passerines and other bird species that found that telomere length was positively correlated with reproductive performance (Le Vaillant et al. 2015, Parolini et al. 2017) or other reproduction related traits, such as colouration (Taff and Freeman-Gallant 2017, Parolini et al. 2017) or antioxidant status (López-Arrabé et al. 2018; although see Bauch et al. 2013, 2016 and 2020 for species in which TL was negatively correlated with reproductive performance). If telomere length acts as a biomarker of the physiological state, quality, and ability to cope with different stressors in magpies, as has been shown in other species (Monaghan 2010), the lack of a significant difference in telomere rates of change between treatments when individual baseline RTL is small could be explained by low-quality individuals being less capable of adjusting their breeding effort to changes in brood requirements. Unfortunately, we did not know the ages of most of the individuals involved in the experimental treatments, and so we cannot know whether the individuals with shorter telomeres in our experiment were also older. Another possibility would be that shorter telomeres tend to shorten more slowly (Salomons et al. 2009) and that

the differences in rates of telomere change are then too small to be apparent in the temporal window we have used here.

In natural circumstances, the effect of parasitism on magpies' telomere dynamics might also depend on whether cuckoos select hosts according to their characteristics (i.e., age or quality). It has been proposed that great spotted cuckoos choose magpies according to their parental abilities and, therefore, their quality (Soler et al. 1995b, although this criteria varies between years depending on the amount of available host nests and the parasitism rate Molina-Morales et al. 2016). We have, however, eliminated this possible effect by randomly assigning the treatments.

Overall, our study indicates that, at least for some individuals, the reproductive costs of brood parasitism in terms of telomere dynamics would be smaller than those associated with rearing a normal host brood, which could entail consequences for their future survival. In any case, our experiment suggests no extra cost of brood parasitism in terms of telomere attrition, since individuals rearing cuckoos did not experience larger telomere attrition than individuals rearing magpies. Our results also suggest that telomere length is related to age and reproductive performance in magpies, and that both age and individual quality may play a role in how individuals deal with the costs of reproduction, and therefore, with the costs of brood parasitism. The relationship between telomere dynamics and survival in magpies has not yet been established, but this experimental study constitutes a first step towards understanding how brood parasitism may affect host lifespan.

Acknowledgments

We thank the staff of the NERC Biomolecular Analysis Facility, especially Natalie dos Remedios for her help during the optimization of the qPCR assay and Mirre Simons for providing the script for the qPCR data analysis and his help with the conceptual aspects of the technique.

Funding

This study was supported by the Spanish Ministry of Science through a project (CGL2014-55362-P), a grant (BES-2015-075675) and a Juan de la Cierva–Incorporación Fellowship (IJC2018-036411-I).

References

Barrett, E. L. B. B., Burke, T. A., Hammers, M., Komdeur, J. and Richardson, D. S. 2013. Telomere length and dynamics predict mortality in a wild longitudinal study. - Mol. Ecol. 22: 249–259.

Bates, D., Mächler, M., Bolker, B. and Walker, S. 2015. Fitting Linear Mixed-Effects Models Using lme4. - J. Stat. Softw. 67: 1-48.

Bateson, M., Eisenberg, D. T. A. A. and Nettle, D. 2019. Controlling for baseline telomere length biases estimates of the rate of telomere attrition. - R. Soc. Open Sci. 6: 190937.

Bauch, C., Becker, P. H. and Verhulst, S. 2013. Telomere length reflects phenotypic quality and costs of reproduction in a long-lived seabird. - Proc. R. Soc. B Biol. Sci. 280: 20122540.

Bauch, C., Riechert, J., Verhulst, S. and Becker, P. H. 2016. Telomere length reflects reproductive effort indicated by corticosterone levels in a long-lived seabird. - Mol. Ecol. 25: 5785–5794.

Bauch, C., Gatt, M. C., Granadeiro, J. P., Verhulst, S. and Catry, P. 2020. Sex-specific telomere length and dynamics in relation to age and reproductive success in Cory's shearwaters. - Mol. Ecol. 29: 1344–1357.

Bize, P., Criscuolo, F., Metcalfe, N. B., Nasir, L. and Monaghan, P. 2009. Telomere dynamics rather than age predict life expectancy in the wild. - Proc. R. Soc. B Biol. Sci. 276: 1679–1683.

Blackburn, E. H. 1991. Structure and function of telomeres. - Nature 350: 569–573.

Boonekamp, J. J., Salomons, M., Bouwhuis, S., Dijkstra, C. and Verhulst, S. 2014a. Reproductive effort accelerates actuarial senescence in wild birds: An experimental study. - Ecol. Lett. 17: 599–605.

Boonekamp, J. J., Mulder, G. A., Salomons, H. M., Dijkstra, C. and Verhulst, S. 2014b. Nestling telomere shortening, but not telomere length, reflects developmental stress and predicts survival in wild birds. - Proc. R. Soc. B Biol. Sci. 281: 20133287.

Boonekamp, J. J., Bauch, C. and Verhulst, S. 2020. Experimentally increased brood size accelerates actuarial senescence and increases subsequent reproductive effort in a wild bird population. - J. Anim. Ecol. 89: 1395–1407.

Burnham, K. P. and Anderson, D. R. 1998. Practical Use of the Information-Theoretic Approach. - In: Model Selection and Inference. Springer New York, pp. 75–117.

Campisi, J. 2005. Senescent cells, tumor suppression, and organismal aging: Good citizens, bad neighbors. - Cell 120: 513–522.

Canestrari, D., Bolopo, D., Turlings, T. C. J., Roder, G., Marcos, J. M. and Baglione, V. 2014. From Parasitism to Mutualism: Unexpected Interactions Between a Cuckoo and Its Host. - Science 343: 1350–1352.

Cawthon, R. M. 2002. Telomere measurement by quantitative PCR. - Nucleic Acids Res. 30: 1–6.

Cawthon, R. M. 2009. Telomere length measurement by a novel monochrome multiplex quantitative PCR method. - Nucleic Acids Res. 37: 1–7.

Chatelain, M., Drobniak, S. M. and Szulkin, M. 2020. The association between stressors and telomeres in non-human vertebrates: a meta-analysis. - Ecol. Lett. 23: 381–398.

Davies, N. B. 2000. Cuckoos, Cowbirds and Other Cheats. - T & AD Poyser.

Dawson, D. A., dos Remedios, N. and Horsburgh, G. J. 2016. A new marker based on the avian spindlin gene that is able to sex most birds, including species problematic to sex with CHD markers. - Zoo Biol. 35: 533–545.

Griffiths, R., Double, M. C., Orr, K. and Dawson, R. J. G. G. 1998. A DNA test to sex most birds. - Mol. Ecol. 7: 1071–1075.

Hamel, S., Gaillard, J.-M. M., Yoccoz, N. G., Loison, A., Bonenfant, C. and Descamps, S. 2010. Fitness costs of reproduction depend on life speed: empirical evidence from mammalian populations. - Ecol. Lett. 13: 915–935.

Hemann, M. T., Strong, M. A., Hao, L. Y. and Greider, C. W. 2001. The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability. - Cell 107: 67–77.

Herborn, K. A., Daunt, F., Heidinger, B. J., Granroth-Wilding, H. M. V., Burthe, S. J., Newell, M. A., Monaghan, P. and Williams, T. 2016. Age, oxidative stress exposure and fitness in a long-lived seabird. - Funct. Ecol. 30: 913–921.

Hoover, J. P. and Reetz, M. J. 2006. Brood parasitism increases provisioning rate, and reduces offspring recruitment and adult return rates, in a cowbird host. - Oecologia 149: 165–173.

Kilner, R. M., Noble, D. G. and Davies, N. B. 1999. Signals of need in parent-offspring communication and their exploitation by the common cuckoo. - Nature 397: 667–672.

Koleček, J., Jelínek, V., Požgayová, M., Trnka, A., Baslerová, P., Honza, M. and Procházka, P. 2015. Breeding success and brood parasitism affect return rate and dispersal distances in the great reed warbler. - Behav. Ecol. Sociobiol. 69: 1845–1853.

Le Vaillant, M., Viblanc, V. A., Saraux, C., Le Bohec, C., Le Maho, Y., Kato, A., Criscuolo, F. and Ropert-Coudert, Y. 2015. Telomere length reflects individual quality in free-living adult king penguins. - Polar Biol. 38: 2059–2067.

Lefcheck, J. 2016. piecewiseSEM: Piecewise structural equation modeling. R package version 1.2.1. - Methods Ecol. Evol. 7: 573–579.

Lenth, R. 2020. emmeans: Estimated Marginal Means, aka Least-Squares Means. - R Packag. version 1.5.0 < https://CRAN.R-project.org/package=emmeans>

Capítulo 2

López-Arrabé, J., Monaghan, P., Cantarero, A., Boner, W., Pérez-Rodríguez, L. and Moreno, J. 2018. Sex-specific associations between telomere dynamics and oxidative status in adult and nestling pied flycatchers. - Physiol. Biochem. Zool. 91: 868–877.

López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M. and Kroemer, G. 2013. The hallmarks of aging. - Cell 153: 1194.

Mark, M. M. and Rubenstein, D. R. 2013. Physiological costs and carry-over effects of avian interspecific brood parasitism influence reproductive tradeoffs. - Horm. Behav. 63: 717–722.

Martínez, J. G., Soler, J. J., Soler, M. and Burke, T. 1998. Spatial patterns of egg laying and multiple parasitism in a brood parasite: A non-territorial system in the great spotted cuckoo (*Clamator glandarius*). - Oecologia 117: 286–294.

Molina-Morales, M., Gabriel Martínez, J., Martín-Gálvez, D., A. Dawson, D., Rodríguez-Ruiz, J., Burke, T. and Avilés, J. M. 2013. Evidence of long-term structured cuckoo parasitism on individual magpie hosts. - J. Anim. Ecol. 82: 389–398.

Molina-Morales, M., Martínez, J. G. and Avilés, J. M. 2016. Criteria for host selection in a brood parasite vary depending on parasitism rate. - Behav. Ecol. 27: 1441–1448.

Monaghan, P. 2010. Telomeres and life histories: The long and the short of it. - Ann. N. Y. Acad. Sci. 1206: 130–142.

Monaghan, P. and Haussmann, M. F. 2006. Do telomere dynamics link lifestyle and lifespan? - Trends Ecol. Evol. 21: 47–53.

Monaghan, P., Metcalfe, N. B. and Torres, R. 2009. Oxidative stress as a mediator of life history trade-offs: Mechanisms, measurements and interpretation. - Ecol. Lett. 12: 75–92.

Nagelkerke, N. 1991. A note on a general definition of the coefficient of determination. - Biometrika. 78: 691–692.

Nakagawa, S., Johnson, P. C. D. D. and Schielzeth, H. 2017. The coefficient of determination R2 and intra-class correlation coefficient from generalized linear mixed-effects models revisited and expanded. - J. R. Soc. Interface 14: 20170213.

Parolini, M., Romano, A., Costanzo, A., Khoriauli, L., Santagostino, M., Nergadze, S. G., Canova, L., Rubolini, D., Giulotto, E. and Saino, N. 2017. Telomere length is reflected by plumage coloration and predicts seasonal reproductive success in the barn swallow. - Mol. Ecol. 26: 6100–6109.

Pineda-Pampliega, J., Herrera-Dueñas, A., Mulder, E., Aguirre, J. I., Höfle, U. and Verhulst, S. 2020. Antioxidant supplementation slows telomere shortening in free-living white stork chicks. - Proc. R. Soc. B Biol. Sci. 287: 1–7.

Požgayová, M., Beňo, R., Procházka, P., Jelínek, V., Abraham, M. M. and Honza, M. 2015. Lazy males and hardworking females? Sexual conflict over parental care in a brood

parasite host and its consequences for chick growth. - Behav. Ecol. Sociobiol. 69: 1053–1061.

Precioso, M., Molina-Morales, M., Sánchez-Tójar, A., Avilés, J. M. and Martínez, J. G. 2020. Brood parasitism, provisioning rates and breeding phenology of male and female magpie hosts. - J. Avian Biol.: jav.02522.

R Core Team 2018. R: A language and environment for statistical computing. R Foundation for Statistical Computing.<https://www.r-project.org/>.

Reichert, S., Stier, A., Zahn, S., Arrivé, M., Bize, P., Massemin, S. and Criscuolo, F. 2014. Increased brood size leads to persistent eroded telomeres. - Front. Ecol. Evol. 2: 1–11.

Richardson, D. S., Jury, F. L., Blaakmeer, K., Komdeur, J. and Burke, T. 2001. Parentage assignment and extra-group paternity in a cooperative breeder: the Seychelles warbler (*Acrocephalus sechellensis*). - Mol. Ecol. 10: 2263–2273.

Ricklefs, R. E. 2010. Life-history connections to rates of aging in terrestrial vertebrates. - PNAS 107: 10314–10319.

Rothstein, S. I. 1990. A Model System for Coevolution: Avian Brood Parasitism. - Annu. Rev. Ecol. Syst. 21: 481–508.

Ruijter, J. M., Ramakers, C., Hoogaars, W. M. H. H., Karlen, Y., Bakker, O., van den Hoff, M. J. B. B. and Moorman, A. F. M. M. 2009. Amplification efficiency: Linking baseline and bias in the analysis of quantitative PCR data. - Nucleic Acids Res. 37: e45–e45.

Salomons, H. M., Mulder, G. A., Van De Zande, L., Haussmann, M. F., Linskens, M. H. K. K. and Verhulst, S. 2009. Telomere shortening and survival in free-living corvids. - Proc. R. Soc. B Biol. Sci. 276: 3157–3165.

Samaš, P., Rutila, J., Honza, M., Kysučan, M. and Grim, T. 2018. Rearing a virulent common cuckoo is not extra costly for its only cavity-nesting host. - Proc. R. Soc. B Biol. Sci. 285: 20181710.

Samaš, P., Grim, T., Jelínek, V., Abraham, M. M., Šulc, M. and Honza, M. 2019. No immediate or future extra costs of raising a virulent brood parasite chick. - Behav. Ecol. 30: 1020–1029.

Schielzeth, H. 2010. Simple means to improve the interpretability of regression coefficients. - Methods Ecol. Evol. 1: 103–113.

Schultner, J., Moe, B., Chastel, O., Bech, C. and Kitaysky, A. S. 2014. Migration and stress during reproduction govern telomere dynamics in a seabird. - Biol. Lett. 10: 3–6.

Sendecka, J., Cicho, M. and Gustafsson, L. 2007. Age-dependent reproductive costs and the role of breeding. Act. Zool. 88: 95–100.

Capítulo 2

Soler, M., Martinez, J. G., Soler, J. J. and Møller, A. P. 1995a. Preferential allocation of food by magpies Pica pica to great spotted cuckoo *Clamator glandarius* chicks. - Behav. Ecol. Sociobiol. 37: 7–13.

Soler, J. J., Soler, M., Møller, A. P. and Martínez, J. G. 1995b. Does the great spotted cuckoo choose magpie hosts according to their parenting ability? - Behav. Ecol. Sociobiol. 36: 201–206.

Soler, M., Martînez, J. G. and Soler, J. J. 1996. Effects of brood parasitism by the great spotted cuckoo on the breeding success of the magpie host: an experimental study. - Ardeola 43: 87–96.

Sudyka, J. 2019. Does Reproduction Shorten Telomeres? Towards Integrating Individual Quality with Life-History Strategies in Telomere Biology. - BioEssays 41: 1900095.

Taff, C. C. and Freeman-Gallant, C. R. 2017. Sexual signals reflect telomere dynamics in a wild bird. - Ecol. Evol. 7: 3436–3442.

Tarwater, C. E. and Arcese, P. 2017. Young females pay higher costs of reproduction in a short-lived bird. - Behav. Ecol. Sociobiol. 71: 84.

Verhulst, S., Aviv, A., Benetos, A., Berenson, G. S. and Kark, J. D. 2013. Do leukocyte telomere length dynamics depend on baseline telomere length? An analysis that corrects for "regression to the mean." - Eur. J. Epidemiol. 28: 859–866.

Wendeln, H. and Becker, P. H. 1999. Effects of parental quality and effort on the reproduction of common terns. - J. Anim. Ecol. 68: 205–214.

Wilbourn, R. V., Moatt, J. P., Froy, H., Walling, C. A., Nussey, D. H. and Boonekamp, J. J. 2018. The relationship between telomere length and mortality risk in non-model vertebrate systems: A meta-analysis. - Philos. Trans. R. Soc. B Biol. Sci. 373: 20160447.

Zou, Y., Sfeir, A., Gryaznov, S. M., Shay, J. W. and Wright, W. E. 2004. Does a sentinel or a subset of short telomeres determine replicative senescence? - Mol. Biol. Cell 15: 3709–3718.

Supplementary Material

Table S1: Results of model selection based on Akaike's information criterion.

Model No.		k	AICc	ΔAICc	AICc Wt	Cum. weight	Multiple R^2	Adjusted R^2
3	TROC ~ Intercept + Treatment+RTL1+ Treatment*RTL1	7	63.76	0	0.995	0.995	0.69	0.62
1	TROC ~ Intercept + Treatment	4	75.14	11.38	0.003	0.998	0.32	0.26
2	TROC ~ Intercept + Treatment + RTL1	5	76.90	13.14	0.001	0.999	0.35	0.26

Models are ordered by their degree of support based on AICc. ΔAICc is the difference between a particular model and the best one. K is the number of estimated parameters (treatment has been dummy coded in all the models, so each treatment is treated as a separate variable compared to the reference one, with its own estimated parameter). Akaike weights (AICcWt) show the relative support a given model has from the data compared with the other models.

Table S2: Evaluation of other variables possibly affecting magpies' TROC.

	β	Lower CI	Higher CL	d.f.	t-value	*p*-value	Multiple R^2	Adjusted R^2
Model 3a:								
Intercept	0.03	-0.22	0.29	20	0.26	0.80	0.72	0.64
Treatment 2	-0.27	-0.95	0.42	20	-0.80	0.43		
Treatment 3	**-1.23**	**-1.83**	**-0.62**	**20**	**-4.24**	**<0.001**		
RTL1	**0.39**	**0.06**	**0.72**	**20**	**2.50**	**0.02**		
Diff. Body condition	0.18	-0.08	0.44	20	1.43	0.17		
Treatment 2*RTL1	-0.28	-1.30	0.73	20	-0.59	0.56		
Treatment 3*RTL1	**-1.28**	**-1.85**	**-0.70**	**20**	**-4.65**	**<0.001**		
Model 3b:								
Intercept	0.03	-0.23	0.30	20	0.28	0.78	0.69	0.60
Treatment 2	-0.27	-0.99	0.46	20	-0.77	0.45		
Treatment 3	**-1.14**	**-1.76**	**-0.52**	**20**	**-3.85**	**<0.001**		
RTL1	**0.44**	**0.08**	**0.81**	**20**	**2.53**	**0.02**		
Sex	-0.08	-0.63	0.48	20	-0.28	0.78		
Treatment 2*RTL1	-0.25	-1.32	0.82	20	-0.49	0.63		
Treatment 3*RTL1	**-1.31**	**-1.92**	**-0.70**	**20**	**-4.50**	**<0.001**		
Model 3c:								
Intercept	0.03	-0.23	0.3	20	0.26	0.79	0.69	0.60
Treatment 2	-0.25	-0.99	0.49	20	-0.71	0.48		
Treatment 3	**-1.13**	**-1.79**	**-0.47**	**20**	**-3.57**	**0.002**		
RTL1	**0.43**	**0.08**	**0.77**	**20**	**2.60**	**0.02**		
Interval of time	0.01	-0.27	0.3	20	0.10	0.92		
Treatment 2*RTL1	-0.27	-1.36	0.81	20	-0.53	0.60		
Treatment 3*RTL1	**-1.31**	**-1.94**	**-0.67**	**20**	**-4.32**	**<0.001**		

Results of models (LMs) based on Model 3 additionally evaluating the effect of: differences in body condition between captures (model 3a), sex (model 3b) and the interval of time past between captures (model 3c) on magpies' corrected telomere rate of change (D/time). RTL1 refers to baseline RTL, that is, RTL of individuals before the experimental treatment. Significant estimates are highlighted in bold.

Table S3: Variables affecting relative telomere length (T/S ratio) in magpies (n=69 observations, 61 individuals; 24 females, 37 males).

	β	Lower CI	Upper CI	t-value	*p*-value
Fixed effects					
Intercept	**-0.19**	**-0.32**	**-0.05**	**-2.75**	**<0.01**
Age	**-0.22**	**-0.34**	**-0.1**	**-3.52**	**<0.001**
Sex	-0.15	-0.42	0.12	-1.08	0.28
Body condition	-0.02	-0.06	0.01	-1.27	0.20
Breeding Success	**0.31**	**0.18**	**0.44**	**4.53**	**<0.001**
Time capture (before/after laying)	-0.01	-0.07	0.05	-0.24	0.81
Random effect	σ	σ^2			
Individual ID	0.35	0.12			
Residual	0.09	0.01			

Results of a GLMM (gamma error distribution, log link function) evaluating the effect of different variables on magpies T/S ratios. Significant estimates are highlighted in bold. 95% CI were calculated by the Wald approximation; parameter estimates were calculated by the Laplace approximation to the maximum likelihood; *p*-values were calculated by a Wald t-test. Marginal R^2: 0.35; Conditional R^2: 0.96 (calculated following Nakagawa, Johnson and Schielzeth (2017); piecewiseSEM package, version 2.1.0, Lefcheck (2016)).

Table S4: Variables affecting relative telomere length (T/S ratio) in magpies (n=69 observations, 62 individuals; 25 females, 37 males).

	β	Lower CI	Upper CI	t-value	*p*-value
Fixed effects					
Intercept	**-0.19**	**-0.35**	**-0.02**	**-2.27**	**0.02**
Age	**-0.34**	**-0.52**	**-0.16**	**-3.74**	**<0.001**
Sex	-0.15	-0.49	0.18	-0.91	0.36
Body condition	**-0.05**	**-0.09**	**-0.01**	**-2.34**	**0.02**
Breeding Success	**0.44**	**0.27**	**0.60**	**5.26**	**<0.001**
Time capture (before/after laying)	-0.05	-0.11	0.01	-1.73	0.08
Random effect	σ	σ^2			
Individual ID	0.44	0.19			
Residual	0.09	0.01			

Results of a GLMM (gamma error distribution, log link function) evaluating the effect of different variables on magpies T/S ratios. Significant estimates are highlighted in bold. 95% CI were calculated by the Wald approximation; parameter estimates were calculated by the Laplace approximation to the maximum likelihood; *p*-values were calculated by a Wald t-test. Marginal R^2: 0.45; Conditional R^2: 0.98 (calculated following Nakagawa et al. (2017); piecewiseSEM package, version 2.1.0, Lefcheck (2016)).

Capítulo 3

Effects of long-term ethanol storage of blood samples on the estimation of telomere length

Effects of long-term ethanol storage of blood samples on the estimation of telomere length

Marta Precioso[1, 2], Mercedes Molina-Morales[1], Deborah A. Dawson[2], Terry Burke[2], Juan G. Martínez[1]

[1] *Departamento de Zoología, Universidad de Granada, Granada, Spain*
[2] *Department of Animal and Plant Sciences, University of Sheffield, Sheffield, United Kingdom*

Abstract

Telomeres, DNA structures located at the end of eukaryotic chromosomes, shorten with each cellular cycle. The shortening rate is affected by factors associated with stress, and, thus telomere length has been used as a biomarker of ageing, disease, and different life history trade-offs. Telomere research has received much attention in the last decades, however there is still a wide variety of factors that may affect telomere measurements and to date no study has thoroughly evaluated the possible long-term effect of storage medium on telomere measurements. In this study we evaluated the long-term effects of ethanol on relative telomere length (RTL) measured by qPCR, using blood samples of magpies collected over twelve years and stored in absolute ethanol at room temperature. During this study, we firstly tested whether storage time had an effect on RTL and secondly we modelled the effect of time of storage (from 1 to 12 years) in differences in RTL from DNA extracted twice in consecutive years from the same blood sample. We propose an equation to correct the effect of storage duration on RTL estimates. Finally, to validate the results we also compared corrected RTL values with RTL values of DNA extracted from ethanol-stored blood samples shortly after sampling. Our study provides evidence of an effect of storage time on telomere length measurements when blood samples are stored in absolute ethanol at room temperature. Importantly, this effect shows a temporal pattern of decreasing loss of telomere sequence with storage time that stops after approximate 4 years of storage, which suggests that telomeres may degrade in blood samples stored in ethanol. Our method to quantify the effect of storage time and correct RTL measurements could be used to evaluate other storage

buffers and methods. Our results highlight the need to evaluate the long-term effects of storage on telomere measurements in particular in long-term studies where samples not initially intended for telomere measurements are used at a later date for this purpose.

Introduction

Telomeres are non-coding repetitive and conserved DNA sequences located at the ends of eukaryotic chromosomes that maintain their integrity (Blackburn 1991). Telomeres in vivo are compacted in a chromatin structure that interacts with modified histones, and their ends are associated with other proteins forming a complex denominated shelterin (De Lange 2005). This complex forms a loop (T-loop, Griffith et al. 1999) that protects the single-stranded 3' overhang end of the chromosome from the DNA damage response machinery that, otherwise, would recognize it as broken DNA and trigger cell replicative senescence or its apoptosis (De Lange 2010).

During the replication process, the DNA polymerase cannot replicate the end of the lagging DNA strand ("the end-replication problem") and telomeres shorten with each cellular cycle (Blackburn 1991). When telomeres shorten to a critical length, the cell enters a state of replicative senescence or dies via apoptosis (Hemann et al. 2001, Zou et al. 2004). Telomeres then also protect the coding DNA sequence from loss during replication and help to maintain the proliferative capacity of cells (Blackburn 2000).

Moreover, telomeres constitute fragile sites that are more susceptible to oxidative damage than other parts of the genome and are repaired less efficiently (Petersen et al. 1998, Rhee et al. 2011, Coluzzi et al. 2014), so that situations that may cause oxidative stress, such as infection or reproduction (Chatelain et al. 2020), may contribute to telomere loss. This loss can result in an accumulation of defects that may affect the function of the different tissues leading to organismal ageing and disease (López-Otín et al. 2013, Blackburn et al. 2015) but may also link lifespan and lifestyle (Monaghan and Haussmann 2006) and help to understand how individuals deal with stressful circumstances (Haussmann and Marchetto 2010, Monaghan 2014). For these reasons, telomere biology has become an important topic in different areas of research, such as epidemiology or evolutionary ecology.

The study of telomeres in natural populations has received considerable attention in the last two decades (Monaghan et al. 2018). Telomeres may play an important role in mediating different life-history trade-offs (Young 2018), and telomere length have been shown to be correlated with, for example, survival (Wilbourn et al. 2018) or reproductive success (Eastwood et al. 2019) in a variety of taxa. Besides, the rate at which telomeres shorten is also related to growth (Monaghan and Ozanne 2018), the costs of reproduction (Sudyka et al. 2019), environmental conditions (Spurgin et al. 2018, Foley et al. 2020) or lifespan (Whittemore et al. 2019), and thus, both telomere length and its dynamics may give us insights into the life history strategies of individuals.

There are different methods available to estimate telomere length (Nakagawa et al. 2004, Nussey et al. 2014), although telomere measurement using real-time quantitative PCR (qPCR, Cawthon 2002, Cawthon 2009) has probably become the most widespread method in all areas of telomere research (Pepper et al. 2018, Morinha et al. 2020a). qPCR is an inexpensive and fast method that only requires a small amount of DNA (Cawthon 2002) and estimates a relative telomere length (RTL) as the ratio between the amount of telomeric sequence (T) and the amount of sequence of a single-copy reference gene (S) amplified from the same sample. However, several factors can impact telomere measurement by qPCR (reviewed in Morinha et al. 2020a) and there is still heterogeneity regarding sample collection, storage and DNA extraction methods. This is especially important in the case of long-term studies, where samples taken at some point can be analysed quite a long time after collection; in some instances, samples may have not been intended for measuring telomeres.

Different studies have evaluated the impact of different storage methods on telomere measurements. For example, Reichert et al. (2017) found differences in telomere length estimations depending on the storage method in short-term and long-term stored samples, but the effect of storage could be confounded by different extraction methods, which have already been shown to impact telomere measurements by qPCR (Cunningham et al. 2013, Tolios et al. 2015, Raschenberger et al. 2016, Eastwood et al. 2018).

Ethanol is widely used for the long-term storage of blood samples (Kilpatrick 2002, Camacho-Sanchez et al. 2013), since it is easy to obtain, found in most labs, inexpensive and maintains DNA quality for a long period at room temperature. It has also been shown to be a good blood preservation method for

telomere measurement by qPCR compared to other storage buffers (Eastwood et al. 2018) and that it yields a high correlation between TL measurements by qPCR and terminal restriction fragment assay (correlation coefficients r ranged from 0.68 to 0.74). However, the suitability of ethanol as a long-term storage medium for samples used in telomere measurements has not previously been tested.

Here, we present a test of the effect of long-term preservation of blood samples in absolute ethanol at room temperature on telomere length measurement by qPCR. We aim, i) to test if the number of years that samples had been stored in ethanol affects telomere length measurements, using samples stored between 1 and 12 years. Secondly, ii) we aim to model how the differences in the estimation of RTL from DNA templates extracted from the same blood sample in two consecutive years depend on the duration of storage (1 to 12 years). Finally, we suggest a way to correct for the effect of storage duration on RTL estimation, and validate this method by comparing corrected RTL values with RTL values from freshly extracted DNA samples.

Methods

Sample collection and Study design

All the samples were collected from a population of Eurasian magpies (*Pica pica*) located in La Calahorra, Granada, South-eastern Spain (37°10'N, 3°03'W). This population has been monitored since 2005, and blood samples (about 100 microliters) from adults and chicks have been collected during each breeding season (March - July) and kept in one millilitre of absolute ethanol (Analytical Reagent Grade) at room temperature for future analysis.

Blood samples were taken from 143 magpies, 118 corresponded to chicks that were sampled soon before fledging (age class= 0), during the breeding seasons from 2005 to 2017; and 25 samples corresponded to adult magpies (age class= 1), that were captured during breeding in the same period. Genomic DNA was extracted from the erythrocytes of the whole blood samples by the ammonium acetate precipitation method (Richardson et al. 2001) between October and November of 2016, 2017 or 2018 and stored at -20ºC. Relative telomere length (RTL) of these samples was estimated by real-time PCR method (see below).

To test whether long-term storage in ethanol could have an effect on the estimates of telomere length we used 91 blood samples that had been stored in

absolute ethanol up to eleven years in 2016 (Fig. 1). We extracted DNA from the blood samples and measured their RTL in 2016, and, one year later (2017), we extracted DNA from the same samples and measured their RTL again. We ensured that ethanol volume was at least ten times larger than sample volume in all cases (Barrett et al. 2012). We could then check whether there were differences in RTL of samples stored in ethanol between consecutive years and whether these differences were related to the time that samples had been stored in ethanol before the second DNA extraction (1 to 12 years). We used between 2 and 23 blood samples taken per year between 2005 and 2016 (mean (SD): 6.4 samples per year (3.7)). Unfortunately, we were unable to extract DNA twice from samples obtained in 2013 and 2012, so we did not have data of samples stored for 4 and 5 years.

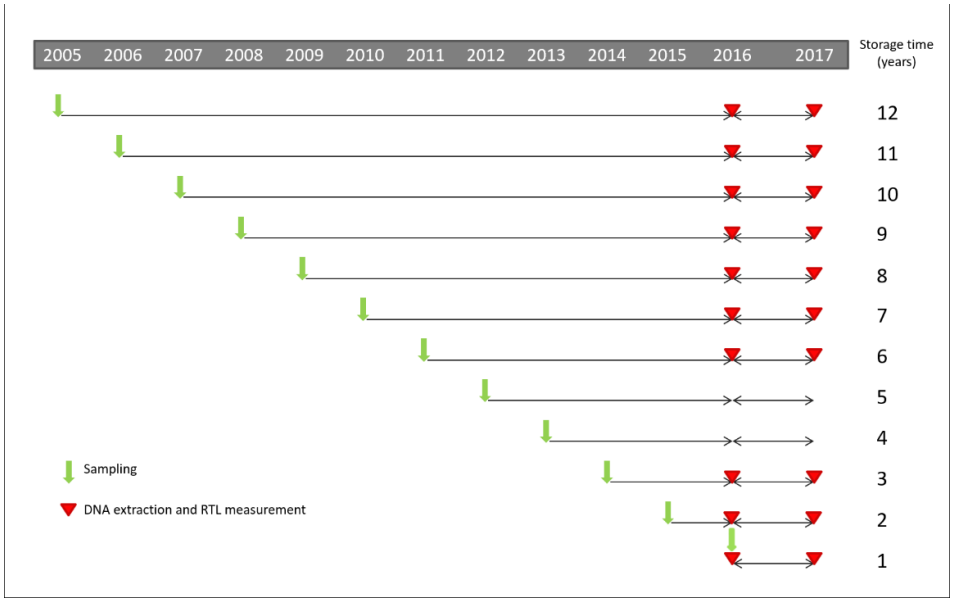


Figure 1: Scheme illustrating the study design: green arrows represent the years when samples were taken; red triangles represent when samples were extracted and their RTL were measured; storage time is the number of years that samples were stored before their second extraction.

Telomere length measurements by qPCR

All laboratory analyses were carried out at the NERC Biomolecular Analysis Facility of the University of Sheffield (United Kingdom).

DNA sample concentrations were measured using a NanoDrop 8000 Spectrophotometer (ThermoScientific); samples were then diluted to 25 nanograms/microliter. DNA quality was assessed by agarose gel electrophoresis in a subset of samples. We additionally performed a high sensitivity D1000 ScreenTape electrophoresis assay with an Agilent 4200 TapeStation system (Agilent Technologies) in genomic DNA from 4 samples that were extracted twice (in two consecutive years) to assure the integrity of the DNA (see Figures S1 to S8, Supplementary material).

Telomere length was assessed by real-time PCR (qPCR) in a monochrome multiplex reaction (Cawthon 2009) with some modifications. We used telc and telg primers for the telomere sequence (Cawthon 2009) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the reference gene. For this gene, we used the pair of specific primers for magpies described in Chapter 2 (p. 89) (Genbank Accession Number: EF052752). This primer set was clamped with two guanine-cytosine tails on their 5' to elevate their melting temperature (Tm), to allow us to separate the amplification of the telomere sequence and the GAPDH sequence within the same reaction (see Cawthon 2009 for further explanations). The size of the product amplified with this primer set was 97 bp, similar to the fixed length telomere product. The sequence of the forward and reverse primers for GAPDH including the clamp are 5'- CGGCGGCGGGCGGCGCGGGCTGGGCGGAGCC-AAAGTGGCTCCAATCCCACT-3' and 5'-GCCCGGCCCGCCGCGCCCGTCCCGCCG-CTGAACTCCCATCCACCCT-3', respectively. Primer concentration was 900 nM for both pairs of primers and DNA concentration was 25 nanograms per reaction. Reaction volume was 20 µl containing 10 µl of SYBR Select Master Mix (Applied Biosystems).

Each sample was run in duplicate, loaded manually in MicroAmp Fast Optical 96 Well Plates and sealed with MicroAmp Optical Adhesive Film (Applied Biosystems). Samples were randomly distributed on 29 different plates (along with samples belonging to a different study) to avoid bias regarding the year of sampling. All qPCR assays were performed in a QuantStudio 12K Flex qPCR instrument (ThermoFisher SCIENTIFIC) and the thermal cycling profile was as follows: Hold

Stage: 2 min at 50ºC, 2 min at 95ºC; Stage 1: 2 cycles of 15 s at 94ºC, 10 s at 49ºC; Stage 2: 40 cycles of 15 s at 94ºC, 10 s of 62ºC, 15 s at 74ºC with signal acquisition (for the telomere amplification), 10 s at 84ºC, 15 s at 86 ºC with signal acquisition (for the GAPDH amplification); Melt curve stage: 15 s at 95ºC, 1 min at 60ºC, 15s at 95ªC. The melting curve allowed us to confirm the presence of both amplicons and to evaluate the specificity of the primers (Morinha et al. 2020b).

Each plate included two no-template controls (NTC) and serial dilutions by duplicate (80, 20, 5, 1.25, 0.3125 nanograms/microliter) of DNA from the same sample to generate a standard curve. This standard curve allows us to calculate the amount of DNA amplified in each reaction from its Cq values (cycle at which amplification crosses a fixed threshold), assess amplification efficiency and account for inter-plate variation. Efficiencies were calculated as $E= (10^{(-1/slope)}-1) \times 100$ for the telomeres and GAPDH separately, using the slope of the standard curve for each amplification. T and S were calculated following the method by Ruijter et al. (2009), and then divided to get a ratio (T/S).

Amplification efficiencies were on average 97.7 % (6.8) for telomeres and 94.2% (4.8) for GAPDH. The average intra-plate standard deviations of Cq values were on average 0.078 (0.014) for telomeres and 0.083 (0.021) for GAPDH. Intra-plate and inter-plate T/S ratio standard deviations were on average 0.037 (0.013) and 0.078 (0.016), respectively. R^2 for standard curves were on average 0.99 (0.004) for telomeres and 0.99 (0.003) for GAPDH.

T/S ratios varied between 0.16 and 1.86. All T/S ratios were standardized to account for inter-run variability by dividing the average T/S ratio of duplicates by the average T/S ratio of the 20 nanograms/microliter standard sample of the plate where the sample was run. This standardized value is our estimate of relative telomere length (RTL).

Statistical analyses

All statistical analyses were performed in R version 4.0.3 (R Core Team 2020).

Sample storage duration and RTL

To explore whether the time (years) that samples had been preserved in ethanol affected the estimation of RTL, we constructed a linear mixed model (LMM, package lme4 version 1.1-23, Bates et al. 2015) in which RTL was the dependent variable and the number of years of storage and date of sampling (1 = 1st April) were

considered continuous fixed effects; the age class of individuals when sampled (chick: 0, adult: 1) was also included as a factor. Date of sampling and age class were highly correlated, so we removed one of them (date of sampling) from the final model. We also included as random factors individual id, to account for the samples that had been extracted twice, and nest id, because there were siblings in our data set. The analysis included 234 DNA samples extracted from 143 blood samples (individuals); these 143 individuals belonged to 117 nests.

Modelling the effect of storage time on RTL estimation

We used the 91 blood samples (extracted twice in two consecutive years) to model how RTL could vary depending on the duration of storage (1 to 12 years). We constructed a linear model (LM) in which the difference in RTLs estimated using two DNA samples extracted in two consecutive years from the same blood sample was the dependent variable. Visual inspection of the raw data suggested that the relationship between the difference in RTL and storage time (number of years elapsed between blood sampling and the second DNA extraction) could be polynomial, so we tested whether including a quadratic and a cubic term of the storage time significantly improved the model fit. The linear model significantly differed from the quadratic model ($F_{1,88} = 59.9$, p-value < 0.001) and the quadratic model significantly differed from the cubic model ($F_{1,87} = 4.2$, p-value $= 0.04$), which represented the best fit (see Table S1, Supplementary material for a comparison of the three models), so we finally included storage time and its polynomials of degree two and three as continuous fixed effects. The first estimate of RTL was also included as a continuous fixed effect and individual age class when sampling (chick: 0, adult: 1) was included as a factor. Age class when sampling was not significant ($\beta = -0.003$; 95% CI = -0.07, 0.06; t = -0.09; $p = 0.93$) and explained little variance, so it was not included in the final model. This model would allow us to estimate for each sample the relative amount of telomere sequence lost (differences in RTLs) in each year of its storage, and, by summing all these differences depending on the number of years the sample was stored, correct its RTL.

The difference in RTLs and the first estimated RTL are not statistically independent, and this dependency can lead to a biased estimate of the relationship between these two variables caused by a statistical artefact called "regression to the mean" (Barnett et al. 2005). For this reason we additionally constructed a LM in which the differences in RTL were corrected for this effect using the equation

described in Verhulst et al. (2013) so that a corrected value for the differences (D) was the dependent variable and the first estimate of RTL was included as continuous fixed effect.

Validation of the correction of RTL estimates

We tested whether there were differences between the corrected RTL estimated for 80 fledglings that had recruited into the population during the last 12 years and whose DNA was extracted years after blood sampling and RTL of 21 fledglings born in 2016 and 2017 whose DNA samples were extracted soon after sampling (3-5 months) and also recruited into the population. We used samples only from recruits to avoid bias due to the possible confounding effect of telomere length on survival. We constructed a LM in which RTL was the dependent variable, whether the RTL had been corrected or not (non-corrected: 0, corrected: 1) was included as a factor. Age (in days, ranging from 11 to 25 days old) and weight (in grams) of the fledglings at sampling were included as continuous fixed effects to control for the effect of these variables in the telomere length (Salomons et al. 2009, Monaghan and Ozanne 2018). We also included as random factors the year of sampling because the number of samples per year was unbalanced, and nest id, because there were siblings in our data set. This analysis included 101 individuals from 83 nests.

Results

Sample storage duration and RTL

There was a significant negative relationship between the number of years a sample had been stored and the RTL estimated from it: samples that had been preserved for more years tended to have shorter RTL (Table 1).

On the other hand, there were no significant differences in RTL between samples from fledglings and adults in our data set (Table 1).

Modelling storage-time effects on RTL

There is a polynomial relationship between RTL estimated from DNA samples extracted in consecutive years from the same blood sample and the number of years that sample had been stored before the second extraction, so that the differences in RTL decrease for samples stored between one and 4 years until they drop to approximately zero (Table 2, Fig. 1). This suggests that there is degradation of

telomeres causing shortening during the first four years of storage in ethanol and then the degradation stops. Our results also suggests that this degradation is dependent on telomere length, as the first RTL is positively and significantly related to the differences in RTL (Table 2) and this relationship is not a result of a regression to the mean effect since the corrected value (D) was significantly explained by RTL1 (see Supplementary material S2).

Table 1: Factors affecting RTL of magpies' blood samples stored in ethanol (N = 234 DNA samples, 143 blood samples (individuals), 117 nests).

Fixed effects	β	Lower CI	Upper CI	df	t	*p*-value
Intercept	**0.79**	**0.68**	**0.82**	**157.6**	**18.41**	**<0.001**
Storage time	**-0.03**	**-0.04**	**-0.02**	**166.1**	**-5.53**	**<0.001**
Age class	-0.05	-0.18	0.08	140.9	-0.78	0.44
Random effects	σ	σ^2				
Individual identity	0.20	0.04				
Nest identity	0.13	0.02				

Results of a LMM testing the effect of the duration of storage (years) in ethanol of blood samples, sampling age class (whether the individual was sampled as a chick or an adult) on the estimated RTL. 95% CI were calculated by parametric bootstrapping; *p*-values and df were calculated by the Kenward-Roger approximation (package lmerTest version 3.1-3 (Kuznetsova et al. 2017)). Marginal R^2: 0.17; Conditional R^2: 0.89 (calculated following Nakagawa and Schielzeth (2017); package piecewiseSEM, version 2.1.0 (Lefcheck 2016)). Reference level for Age class = chick. Significant estimates are highlighted in bold.

The results of the previous model led to the design of the following equation that will make possible to correct for the effect of storage duration for each sample:

$$\text{Difference RTL} = 0.302 - 0.130 \times \text{storage time} + 0.015 \times \text{storage time}^2 - 0.001 \times \text{storage time}^3 + 0.071 \times \text{RTL}_1$$

Following this equation we corrected RTLs by estimating for each sample the relative amount of telomere sequence lost in each year of its storage and summing all the losses depending on the number of years the sample was stored.

Table 2: Model evaluating the effect of ethanol storage duration on differences in RTLs from the same blood sample extracted in two consecutive years (N = 91 samples).

Fixed effects	β	Lower CI	Upper CI	df	t	*p*-value
Intercept	**0.302**	**0.224**	**0.380**	**4,86**	**7.68**	**<0.001**
Storage time	**-0.130**	**-0.187**	**-0.074**	**4,86**	**-4.59**	**<0.001**
Storage time ^{2}	**0.015**	**0.005**	**0.026**	**4,86**	**2.87**	**0.005**
Storage time ^{3}	**-0.001**	**-0.001**	**-0.00001**	**4,86**	**-2.05**	**0.044**
RTL_1	**0.071**	**0.017**	**0.125**	**4,86**	**2.61**	**0.011**

Results of a LM modelling the effect of the ethanol storage time and the first RTL (RTL_1) on the difference in RTLs from the same blood sample extracted in two consecutive years. Multiple R^2: 0.80; adjusted R^2: 0.80. Significant estimates are highlighted in bold.

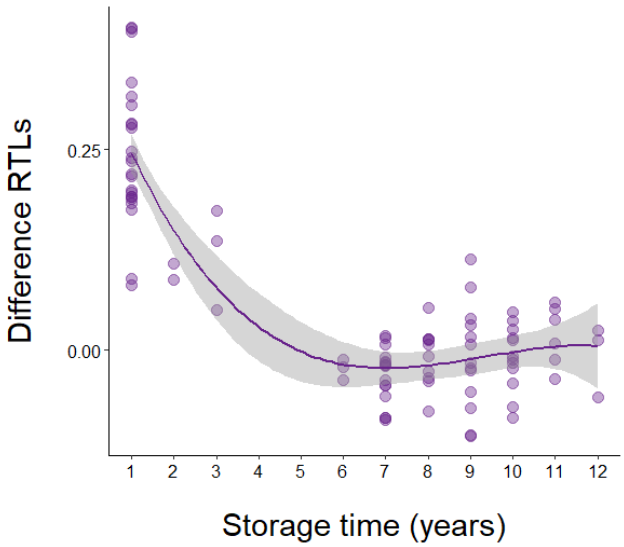


Figure 2: Differences in RTLs from the same ethanol stored blood sample extracted in two consecutive years depending on the storage time (number of years) before the second extraction (N= 91). Solid line represents the regression line and shaded area represents the 95% confidence interval.

Validation of the correction of RTL estimates

Correction had no effect on RTL (Table 3). Nevertheless, the age and the weight of the fledglings at sampling were negatively and positively related to their RTLs, respectively (Table 3).

Table 3: Factors affecting RTLs of magpie fledglings that had recruited into the population (N = 101 individuals, 83 nests).

Fixed effects	β	Lower CI	Upper CI	df	t	*p*-value
Intercept	0.58	-0.05	1.15	54.2	1.77	0.083
Correction	0.08	-0.15	0.35	7.3	0.59	0.572
Age (days)	**-0.04**	**-0.06**	**-0.02**	**76.5**	**-3.21**	**0.002**
Weight (grams)	**0.01**	**0.003**	**0.01**	**91.4**	**3.66**	**<0.001**
Random effects	σ	σ^2				
Nest identity	0.07	0.01				
Year of blood sampling	0.14	0.02				

Results of a LMM evaluating whether there are differences between corrected and non-corrected RTLs of fledglings recruited into the population, controlling for their age and weight at sampling. 95% CI were calculated by parametric bootstrapping; p-values and df were calculated by the Kenward-Roger approximation. Marginal R^2: 0.17; Conditional R^2: 0.40 (calculated following Nakagawa and Schielzeth (2017); piecewiseSEM, version 2.1.0 (Lefcheck 2016). Reference level for Correction = No. Significant estimates are highlighted in bold.

Discussion

Our study presents the first comprehensive evaluation of the long-term effect of a storage medium on the estimation of relative telomere length. We show that the telomeres of blood samples stored in ethanol at room temperature suffer from degradation during the first 3 or 4 years of storage, and after this time further degradation appears to halt (Fig. 2). Moreover, we have also suggested a way of quantifying this degradation and correct RTL measurements of samples that have been stored for up to 12 years.

The loss of telomere sequence between consecutive DNA extractions is not likely to be due to genomic DNA degradation, since in that case, samples stored for larger periods of time should experience higher levels of telomere sequence loss; however, our data show the opposite pattern: samples stored for fewer years before the first RTL estimation lost more telomere sequence between two consecutive

DNA extractions and RTL estimations. Indeed, high sensitivity D1000 ScreenTape electrophoresis didn't show degradation in samples that were stored one year before the second extraction (Supplementary material, Figs. S1 to S8).

One possible explanation for this phenomenon could be related to telomere composition and structure itself: telomere repeats are rich in guanine, which is the base most susceptible to oxidation (Wang et al. 2010), and they constitute fragile sites that are susceptible to accumulate oxidative damage (Petersen et al. 1998). This damage can be caused by exogenous sources and/or cell metabolism (De Bont and van Larebeke 2004). Moreover, telomere damage is repaired less frequently than other parts of the genome (Coluzzi et al. 2014, Rhee et al. 2011) and the 3' overhang lacks a complementary template to be repaired from (Ahmed and Lingner 2018). In vivo, telomeres are usually protected by the shelterin complex until damage can be repaired, and this complex also regulates their repair (De Lange 2005). However, when cells (erythrocytes in this case) are placed in ethanol shelterin proteins lose their tertiary structure and precipitate and t-loops are disarranged, leaving the telomere sequence and its fragile sites exposed. It could be also possible that, due to the polar nature of its hydroxyl group, ethanol could dissolve reactive oxygen species (ROS) already present in the cell that would continue damaging the exposed telomere sequence until ROS are reduced. This could explain the temporal pattern of telomere degradation that we have found (Fig. 2). Moreover, the size of the t-loop correlates with telomere length (Griffith et al. 1999), which could also explain the length dependent degradation (Table 2). This explanation also suggests that the amount of telomere shortening that a sample suffers would also depend on the redox balance of their cells in the time of sampling, what would explain the percentage of variance not explained by our model (Table 2).

Storage-time effect and RTL correction

Our design allowed us to evaluate the long term effect of storage (up to 12 years) in only two years. This kind of design promises to be a useful tool to evaluate this kind of effects in a short period of time and could be applied to any other storage conditions and methods for telomere measurement. We have also showed that storage in absolute ethanol at room temperature is not a suitable storage method for telomere measurement, contrary to what was suggested by a previous study (Eastwood et al. 2018). In the cited article, authors compared RTLs obtained from the same samples stored in absolute ethanol and 2 other storage media for 7 months

at 4ºC; they also compared those RTLs with absolute measures of telomeres obtained by pulse gel electrophoresis terminal restriction fragment analysis and concluded that ethanol was the best preservative among the 3 tested. Our study differs on the time that samples were kept in ethanol and the temperature at which they were stored, what could be a key factor in the degradation process. Even if ethanol at room temperature is not a good long-term storage medium for telomere measurements, our systematic and consistent method of sampling and storage over the years (Nussey et al. 2014, Reichert et al. 2017) and the existence of a pattern in the degradation process has allowed us to correct the telomere measurements.

Moreover, our correction of RTL values seems to be appropriate since corrected RTLs did not differed from RTLs of fresh extracted samples. The only way to experimentally determine a correction coefficient would be to obtain RTL measurements from freshly collected samples, and after a few years re-estimate RTL and then validate the correction. Although our experimental design show some flaws, such as lack of data of samples stored for 4 and 5 years and low sample size in samples stored for 2 and 3 years, the explanatory power of the model indicates that the results are consistent (R^2, Table 2). The model showed that fledglings' telomere length is negatively correlated with their age (Table 3), which is a common pattern in telomere biology: telomere shortening is more marked during growth and development in a wide range of taxa (Zeichner et al. 1999, Brümmendorf et al. 2002, Salomons et al. 2009), including magpies (Soler et al. 2015). Fledgling weight was also positively correlated with RTL, which is consistent with Chapter 2 (p. 89), which found that relative telomere length is related to quality in adult magpies and Molina-Morales et al. (2012) which found that fledglings' weight is related to their recruitment probability, although the relation between telomere length and survival in magpies needs to be elucidated.

Consequences of telomere degradation

Different longitudinal studies have reported that a small percentage of individuals show telomere elongation in tissues in which telomeres are commonly not elongated (Gorbunova and Seluanov 2009). In most cases, this anomaly has been attributed to measurement error (e.g. Salomons et al. 2009, Chen et al. 2011, Steenstrup et al. 2013). However, the possible degradation of telomeres during preservation has been rarely considered, though, a length dependent shortening of telomeres during sampling storage along with inconsistencies in the time past between sampling and

DNA extraction could be responsible of an apparent elongation of telomeres: if some time pass between the sampling of blood and DNA extraction in a set of individuals, which are later re-sampled and their DNA extracted straight away, we expect telomere degradation in the first set of samples (first telomere length measurement), but not in the second set of samples (non-degraded telomeres), in such a way that the second measurement could produce larger telomeres estimates than the first measurement, in particular in individuals with larger telomeres, because long telomeres would shorten during storage more that short telomeres; this would result in a false elongation effect in the second sampling only apparent in a subset of individuals with longer telomeres.

On the other hand, the effect of telomere shortening during storage could also be confounded to some extent with cohort effects in longitudinal studies if telomeres are not measured soon after sampling. In this type of studies in particular, the possible effect of long-term storage on telomere measurements should be carefully evaluated and experimental designs like the one we proposed here would help to quantify, correct and disentangle this effect from cohort effects.

Conclusions

Our study provides evidence that storage time affects telomere length measurements when blood samples are stored in ethanol at room temperature. Specifically, this effect shows a temporal pattern of loss of telomere sequence during the first 3 or 4 years of storage, after which the loss halts. We also present a method to quantify the effect of storage time and correct RTL measurements. This approach could be used to evaluate other methods of storage and types of storage media. Our results stress the need to evaluate long-term effects of sample storage on telomere measurements. This is an important consideration in long-term studies where samples were not originally intended for telomere measurements but stored and are used for telomere analysis at a later date.

Acknowledgments

We thank the staff of the NERC Biomolecular Analysis Facility and Molecular Ecology Laboratory, especially Natalie dos Remedios for her help during the optimization of the qPCR assay and Mirre Simons for the script for the qPCR data analysis and his help with the theoretical/conceptual corpus of the telomere analysis.

Funding

This study was supported by the Spanish Ministry of Science through a project (CGL2014-55362-P), a predoctoral spanish grant (BES-2015-075675) and a Juan de la Cierva –Incorporación Fellowship (IJC2018-036411-I).

References

Ahmed, W. and Lingner, J. 2018. Impact of oxidative stress on telomere biology. - Differentiation 99: 21–27.

Barnett, A. G., van der Pols, J. C. and Dobson, A. J. 2005. Regression to the mean: What it is and how to deal with it. - Int. J. Epidemiol. 34: 215–220.

Barrett, E. L. B., Boner, W., Mulder, E., Monaghan, P., Verhulst, S. and Richardson, D. S. 2012. Absolute standards as a useful addition to the avian quantitative PCR telomere assay. - J. Avian Biol. 43: 571–576.

Bates, D., Mächler, M., Bolker, B. and Walker, S. 2015. Fitting Linear Mixed-Effects Models Using lme4. - J. Stat. Softw. 67: 1-48.

Blackburn, E. H. 1991. Structure and function of telomeres. - Nature 350: 569–573.

Blackburn, E. H. 2000. Telomere states and cell fates. - Nature 408: 53–56.

Blackburn, E. H., Epel, E. S. and Lin, J. 2015. Human telomere biology: A contributory and interactive factor in aging, disease risks, and protection. - Science 350: 1193–1198.

Brümmendorf, T. H., Mak, J., Sabo, K. M., Baerlocher, G. M., Dietz, K., Abkowitz, J. L. and Lansdorp, P. M. 2002. Longitudinal studies of telomere length in feline blood cells: Implications for hematopoietic stem cell turnover in vivo. - Exp. Hematol. 30: 1147–1152.

Camacho-Sanchez, M., Burraco, P., Gomez-Mestre, I. and Leonard, J. A. 2013. Preservation of RNA and DNA from mammal samples under field conditions. - Mol. Ecol. Resour. 13: 663–673.

Cawthon, R. M. 2002. Telomere measurement by quantitative PCR. - Nucleic Acids Res. 30: 1–6.

Cawthon, R. M. 2009. Telomere length measurement by a novel monochrome multiplex quantitative PCR method. - Nucleic Acids Res. 37: 1–7.

Chatelain, M., Drobniak, S. M. and Szulkin, M. 2020. The association between stressors and telomeres in non-human vertebrates: a meta-analysis. - Ecol. Lett. 23: 381–398.

Chen, W., Kimura, M., Kim, S., Cao, X., Srinivasan, S. R., Berenson, G. S., Kark, J. D. and Aviv, A. 2011. Longitudinal versus cross-sectional evaluations of leukocyte telomere length dynamics: Age-dependent telomere shortening is the rule. - Journals Gerontol. - Ser. A Biol. Sci. Med. Sci. 66 A: 312–319.

Coluzzi, E., Colamartino, M., Cozzi, R., Leone, S., Meneghini, C., O'Callaghan, N., and Sgura, A. 2014. Oxidative Stress Induces Persistent Telomeric DNA Damage Responsible for Nuclear Morphology Change in Mammalian Cells. - PLoS One 9: e110963.

Capítulo 3

Cunningham, J. M., Johnson, R. A., Litzelman, K., Skinner, H. G., Seo, S., Engelman, C. D., Vanderboom, R. J., Kimmel, G. W., Gangnon, R. E., Riegert-Johnson, D. L., Baron, J. A., Potter, J. D., Haile, R., Buchanan, D. D., Jenkins, M. A., Rider, D. N., Thibodeau, S. N., Petersen, G. M. and Boardman, L. A. 2013. Telomere Length Varies By DNA Extraction Method: Implications for Epidemiologic Research. - Cancer Epidemiol. Biomarkers Prev. 22: 2047–2054.

De Bont, R. and van Larebeke, N. 2004. Endogenous DNA damage in humans: A review of quantitative data. - Mutagenesis 19: 169–185.

De Lange, T. 2005. Shelterin: The protein complex that shapes and safeguards human telomeres. - Genes Dev. 19: 2100–2110.

De Lange, T. 2010. How shelterin solves the telomere end-protection problem. - Cold Spring Harb. Symp. Quant. Biol. 75: 167–177.

Eastwood, J. R., Mulder, E., Verhulst, S. and Peters, A. 2018. Increasing the accuracy and precision of relative telomere length estimates by RT qPCR. - Mol. Ecol. Resour. 18: 68–78.

Eastwood, J. R., Hall, M. L., Teunissen, N., Kingma, S. A., Hidalgo Aranzamendi, N., Fan, M., Roast, M., Verhulst, S. and Peters, A. 2019. Early-life telomere length predicts lifespan and lifetime reproductive success in a wild bird. - Mol. Ecol. 28: 1127–1137.

Foley, N. M., Petit, E. J., Brazier, T., Finarelli, J. A., Hughes, G. M., Touzalin, F., Puechmaille, S. J. and Teeling, E. C. 2020. Drivers of longitudinal telomere dynamics in a long-lived bat species, Myotis myotis. - Mol. Ecol. 29: 2963–2977.

Gorbunova, V. and Seluanov, A. 2009. Coevolution of telomerase activity and body mass in mammals: From mice to beavers. - Mech. Ageing Dev. 130: 3–9.

Griffith, J. D., Comeau, L., Rosenfield, S., Stansel, R. M., Bianchi, A., Moss, H. and De Lange, T. 1999. Mammalian telomeres end in a large duplex loop. - Cell 97: 503–514.

Haussmann, M. F. and Marchetto, N. M. 2010. Telomeres: Linking stress and survival, ecology and evolution. - Curr. Zool. 56: 714–727.

Kilpatrick, C. W. 2002. Noncryogenic preservation of mammalian tissues for DNA extraction: An assessment of storage methods. - Biochem. Genet. 40: 53–62.

Kuznetsova, A., Brockhoff, P. B. and Christensen, R. H. B. 2017. lmerTest Package: Tests in Linear Mixed Effects Models. - J. Stat. Softw. 82: 1-26.

Lefcheck, J. 2016. piecewiseSEM: Piecewise structural equation modeling. R package version 1.2.1. - Methods Ecol. Evol. 7: 573–579.

López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M. and Kroemer, G. 2013. The hallmarks of aging. - Cell 153: 1194.

Molina-Morales, M., Martínez, J. G. and Avilés, J. M. 2012. Factors affecting natal and breeding magpie dispersal in a population parasitized by the great spotted cuckoo. - Anim. Behav. 83: 671–680.

Monaghan, P. 2014. Organismal stress, telomeres and life histories. - J. Exp. Biol. 217: 57–66.

Monaghan, P. and Haussmann, M. F. 2006. Do telomere dynamics link lifestyle and lifespan? - Trends Ecol. Evol. 21: 47–53.

Monaghan, P. and Ozanne, S. E. 2018. Somatic growth and telomere dynamics in vertebrates: Relationships, mechanisms and consequences. - Philos. Trans. R. Soc. B Biol. Sci. 373: 20160446.

Monaghan, P., Eisenberg, D. T. A., Harrington, L. and Nussey, D. 2018. Understanding diversity in telomere dynamics. - Phil. Trans. R. Soc. B 373: 20160435.

Morinha, F., Magalhães, P. and Blanco, G. 2020a. Standard guidelines for the publication of telomere qPCR results in evolutionary ecology. - Mol. Ecol. Resour. 20: 635–648.

Morinha, F., Magalhães, P. and Blanco, G. 2020b. Different qPCR master mixes influence telomere primer binding within and between bird species. - J. Avian Biol. 51: 1–9.

Nakagawa, S. and Schielzeth, H. 2013. A general and simple method for obtaining R2 from generalized linear mixed-effects models. - Methods Ecol. Evol. 4: 133–142.

Nakagawa, S., Gemmell, N. J. and Burke, T. 2004. Measuring vertebrate telomeres: Applications and limitations. - Mol. Ecol. 13: 2523–2533.

Nussey, D. H., Baird, D., Barrett, E., Boner, W., Fairlie, J., Gemmell, N., Hartmann, N., Horn, T., Haussmann, M., Olsson, M., Turbill, C., Verhulst, S., Zahn, S. and Monaghan, P. 2014. Measuring telomere length and telomere dynamics in evolutionary biology and ecology. - Methods Ecol. Evol. 5: 299–310.

Pepper, G. V., Bateson, M. and Nettle, D. 2018. Telomeres as integrative markers of exposure to stress and adversity: A systematic review and meta-analysis. - R. Soc. Open Sci. 5: 180744.

Petersen, S., Saretzki, G., Zglinicki, T. Von and Von Zglinicki, T. 1998. Preferential Accumulation of Single-Stranded Regions in Telomeres of Human Fibroblasts. - Exp. Cell Res. 239: 152–160.

R Core Team 2020. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. <https://www.r-project.org/>.

Raschenberger, J., Lamina, C., Haun, M., Kollerits, B., Coassin, S., Boes, E., Kedenko, L., Köttgen, A. and Kronenberg, F. 2016. Influence of DNA extraction methods on relative telomere length measurements and its impact on epidemiological studies. - Sci. Rep. 6: 1–10.

Reichert, S., Froy, H., Boner, W., Burg, T. M., Daunt, F., Gillespie, R., Griffiths, K., Lewis,

S., Phillips, R. A., Nussey, D. H. and Monaghan, P. 2017. Telomere length measurement by qPCR in birds is affected by storage method of blood samples. - Oecologia 184: 341–350.

Rhee, D. B., Ghosh, A., Lu, J., Bohr, V. A. and Liu, Y. 2011. Factors that influence telomeric oxidative base damage and repair by DNA glycosylase OGG1. - DNA Repair 10: 34–44.

Richardson, D. S., Jury, F. L., Blaakmeer, K., Komdeur, J. and Burke, T. 2001. Parentage assignment and extra-group paternity in a cooperative breeder: the Seychelles warbler (*Acrocephalus sechellensis*). - Mol. Ecol. 10: 2263–2273.

Ruijter, J. M., Ramakers, C., Hoogaars, W. M. H. H., Karlen, Y., Bakker, O., van den Hoff, M. J. B. B. and Moorman, A. F. M. M. 2009. Amplification efficiency: Linking baseline and bias in the analysis of quantitative PCR data. - Nucleic Acids Res. 37: e45–e45.

Salomons, H. M., Mulder, G. A., Van De Zande, L., Haussmann, M. F., Linskens, M. H. K. K. and Verhulst, S. 2009. Telomere shortening and survival in free-living corvids. - Proc. R. Soc. B Biol. Sci. 276: 3157–3165.

Soler, J. J., Ruiz Castellano, C., Martínez-de la Puente, J., Tomás, G., Ruiz-Rodríguez, M. and Figuerola, J. 2015. Telomere dynamics in parasitic great spotted cuckoos and their magpie hosts. - J. Evol. Biol. 28: 1610–1617.

Spurgin, L. G., Bebbington, K., Fairfield, E. A., Hammers, M., Komdeur, J., Burke, T., Dugdale, H. L. and Richardson, D. S. 2018. Spatio-temporal variation in lifelong telomere dynamics in a long-term ecological study. - J. Anim. Ecol. 87: 187–198.

Steenstrup, T., Hjelmborg, J. V. B., Kark, J. D., Christensen, K. and Aviv, A. 2013. The telomere lengthening conundrum - Artifact or biology? - Nucleic Acids Res. 41: 1–7.

Sudyka, J., Arct, A., Drobniak, S. M., Gustafsson, L. and Cichon, M. 2019. Birds with high lifetime reproductive success experience increased telomere loss. - Biol. Lett. 15: 9–12.

Tolios, A., Teupser, D. and Holdt, L. M. 2015. Preanalytical conditions and DNA isolation methods affect telomere length quantification in whole blood. - PLoS One 10: 1–14.

Verhulst, S., Aviv, A., Benetos, A., Berenson, G. S. and Kark, J. D. 2013. Do leukocyte telomere length dynamics depend on baseline telomere length? An analysis that corrects for "regression to the mean." - Eur. J. Epidemiol. 28: 859–866.

Wang, Z., Rhee, D. B., Lu, J., Bohr, C. T., Zhou, F., Vallabhaneni, H., de Souza-Pinto, N. C. and Liu, Y. 2010. Characterization of oxidative guanine damage and repair in mammalian telomeres. - PLoS Genet. 6: 28.

Whittemore, K., Vera, E., Martínez-Nevado, E., Sanpera, C. and Blasco, M. A. 2019. Telomere shortening rate predicts species life span. - Proc. Natl. Acad. Sci. U. S. A. 116: 15122–15127.

Wilbourn, R. V., Moatt, J. P., Froy, H., Walling, C. A., Nussey, D. H. and Boonekamp, J. J. 2018. The relationship between telomere length and mortality risk in non-model vertebrate systems: A meta-analysis. - Philos. Trans. R. Soc. B Biol. Sci. 373: 20160447.

Young, A. J. 2018. The role of telomeres in the mechanisms and evolution of life-history trade-offs and ageing. - Philos. Trans. R. Soc. B Biol. Sci. 373: 20160452.

Zeichner, S. L., Palumbo, P., Feng, Y. R., Xiao, X., Gee, D., Sleasman, J., Goodenow, M., Biggar, R. and Dimitrov, D. 1999. Rapid telomere shortening in children. - Blood 93: 2824–2830.

Zou, Y., Sfeir, A., Gryaznov, S. M., Shay, J. W. and Wright, W. E. 2004. Does a sentinel or a subset of short telomeres determine replicative senescence? - Mol. Biol. Cell 15: 3709–3718.

Supplementary material

Table S1: Comparison of models with different functions of the storage time.

Function of the storage time	Model	k	AICc	ΔAICc	AICcWt	Cumulative weight	Multiple R^2	Adjusted R^2
cubic	~ storage time + storage time^2 + storage time^3	5	-250.4	0	0.74	0.74	0.79	0.78
quadratic	~ storage time + storage time^2	4	-248.3	2.10	0.26	1.00	0.78	0.77
linear	~ storage time	3	-203.2	47.2	0.00	1.00	0.63	0.62

Models are ordered by their degree of support based on AICc and R^2. ΔAICc is the difference between a particular model and the best one. K is the number of estimated parameters. Akaike weights (AICcWt) show the relative support a given model has from the data compared with the other models.

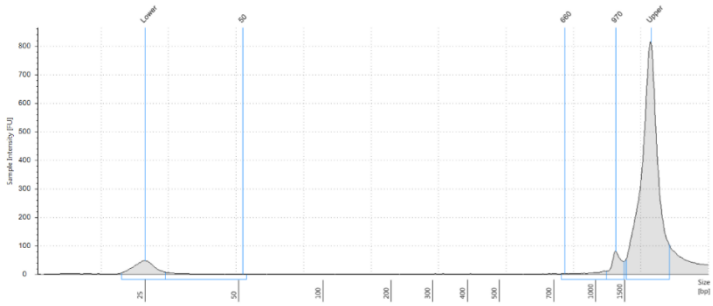
Table S2: Model evaluating the regression to the mean effect on the estimate of RTL_1 (N = 91 samples).

Fixed effects	β	Lower CI	Upper CI	df	t	*p*-value
Intercept	**-0.083**	**0.248**	**0.404**	**1, 89**	**-3.01**	**0.003**
RTL_1	**0.138**	**-0.122**	**-0.145**	**1, 89**	**3.28**	**0.001**

Results of a LM evaluating the relationship between the differences in RTLs after correcting for the regression to the mean effect (D) from the same blood sample extracted in two consecutive years and RTL_1. Multiple R^2: 0.11; adjusted R^2: 0.10. Significant estimates are highlighted in bold.

Figure S1: Result of the high sensitivity D1000 ScreenTape electrophoresis assay with an Agilent 4200 TapeStation system (Agilent Technologies) of genomic DNA from one of the samples of the study (315).

B1: 315



Sample Table

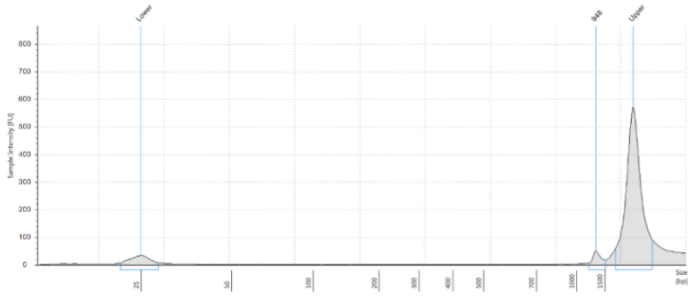
Well	Conc. [pg/µl]	Sample Description	Alert	Observations
B1	19.1	315		

Peak Table

Size [bp]	Calibrated Conc. [pg/µl]	Assigned Conc. [pg/µl]	Peak Molarity [pmol/l]	%Integrated Area	Peak Comment	Observations
25	19.4	-	1190	-		Lower Marker
50	1.94	-	60.0	10.15		
660	3.19	-	7.44	16.71		
970	14.0	-	22.2	73.14		
1500	250	250	256	-		Upper Marker

Figure S2: Result of the high sensitivity D1000 ScreenTape electrophoresis assay with an Agilent 4200 TapeStation system (Agilent Technologies) of genomic DNA from the sample 315 extracted one year later.

C1: 315A



Sample Table

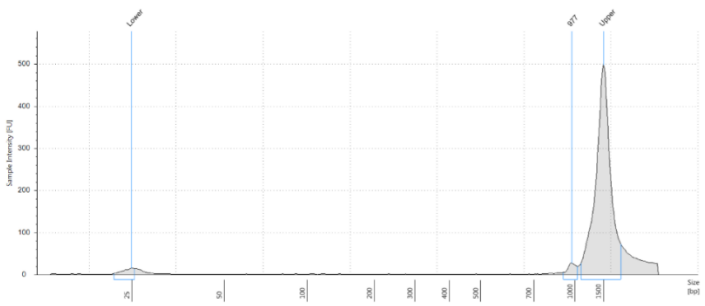
Well	Conc. [pg/µl]	Sample Description	Alert	Observations
C1	12.2	315A		

Peak Table

Size [bp]	Calibrated Conc. [pg/µl]	Assigned Conc. [pg/µl]	Peak Molarity [pmol/l]	%Integrated Area	Peak Comment	Observations
25	20.4	-	1260	-		Lower Marker
948	12.2	-	19.8	100.00		
1500	250	250	256	-		Upper Marker

Figure S3: Result of the high sensitivity D1000 ScreenTape electrophoresis assay with an Agilent 4200 TapeStation system (Agilent Technologies) of genomic DNA from one of the samples of the study (320).

D1: 320



Sample Table

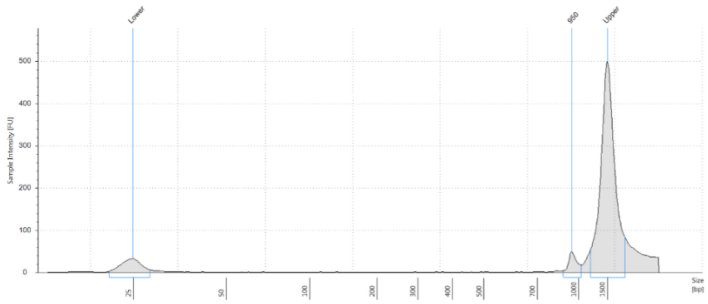
Well	Conc. [pg/µl]	Sample Description	Alert	Observations
D1	7.65	320		

Peak Table

Size [bp]	Calibrated Conc. [pg/µl]	Assigned Conc. [pg/µl]	Peak Molarity [pmol/l]	%Integrated Area	Peak Comment	Observations
25	5.74	-	353	-		Lower Marker
977	7.65	-	12.1	100.00		
1500	250	250	256	-		Upper Marker

Figure S4: Result of the high sensitivity D1000 ScreenTape electrophoresis assay with an Agilent 4200 TapeStation system (Agilent Technologies) of genomic DNA from the sample 320 extracted one year later.

E1: 320a



Sample Table

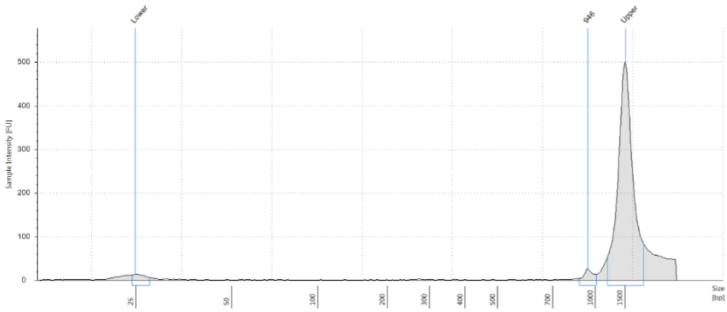
Well	Conc. [pg/µl]	Sample Description	Alert	Observations
E1	14.3	320a		

Peak Table

Size [bp]	Calibrated Conc. [pg/µl]	Assigned Conc. [pg/µl]	Peak Molarity [pmol/l]	% Integrated Area	Peak Comment	Observations
25	23.6	-	1450	-		Lower Marker
950	14.3	-	23.2	100.00		
1500	250	250	256	-		Upper Marker

Figure S5: Result of the high sensitivity D1000 ScreenTape electrophoresis assay with an Agilent 4200 TapeStation system (Agilent Technologies) of genomic DNA from one of the samples of the study (336).

H1: 336



Sample Table

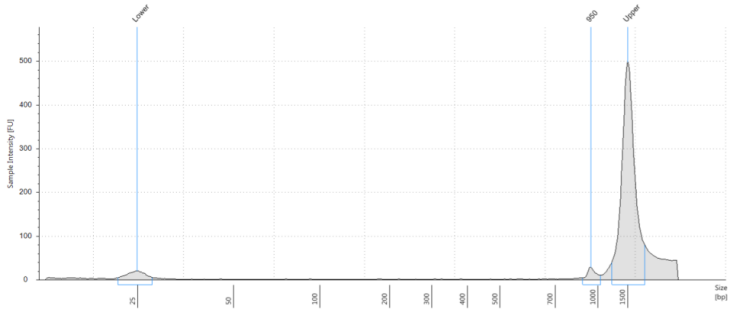
Well	Conc. [pg/µl]	Sample Description	Alert	Observations
H1	7.61	336		

Peak Table

Size [bp]	Calibrated Conc. [pg/µl]	Assigned Conc. [pg/µl]	Peak Molarity [pmol/l]	% Integrated Area	Peak Comment	Observations
25	5.83	-	359	-		Lower Marker
946	7.61	-	12.4	100.00		
1500	250	250	256	-		Upper Marker

Figure S6: Result of the high sensitivity D1000 ScreenTape electrophoresis assay with an Agilent 4200 TapeStation system (Agilent Technologies) of genomic DNA from the sample 336 extracted one year later.

A2: 336a



Sample Table

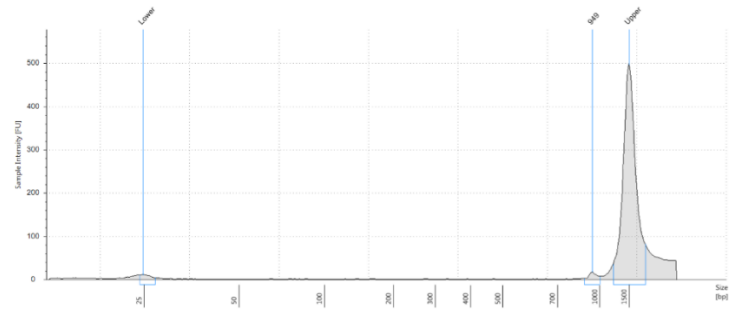
Well	Conc. [pg/µl]	Sample Description	Alert	Observations
A2	9.12	336a		

Peak Table

Size [bp]	Calibrated Conc. [pg/µl]	Assigned Conc. [pg/µl]	Peak Molarity [pmol/l]	% Integrated Area	Peak Comment	Observations
25	14.2	-	871	-		Lower Marker
950	9.12	-	14.8	100.00		
1500	250	250	256	-		Upper Marker

Figure S7: Result of the high sensitivity D1000 ScreenTape electrophoresis assay with an Agilent 4200 TapeStation system (Agilent Technologies) of genomic DNA from one of the samples of the study (337).

B2: 337



Sample Table

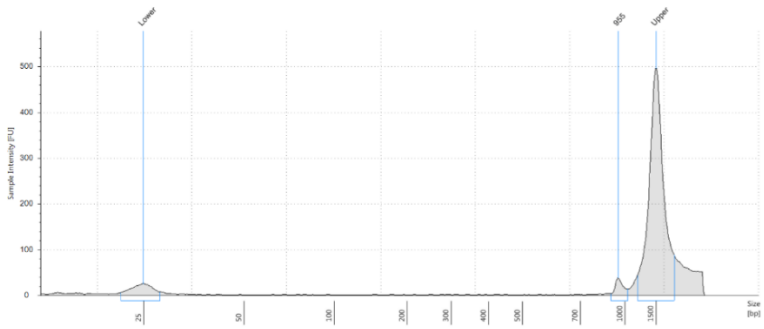
Well	Conc. [pg/µl]	Sample Description	Alert	Observations
B2	5.29	337		

Peak Table

Size [bp]	Calibrated Conc. [pg/µl]	Assigned Conc. [pg/µl]	Peak Molarity [pmol/l]	% Integrated Area	Peak Comment	Observations
25	4.51	-	278	-		Lower Marker
949	5.29	-	8.58	100.00		
1500	250	250	256	-		Upper Marker

Figure S8: Result of the high sensitivity D1000 ScreenTape electrophoresis assay with an Agilent 4200 TapeStation system (Agilent Technologies) of genomic DNA from the sample 337 extracted one year later.

C2: 337a



Sample Table

Well	Conc. [pg/µl]	Sample Description	Alert	Observations
C2	10.6	337a		

Peak Table

Size [bp]	Calibrated Conc. [pg/µl]	Assigned Conc. [pg/µl]	Peak Molarity [pmol/l]	%Integrated Area	Peak Comment	Observations
25	19.4	-	1190	-		Lower Marker
955	10.6	-	17.1	100.00		
1500	250	250	256	-		Upper Marker

Capítulo 4

Lifetime costs of parasitism: Parasitized hosts live longer and have the same lifetime reproductive success than non-parasitized ones

Lifetime costs of parasitism: Parasitized hosts live longer and have the same lifetime reproductive success than non-parasitized ones

Marta Precioso[1], Mercedes Molina-Morales[1], Jesús Avilés[2] & Juan Gabriel Martínez[1]

[1]Departamento de Zoología, Facultad de Ciencias, Universidad de Granada, Spain
[2]Departamento de Ecología Funcional y Evolutiva, Estación Experimental de Zonas Áridas (EEZA-CSIC), Almería, Spain.

Abstract

Brood parasites leave all the costs of rearing their offspring to their hosts. Parasitism may have long-term consequences on hosts' longevity and lifetime reproductive success that are unknown for most host species. Here, we used longitudinal data of individual magpies monitored during their lifetime to investigate the survival and LRS consequences of parasitism on magpie (*Pica pica*) hosts parasitized by the great spotted cuckoo (*Clamator glandarius*). We also explored whether the effect of parasitism on survival differed at younger compared to older ages and whether telomere length at fledging is related to magpies' lifespan. Individuals that were parasitized during their lifetime had a similar lifetime reproductive success that individuals never parasitized, probably mediated by the longer lifespan of parasitized individuals, which showed reduced mortality across their lifetime compared to non-parasitized ones. This may be due to a larger effect over mortality of breeding non-parasitized broods early in life. Although relative telomere length at fledging was not related to individuals' mortality, a role of telomere dynamics cannot be discarded and further research would be necessary to explore this possibility. This study provides new insights into the real costs of brood parasitism for a host suggesting that the lifetime costs of parasitism are small, and thus the strength of cuckoo selection on host defences might be also small, which helps to explain the maintenance of cuckoo parasitism in the long-term.

Introduction

Avian brood parasites lay their eggs in the nest of their hosts, which assume all the costs of rearing the parasitic offspring (Davies 2000), at the expenses of reducing their own breeding success (Rothstein 1990). This supposes a selective pressure for hosts to evolve defences against brood parasites and may drive changes in different host life history traits (Rothstein 1990, Davies 2000, Krüger 2007). The costs of brood parasitism across hosts' lives and the impact of parasitism on hosts´ lifetime reproductive success will modulate this pressure and the evolution of defences. It has been suggested that extra costs, other than those related directly to breeding success, such as effects on host survival probability, should be considered (Hoover and Reetz 2006, Koleček et al. 2015, Samaš et al. 2019). However, the lifetime consequences of parasitism have rarely been evaluated, probably due to the difficulty to perform long-term, individually-based studies. For example, most of the studies evaluating the effect of parasitism on hosts survival are short-term studies (two or three breeding seasons), and thus we know very little about how brood parasites may affect hosts lifespan or lifetime reproductive success (Krüger 2007).

Brood parasitism may affect hosts' lifespan in different ways. On the one hand, an increase in longevity could be selected for if anti-parasite hosts defences are somehow limited to older ages (Lotem et al. 1992, Lotem et al. 1995). For example, recognition and rejection of parasitic eggs may require a more or less prolonged learning period (Rothstein 1978, Stokke et al. 2007), so that in some hosts species young individuals do not reject eggs, though they acquire the capacity to do it later in life (Molina-Morales et al. 2014). Consequently, individuals that live longer are more likely to escape from parasitism (Martínez et al. 2020).

On the other hand, brood parasitism could affect host longevity through changes in hosts' baseline mortality and/or their rates of actuarial senescence. Parasites could modify hosts baseline mortality (that is, mortality probability independent of age) in two ways. Firstly, parasitism could induce changes in hosts' predation risk through changes in brood size and nest visiting rates: if predators use nest visits as a cue to find nests (Martin et al. 2000), a change in the rate of visits due to parasitism could change the probability of being predated while defending the brood. This way and depending on the brood parasite-host system, predation risk could be increased in hosts in which parasitized broods are larger than no parasitized ones (e.g. hosts of some *Molothrus* species, Hoover (2003)), or it could be

reduced in species in which parasitized brood are smaller than non-parasitized ones (e.g. most hosts of parasitic cuckoos). And secondly, brood parasitism could also affect hosts' baseline mortality if parasites do choose hosts according to any characteristic that could be correlated with survival probability or lifespan. The non-randomness of parasitism has been documented in different systems. For example, young splendid fairy-wrens (*Malurus splendens*) are more frequently parasitized by the Horsfield's bronze-cuckoo (*Chrysococcyx basalis*, Brooker and Brooker 1996), while older song sparrow females (*Melospiza melodia*) are more likely parasitized by brown-headed cowbirds (*Molothrus ater* (Smith et al. 1984)). Different life-history traits can be correlated (Hamel et al. 2009), and thus the selection of parasites of one or several of these traits could indirectly modulate changes in hosts life-history.

Brood parasites may affect hosts' longevity also through changes in hosts' actuarial senescence rates (that is, age-dependent mortality probability) related to the costs of reproduction. The direction of these changes would depend on whether rearing parasitic broods entails extra costs compared to rearing host broods or not. In this vein, some studies have found a negative effect of parasitism on hosts future reproductive performance (Mark and Rubenstein 2013) or survival (Hoover and Reetz 2006, Koleček et al. 2015), while others have failed to find such an effect (Brooker and Brooker 1996, Payne and Payne 1998, Hauber 2006, Samaš et al. 2019).

Moreover, it has been proposed that the costs of reproduction are higher at younger ages in different species of vertebrates but especially in birds (Proaktor et al. 2007). Younger individuals are inexperienced at selecting nest sites, acquiring and allocating resources (Robertson and Rendell 2001, Rebke et al. 2010), which may imply to incur in higher reproductive costs and earlier reproductive senescence or reduced survival (Nussey et al. 2006, Descamps et al. 2009, Reed et al. 2008, Bouwhuis et al. 2010, Tarwater and Arcese 2017). Similarly, the costs or consequences of brood parasitism, in particular those different to fitness losses, may be not constant across hosts lifespan (Krüger 2007), a possibility that has rarely been considered.

The only study that has evaluated the effect of brood parasitism on hosts lifetime reproductive success (LRS) found that parasitism had no effect (Brooker and Brooker 1996); however, in this study only females' survival from one year to another was analysed. To fully understand the evolutionary interactions between brood parasites and their hosts we need to evaluate the costs of parasitism across hosts' lifespan.

One of the most widely proposed molecular mechanism to link organismal ageing and lifespan is telomere length and its dynamics (Haussmann and Marchetto 2010). Telomeres are non-coding repetitive DNA sequences located in the extremes of eukaryotic chromosomes that maintain the integrity of chromosomes (Blackburn 1991). However, telomeres shorten with each cellular cycle (Blackburn 1991) until they reach a critical length, commonly causing the death of the cell (Hemann et al. 2001) and the accumulation of defects in tissues that are related to ageing (Campisi 2005). Early life telomere length has been shown to predict life expectancy in different bird species (Heidinger et al. 2012, Barrett et al. 2013), and telomere length and dynamics could be the subject of selection, playing a role in the evolution of lifespan (Young 2018).

Here, we investigated the survival and LRS consequences of parasitism on magpie (*Pica pica*) hosts parasitized by the great spotted cuckoo (*Clamator glandarius*), explored whether the survival consequences of parasitism differed at younger compared to older ages and whether telomere length at fledging is related to individuals lifespan. For that, we used longitudinal data of individual magpies monitored during the lifetime in a population of magpies that has been monitored and sampled for 15 years. Firstly, we aim to test whether the parasitism status across individuals' lifespan has an effect on mortality and LRS. We expect that parasitized individuals have longer lifespan because parasitism should diminish their mortality likelihood. Moreover, if parasitized individuals live longer, their probabilities of escaping from parasitism by rejecting cuckoo eggs increases at older ages (Molina-Morales et al. 2014, Martínez et al. 2020), which may compensate the losses in reproductive success due to parasitism, allowing them to obtain similar LRS than individuals never parasitized. Secondly, we aim to test whether relative telomere length (RTL) of individuals predicts their lifespan, as it has been shown previously in other animal species (Heidinger et al. 2012, Seeker et al. 2018). If this is so, RTL at fledging of magpies that recruited into the population should be negatively related to their mortality probabilities. Finally, we aim to explore whether the consequences of reproduction and parasitism on survival differed between younger and older ages. If the costs of reproduction are higher for younger individuals and parasitism reduces hosts' mortality, individuals parasitized earlier in their life (up to three years old) will live longer than individuals non-parasitized in early life. However, these differences would disappear at older ages (four years or older).

Methods

Study area and system

This study was conducted in La Calahorra (37°10'N, 3°03'W, Granada, Southern Spain) during the breeding seasons (March to July) of 2006 — 2019. The study area is comprised of cereal fields patched with groves of almond trees in which magpies preferentially build their nests. Magpies are monogamous, sedentary and long-lived passerines that may start breeding when they are one or two years old (Birkhead 1991). They are the main hosts of the great spotted cuckoo (hereafter cuckoo) in Europe. Cuckoo parasitism frequently reduces magpies' breeding success to zero through early hatching of cuckoo eggs and effective competition of cuckoo nestlings for parental care (Soler et al. 1996), and in most parasitized nests cuckoos are raised alone or with few other cuckoo nestlings (Martínez et al. 1998). This host-brood parasite system has selected for host recognition and rejection of cuckoo eggs (Soler and Møller 1990), though only a small percentage of individuals in the population reject cuckoo eggs, and the ability of females to reject foreign eggs in the nests is related to their age (Martínez et al. 2020).

Individual marking and monitoring

Magpies start building their nests in March and from that moment, nests are located, GPS positioned and monitored until chicks fledge. Nest are observed with a telescope during the nest building stage to identify the adults (which are ringed, see below) and visited every four-five days to record different reproductive variables until chicks fledge (laying date, parasitism status, hatching date and fledging success). Chicks are ringed with a unique combination of coloured rings before fledging (at about 15—18 days old); some fledglings banded during the study have recruited into the population. Besides, during the study period adult magpies were captured with corvid traps and coloured-ringed. First-year magpies differ from older individuals in some plumage characteristics (see Birkhead 1991, Martínez et al. 2020), so for some of the individuals captured as adults we could assign their age as one year old. At the time of ringing, a blood sample (approximately 100 microlitres) was collected from each individual by puncturing the brachial vein with a sterile needle and placed in a microcentrifuge tube containing 1 ml of analytical grade Absolute ethanol (Fisher) and stored in a rubber sealed screw-topped microfuge tube at room temperature. Magpie sexes only differ slightly in size, so individual sex was assigned using

molecular markers, extracting DNA from each blood sample and amplifying with two sex-specific primer sets, P2/P8 (Griffiths et al. 1998) and Z43B (Dawson et al. 2016) through PCR.

Telomere Length Measurements

All laboratory analysis where carried out in the NERC Biomolecular Analysis Facility at the University of Sheffield (United Kingdom). Telomere length was assessed by real-time PCR in a monochrome multiplex reaction (Cawthon 2009) with some modifications. Relative telomere length (RTL) was estimated as the ratio (T/S) between the amount of telomeric sequence (T) and the amount of a reference gene of single copy number (S) amplified within the same reaction. We used the telc-telg primer set to amplify the telomere sequence (Cawthon 2009) and another set of primers to amplify part the reference gene (Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)). The detailed methodology followed for RTL measurements is described in Chapter 2 (p. 89) and Chapter 3 (p. 117).

Samples were randomly distributed and run on 18 different plates (along with samples belonging to a different study). Amplification efficiencies were on average (SD) 98.6 % (6.7) for telomeres and 94.6% (4.5) for GAPDH. The average intra-plate standard deviations of Cq values were on average 0.074 (0.014) for telomeres and 0.080 (0.019) for GAPDH. Intra-plate and inter-plate T/S ratio standard deviations were on average 0.035 (0.010) and 0.070 (0.024), respectively. R^2 for standard curves were on average 0.99 (0.004) for telomeres and 0.99 (0.002) for GAPDH.

T/S ratios varied between 0.25 and 1.80. All T/S ratios were standardized to account for inter-run variability by dividing the average T/S ratio of duplicates by the average T/S ratio of the 20 nanograms/microliter standard sample of the plate where the sample was run.

Statistical analyses

We used data of each ringed individual breeding in the population to create a capture-recapture matrix that included 287 individuals (154 males and 133 females) with 746 detections. For 189 of those individuals we knew their year of birth. We used different subsets of data to perform different analysis (see below). Additionally we tested whether there were differences in the LRS and the age of the last

reproduction recorded between never parasitized individuals and individuals parasitized at least once during their lifetime.
All statistical analyses were performed in R version 4.0.3 (R Core Team 2020).

Age-specific mortality trajectory analyses

We performed several age-dependent survival trajectory analyses using BaSTA package (version 1.9.5, (Colchero et al. 2012)). This package allows to estimate age-specific survival and mortality from capture-recapture data in a Bayesian framework using Markov chain Monte Carlo simulations with Metropolis sampling (Colchero and Clark 2012). Moreover, BaSTA can deal with left-truncated (unknown birth dates) and right-censored data (unknown death dates) and low recapture probabilities. Survival estimates are adjusted to recapture probabilities, and missing dates of birth and death are calculated from the population mean. BaSTA includes four parametric mortality functions to explore age-specific mortality and survival: exponential, Gompertz, Weibull and logistic. In all the subsets of data (see below) the model that best fitted the data based on deviance information criterion (DIC, Spiegelhalter et al. 2002) was a Gompertz model, except in the *Mortality trajectories and parasitism status across individuals' life* analysis, in which a Weibull function had a smaller DIC; however differences in DIC were small (Table S2, Supplementary material) and we decided to use a Gompertz function in this analysis because it may better explain mortality trajectories in magpies and to maintain the consistency in all the analyses (see Supplementary material Tables S1 to S5 for model selection on the complete dataset and on each subanalysis). The Gompertz mortality model is given by the following equation:

$$\mu(x) = \exp(b_0 + b_1 x)$$

where b_0 is the baseline mortality and b_1 determinates the pattern of mortality dependent of age (x), or actuarial senescence. Categorical covariates were included as linear function of the mortality parameters and continuous covariates were included as proportional hazard covariates.

For each model we run eight parallel chains with 100 000 iterations, a burn-in of 3000 and thinned every 300 iterations. Recapture probability was set as fully time dependent in all the analyses. All model parameters converged appropriately based on their potential scale reduction ($\hat{R}$- values between 0.99 and 1.1 (Gelman et al. 2013)) and visual inspection of trace plots (chains mixed appropriately), and

did not affect parameters estimation. Differences in posterior distributions of mortality parameters between categorical covariates were assessed by Kullback-Lleiber discrepancy calibration (KLD, Kullback and Leibler 1951) as proposed by McCulloch (1989); KLD values of 0.5 indicates a complete overlap between distributions and KLD values of 1 means that there is no overlap between them. Life expectancy at age 3 was assessed by the life tables provided by BaSTA. An analysis of the complete dataset revealed no differences between sexes in mortality parameters (KLD_{b_0}= 0.51 and KLD_{b_1}= 0.57), thus sex was not included in further analyses. Each sub-analysis is described in detail below.

Mortality trajectories and parasitism status across individuals' life

We tested whether mortality trajectories differed between individuals that were parasitized and individuals that were not parasitized across their whole lifetime. We used a subset of 71 individuals for which we knew most of their reproductive life and that successfully raised chicks until fledging in all the breeding events recorded (a minimum 70% of their reproductive events, on average (SD): 96% (9.04)). We included lifetime parasitism as a categorical covariate: non-parasitized (individuals that were never parasitized (n= 34)) and parasitized (individuals that were parasitized at least once during their life (n = 37)).

LRS and age at last reproduction

Additionally, we empirically tested whether LRS and the age at the last reproduction recorded differed between parasitized and non-parasitized individuals. We constructed two generalized linear models (GLMs) with a negative binomial distribution (to account for the over-dispersion in the data) and log link function (MASS package, version 7.3-53 (Venables and Ripley 2002)). The first GLM included LRS (number of magpie chicks fledged during their lifetime) as a dependent variable and parasitism status across their life (non-parasitized = 0, parasitized = 1) as a categorical covariate; the second GLM included the age at last reproduction as the dependent variable and parasitism status across their life as a covariate. Some of the individuals used in the previous analysis could be still alive by the end of the study period, so we used the estimated death dates provided by BaSTA from the mortality trajectory analysis of our complete dataset to assess the death date of each individual and only included those that were most likely dead by 2019. These

analyses finally included 47 individuals, from which 22 were non-parasitized and 25 were parasitized at least once during their life.

Mortality trajectories and fledglings' RTL

We analysed whether magpie RTL at fledging was related to their mortality and survival trajectories. We used RTL of 112 fledglings that were born between 2005—2017 and recruited into the population. Fledglings' blood samples had been stored in ethanol at room temperature since collected, and we have previously shown (Chapter 3 (p. 117)) that storage time has an effect on RTL under these conditions. We have corrected this effect using the method proposed in Chapter 3. (p. 117). Fledgling ages at sampling were rather variable in our dataset (varied from 11 to 24 days) and RTL may be affected by age, especially early in life (Hall et al. 2004), thus we regressed the corrected RTL by fledgling age and used the residuals of this regression as a proportional hazard covariate in the analysis.

Mortality trajectories and parasitism status early in life vs late in life

To explore whether parasitism early in life (in the first 3 years of life) affected differently the mortality trajectories of individuals and also whether there was an effect of parasitism later in life (when individuals were 4 years or older), we analysed two subsets of data. One subset included 72 individuals for which we had complete reproductive records of their first 3 years (including individuals that lived less than 3 years) and that successfully raised chicks until fledging but for which had incomplete records of their later reproduction and the other subset included 71 individuals for which we knew most of their reproductive life from the age of 4 and that successfully raised chicks until fledging in all the breeding events recorded (a minimum of 70% of their reproductive events, on average (SD): 97% (1)), but for which we had incomplete records of their earlier life reproduction. This last subset included 44 individuals that were captured with adult plumage (we didn't know their exact age) for which we assigned the estimated birth dates provided by BaSTA (from the mortality trajectory analysis of our complete dataset) to increase sample size, given that fewer individuals reached older ages. We set the threshold of early vs old age in 3 years because the average age of adults in the population is 3.5 years (Martínez et al. 2020). We included parasitism status as a categorical covariate in each analysis depending on which period of the life we were considering: early in life

(parasitized at least once in their first three years of life (n=37), individuals non-parasitized in their first three years of life (n=35)); or late in life (parasitized at least once when they were 4 years or older (n=37), individuals not parasitized when they were 4 years or older (n=34))

Results

Mortality trajectories and parasitism status across individuals' life

Survival decreased as individuals aged more quickly in individuals that were never parasitized across their life compared to individuals that were parasitized (Fig. 1). Mortality trajectories differed between both groups due to differences in both mortality parameters ($KLD_{b0} = 0.90$, $KLD_{b1} = 0.75$; Table 1). Life expectancy at age 3 was smaller for non-parasitized individuals than for parasitized individuals (life expectancy at age 3: non-parasitized = 1.84, parasitized = 4.12).

Table 1: Parameter estimates of mortality trajectory analysis in relation to the parasitism status across individuals' life (n = 71)

Parameter	Parasitism status	Est.	SE	Lower CI	Upper CI	Autocor.	$\hat{R}$	KLD
b_0	Non-parasitized	-1.70	0.38	-2.50	-0.99	0.01	1.00	0.90
	Parasitized	-2.40	0.40	-3.25	-1.66	0.05	1.00	
b_1	Non-parasitized	0.30	0.12	0.06	0.52	0.02	1.00	0.75
	Parasitized	0.19	0.09	0.01	0.36	0.16	1.00	

Posterior means, SE, 95% CI, serial autocorrelation coefficients and potential scale reduction ($\hat{R}$) for the parameters estimated from a Gompertz mortality function. Parameters are estimated for both levels of the categorical covariate parasitism status. b_0 is the baseline mortality and b_1 is the mortality dependent of age. KLD evaluates the amount of overlap between posterior distributions of the parameters for the levels of the covariate.

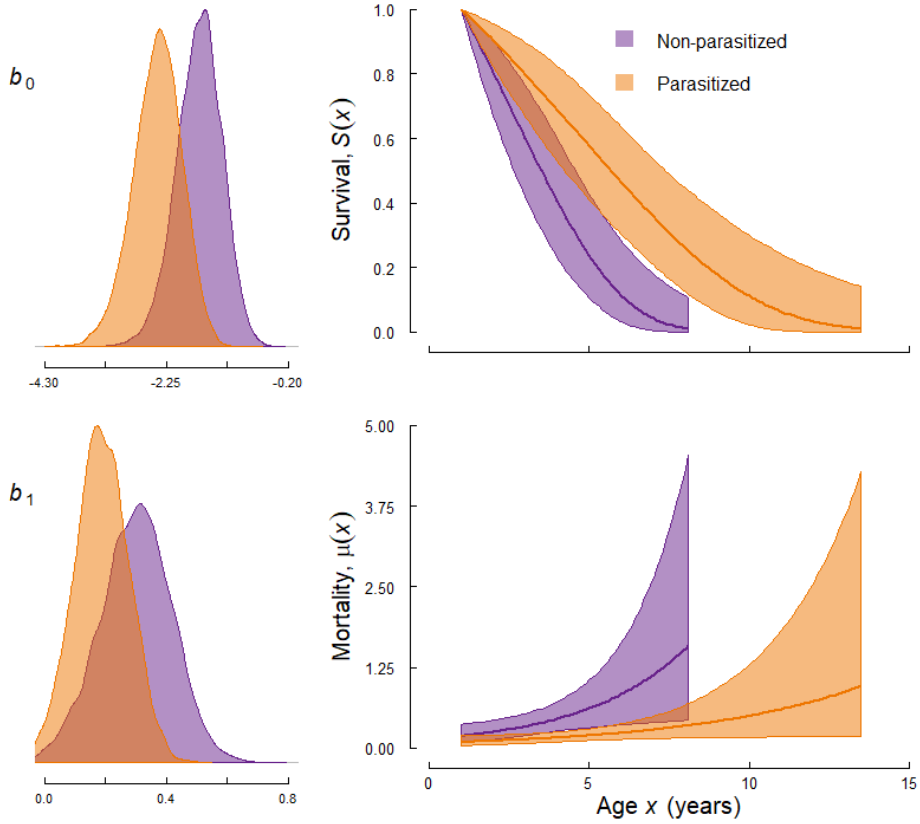


Figure 1: Posterior distributions of mortality the parameters (b_0 and b_1, right side) and age-specific survival and mortality trajectories (left side) of magpies depending on their parasitism status across their life (non-parasitized = 34; parasitized = 37). Coloured areas represent 95% confidence intervals.

LRS and age at last reproduction

LRS did not differed between individuals that were parasitized at least once during their lifetime (mean ± SE = 5.3 ± 1.4) compared to individuals that were never parasitized (mean ± SE = 6.1 ± 1.2; Table 2). On the other hand, the age of the last reproduction recorded was significantly greater for individuals that were parasitized at least once during their lifetime (mean ± SE = 3.9 ± 0.6) compare to non-parasitized individuals (mean ± SE = 2.1 ± 0.4; Table 2).

Table 2: Effect of parasitism status on individuals LRS and their age at last reproduction (n=47 individuals).

	LRS			Age at last reproduction		
	β (95 % CI)	z-value	*p*-value	β (95 % CI)	z-value	*p*-value
Intercept	**1.81 (1.35,2.33)**	**7.31**	**<0.001**	**0.76 (0.41,1.10)**	**4.32**	**<0.001**
Parasitism status	-0,14 (-0.82,0.53)	-0.42	0.68	**0.60 (0.16,1.04)**	**2.68**	**<0.01**

Results of GLMs (Negative Binomial distribution, log link function) testing the effect of parasitism status on individuals LRS and their age at last reproduction. Significant estimates are highlighted in bold. 95% CI were calculated by a profile likelihood ratio; parameter estimates were calculated by iteratively reweighted least squares to the maximum likelihood; p-values were calculated by a Wald z-test. Reference level for parasitism status = non-parasitized.

Mortality trajectories and fledglings' RTL

Relative telomere length at fledging is not correlated with individual mortality trajectory (Table 3).

Table 3: Parameter estimates of mortality trajectory analysis in relation to fledglings' RTL (n= 112).

Parameter	Estimate	SE	Lower CI	Upper CI	Autocor.	$\hat{R}$
b_0	-2.81	0.28	-3.35	-2.29	-0.02	1.00
b_1	0.28	0.05	0.18	0.38	-0.02	1.00
γ_{RTL}	-0.12	0.36	-0.84	0.58	0.02	1.00

Posterior means, SE, 95% CI, serial autocorrelation coefficients and potential scale reduction ($\hat{R}$) for the parameters estimated from a Gompertz mortality function including fledglings' RTL as a proportional hazard covariate. b_0 is the baseline mortality, b_1 is the mortality dependent of age and γ_{RTL} is the proportional effect of RTL on the mortality function.

Mortality trajectories and parasitism early in life vs late in life

Baseline probability of mortality was smaller for individuals parasitized early in life in compared to those non-parasitized (Table 4, Fig. 2a)): posterior distributions of the parameter b_0 for both groups did not overlap, as indicated by a high KLD value (KLD_{b0}= 0.85). Age-dependent mortality also differed between both groups (KLD_{b1}= 0.90), with parasitized individuals suffering less actuarial senescence (Table 4). Life expectancies were higher for parasitized individuals (life expectancy at age 3: non-parasitized = 1.87, parasitized = 4.75). However, mortality trajectories did not differ between individuals that were not parasitized and individuals that were parasitized when we only considered their late-life reproduction (Fig. 2 b)). Baseline mortality and mortality dependent of age were similar for both groups (KLD_{b0} = 0.56, KLD_{b1}= 0.60; Table 4) and life expectancies were similar (life expectancy at age 3: non-parasitized = 4.44, parasitized = 4.45).

Table 4: Parameter estimates of mortality trajectory analyses in relation to the parasitism status early in life (n = 72) and late in life (n = 71).

	Param.	Parasitism status	Est.	SE	Lower CI	Upper CI	Autocor.	$\hat{R}$	KLD
Early in life	b_0	Non-parasitized	-1.76	0.38	-2.52	-1.08	0.01	1.00	0.85
		Parasitized	-2.35	0.39	-3.17	-1.64	0.06	1.00	
	b_1	Non-parasitized	0.31	0.11	0.08	0.53	0.02	1.00	0.90
		Parasitized	0.14	0.08	-0.01	0.3	0.14	1.00	
Late in life	b_0	Non-parasitized	-4.6	0.61	-5.89	-3.46	0.09	1.00	0.56
		Parasitized	-4.33	0.54	-5.41	-3.33	0.05	1.00	
	b_1	Non-parasitized	0.57	0.11	0.36	0.81	0.1	1.00	0.60
		Parasitized	0.51	0.09	0.34	0.69	0.06	1.00	

Posterior means, SE, 95% CI, serial autocorrelation coefficients and potential scale reduction ($\hat{R}$) for the parameters estimated from a simple Gompertz mortality function. Parameters are estimated for both levels of the categorical covariate parasitism. b_0 is the baseline mortality and b_1 is the mortality dependent of age. KLD evaluates the amount of overlap between posterior distributions of the parameters for the levels of the covariate.

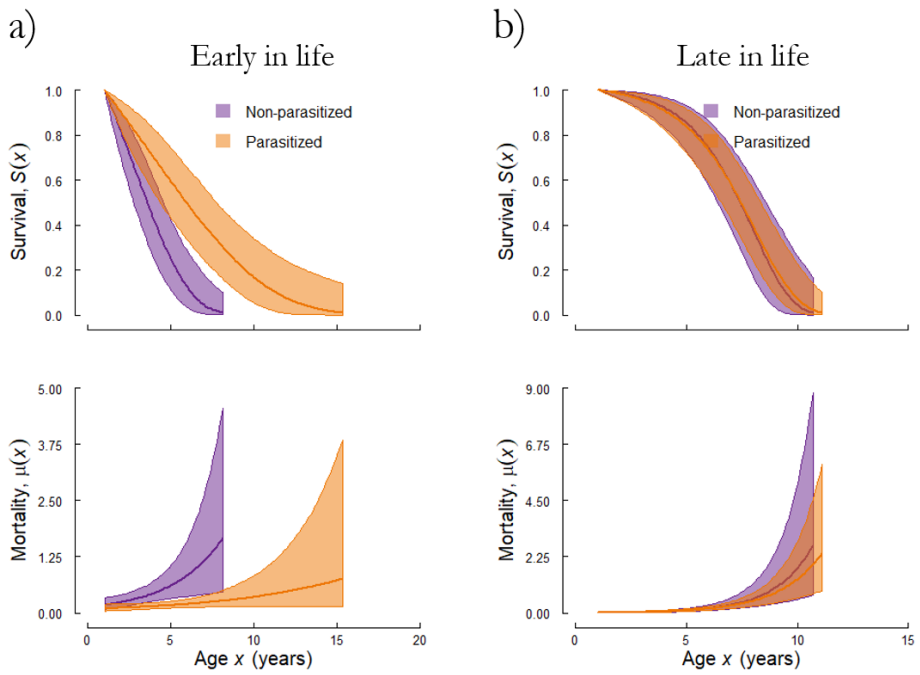


Figure 2: Posterior distributions of mortality parameters (b_0 and b_1) and age-specific survival and mortality trajectories of magpies depending on their parasitism status a) early in life (up to 3 years old, n= 72) and b) late in life (from 4 years old, n = 71). Coloured areas represent 95% confidence intervals.

Discussion

Parasitism status across individuals' life and LRS

Our results indicate that parasitized magpies have lower baseline mortality (Table 1). There are different not mutually exclusive explanations for this pattern. On the one hand, predation risk could be smaller in parasitized nests as a result of a reduced parental activity (Martin et al. 2000) related to brood size. Parasitized nests contain significantly fewer number of chicks than non-parasitized ones (Soler et al. 1996, Precioso et al. 2020), and the number of visits to the nest is significantly smaller in the majority of parasitized nests (containing one or two cuckoo nestlings; 83% of parasitized nests) than in non-parasitized nests (Precioso et al. 2020); thus, if predators use the rate of visits to locate nests, parasitized nests would be less

frequently predated and adult magpies would risk themselves less while defending the nest (Buitron 1983).

On the other hand, the pattern found could also be the result of cuckoos selecting hosts of better individual quality, as suggested by Soler et al. (1995), (see also, De Neve et al. 2004). Parental ability could be positive correlated with other life-history traits, such as lifespan (Hamel et al. 2009).

Other host characteristics could also explain the differences in baseline mortality between parasitized and non-parasitized individuals. For example, differences in personality, more specifically in boldness and aggressiveness, could provide a good explanation (Avilés and Parejo 2011). Boldness can be related to basal mortality (Smith and Blumstein 2008) since bolder individuals commonly experience higher predation risk (Sih et al. 2003, Hall et al. 2015, Ballew et al. 2017). It has also been shown that personality features related to nest defence against parasites and predators are repeatable and correlated in other hosts species (Trnka et al. 2013, Trnka and Grim 2014). Bolder individuals would defence their nest more aggressively against parasites and could more probably scape from parasitism by rejecting cuckoo eggs (Avilés et al. 2014), but at the same time would experience a higher predation risk, leading to a higher baseline mortality. Both boldness and shyness have shown to be evolutionary stable strategies and might be under disruptive natural selection (Dingemanse et al. 2004, Both et al. 2005) thus both behavioural types are commonly maintain in natural populations (Réale et al. 2007).

In addition to differences in baseline mortality, the analysis of parasitism status across individual's life also reveals differences in actuarial senescence between parasitized and non-parasitized individuals (Table 1, Fig. 1). This result may be a consequence of different costs of reproduction between parasitized and non-parasitized broods, assuming smaller rearing costs for parasitized broods (Precioso et al. 2020). An experimental increase in reproductive effort accelerated rates of senescence in jackdaws (Boonekamp et al. 2014b, Boonekamp et al. 2020). Our results point in that direction because of the smaller brood size of parasitized clutches (see above), although our correlational approximation does not allow us to discard that the smaller rates of senescence in parasitized individuals are also due to a higher individual quality of those individuals.

Altogether, our results indicates that magpies that had been parasitized during their life lived longer and this was confirmed by the age of the last reproduction recorded (Table 2). Surprisingly, parasitized individuals have similar

LRS than non-parasitized ones (Table 2), probably because they live longer and are capable of compensating the losses in reproductive success in those breeding events that are parasitized. Although our results contrast with those of Molina-Morales et al. (2013), which found that never parasitized magpies have double reproductive success than parasitized ones, they evaluated the fitness costs of parasitism in a very short period of time for individual magpies (5 years maximum).

In fact, Brooker and Brooker (1996) found similar results in splendid fairy-wrens parasitized by Horsfield's bronze cuckoos, where the small costs of parasitism across individuals' lifetime were proposed to explain the lack of defences (foreign egg rejection) against parasitism.

In the same vein, our results suggest that the lifetime costs of parasitism are small, and thus the strength of cuckoo selection on host defences might be also small, which helps to explain the maintenance of cuckoo parasitism in the long-term (Lotem et al. 1992), together with the fact that not all the individuals in the population suffer the selective pressure of parasitism, which would slow the expansion of anti-parasite defences in the population (Hauber et al. 2004, Molina-Morales et al. 2013).

Early life telomere length and lifespan

Early life telomere length is correlated with lifespan in several bird species (Haussmann et al. 2005, Bize et al. 2009, Heidinger et al. 2012, Barrett et al. 2013) and a recent meta-analysis found that short telomeres are associated with higher mortality risk, especially in birds (Wilbourn et al. 2018), although this meta-analysis did not find a relationship between TL and maximum lifespan.

Our results showed that, contrary to our prediction, telomere length at fledging of individuals that recruited into the population was not related to their lifespan (Table 3), though we cannot discard that telomere length could be related to some extend to fledglings survival but not with later-life survival (Haussmann and Marchetto 2010). Recent studies are finding growing evidence that the rate of telomere loss, rather than telomere length, might better explain individuals lifespan in both adults (Salomons et al. 2009, Bize et al. 2009) and chicks (Boonekamp et al. 2014a). Unfortunately, we don't have data on individuals´ telomere dynamics to test this, but our results would suggest that even if TL had an effect on mortality trajectories, that could be masked by the effects of different reproductive histories during lifetime. In accordance with this we have previously shown that RTL

dynamics may differ between non-parasitized and parasitized individuals (Chapter 2 (p. 89), see below).

Survival consequences of parasitism in early vs late life

Early-life reproduction is commonly challenging in many species and can cause that, with a similar level of effort, younger individuals suffer higher costs than older ones (Tarwater and Arcese 2017). This way, individuals that invest heavily in the reproduction early in life could suffer carry over effects and compromise their later survival and reproductive performance (Reed et al. 2008, Bouwhuis et al. 2010). Magpies not parasitized in their early life showed increased mortality compared to parasitized ones (Table 4, Fig. 2a) in a similar way than when we consider their whole life (see above). However, the differences in age-dependent mortality trajectories disappeared when we consider whether individuals are parasitized or not after 4 years of age (Table 4, Fig. 2b). Apparently, once individuals reached older ages a differential effort (like being parasitized or not) did not incurred in differences in mortality or senescence. These results are consistent with Chapter 2 (p. 89), which found that experimentally manipulated breeding effort (differences in the number of chicks raised) affected telomere loss in magpies with longer telomeres but not in individuals with shorter telomeres. Because younger magpies have longer telomeres than older ones, this suggests that the effects of reproductive effort on telomere dynamics are age-dependent. If telomere dynamics were related to survival in magpies, as it happens in other bird species (Bize et al. 2009, Salomons et al. 2009, Spurgin et al. 2018), the survival costs of reproduction would be higher at younger ages.

Though we are aware that this is not a formal test of differential costs of reproduction with age, the difference in patterns between early life and late life suggests differential survival consequences or rearing parasites in different moments of life, what could affect host-parasite evolutionary dynamics and deserve further research.

Conclusions

We have shown that individuals being parasitized sometimes during their lifetime have a similar lifetime breeding success that individuals never parasitized, and that this may be mediated by a larger lifespan of sometimes parasitized individuals, that show smaller mortality probabilities across their lives than individuals never

parasitized. We also suggest that this effect comes from a larger effect over mortality of breeding non-parasitized broods early in life.

Acknowledgments

We thank the staff of the NERC Biomolecular Analysis Facility. We also thank Deseada Parejo, Juan Rodriguez-Ruiz, Mónica Expósito, and A. Sánchez-Tójar for their help during fieldwork.

Funding

This study was supported by the Spanish Ministry of Science through different projects (CGL2011-27561 and CGL2014-55362-P), a grant (BES-2015-075675) and a Juan de la Cierva–Incorporación Fellowship (IJC2018-036411-I) and by Junta de Andalucía through a project (P06-RNM-01862).

References

Avilés, J. M. and Parejo, D. 2011. Host personalities and the evolution of behavioural adaptations in brood parasitic-host systems. - Anim. Behav. 82: 613–618.

Avilés, J. M., Bootello, E. M., Molina-Morales, M. and Martínez, J. G. 2014. The multidimensionality of behavioural defences against brood parasites: Evidence for a behavioural syndrome in magpies? - Behav. Ecol. Sociobiol. 68: 1287–1298.

Ballew, N. G., Mittelbach, G. G. and Scribner, K. T. 2017. Fitness Consequences of Boldness in Juvenile and Adult Largemouth Bass. - Am. Nat. 189: 396–406.

Barrett, E. L. B. B., Burke, T. A., Hammers, M., Komdeur, J. and Richardson, D. S. 2013. Telomere length and dynamics predict mortality in a wild longitudinal study. - Mol. Ecol. 22: 249–259.

Birkhead, T. R. 1992. The Magpies: The Ecology and Behaviour of Black-Billed and Yellow-Billed Magpies. - T & AD Poyser.

Bize, P., Criscuolo, F., Metcalfe, N. B., Nasir, L. and Monaghan, P. 2009. Telomere dynamics rather than age predict life expectancy in the wild. - Proc. R. Soc. B Biol. Sci. 276: 1679–1683.

Blackburn, E. H. 1991. Structure and function of telomeres. - Nature 350: 569–573.

Boonekamp, J. J., Mulder, G. A., Salomons, H. M., Dijkstra, C. and Verhulst, S. 2014a. Nestling telomere shortening, but not telomere length, reflects developmental stress and predicts survival in wild birds. - Proc. R. Soc. B Biol. Sci. 281: 20133287.

Boonekamp, J. J., Salomons, H. M., Bouwhuis, S., Dijkstra, C. and Verhulst, S. 2014b. Reproductive effort accelerates actuarial senescence in wild birds: An experimental study. - Ecol. Lett. 17: 599–605.

Boonekamp, J. J., Bauch, C. and Verhulst, S. 2020. Experimentally increased brood size accelerates actuarial senescence and increases subsequent reproductive effort in a wild bird population. - J. Anim. Ecol. 89: 1395–1407.

Both, C., Dingemanse, N. J., Drent, P. J. and Tinbergen, J. M. 2005. Pairs of extreme avian personalities have highest reproductive success. - J. Anim. Ecol. 74: 667–674.

Bouwhuis, S., Charmantier, A., Verhulst, S. and Sheldon, B. C. 2010. Individual variation in rates of senescence: Natal origin effects and disposable soma in a wild bird population. - J. Anim. Ecol. 79: 1251–1261.

Brooker, M. and Brooker, L. 1996. Acceptance by the splendid fairy-wren of parasitism by Horsfield's bronze-cuckoo: further evidence for evolutionary equilibrium in brood parasitism. - Behav. Ecol. 7: 395–407.

Buitron, D. 1983. Variability in the responses of black-billed magpies to natural predators. - Behaviour 87: 209–236.

Campisi, J. 2005. Senescent cells, tumor suppression, and organismal aging: Good citizens, bad neighbors. - Cell 120: 513–522.

Cawthon, R. M. 2009. Telomere length measurement by a novel monochrome multiplex quantitative PCR method. - Nucleic Acids Res. 37: 1–7.

Colchero, F. and Clark, J. S. 2012. Bayesian inference on age-specific survival for censored and truncated data. - J. Anim. Ecol. 81: 139–149.

Colchero, F., Jones, O. R. and Rebke, M. 2012. BaSTA: An R package for Bayesian estimation of age-specific survival from incomplete mark-recapture/recovery data with covariates. - Methods Ecol. Evol. 3: 466–470.

Davies, N. B. 2000. Cuckoos, Cowbirds and Other Cheats. - T & AD Poyser.

Dawson, D. A., dos Remedios, N. and Horsburgh, G. J. 2016. A new marker based on the avian spindlin gene that is able to sex most birds, including species problematic to sex with CHD markers. - Zoo Biol. 35: 533–545.

De Neve, L., Soler, J. J., Soler, M. and Pérez-Contreras, T. 2004. Nest size predicts the effect of food supplementation to magpie nestlings on their immunocompetence: An experimental test of nest size indicating parental ability. - Behav. Ecol. 15: 1031–1036.

Descamps, S., Boutin, S., McAdam, A. G., Berteaux, D. and Gaillard, J. M. 2009. Survival costs of reproduction vary with age in North American red squirrels. - Proc. R. Soc. B Biol. Sci. 276: 1129–1135.

Dingemanse, N. J., Both, C., Drent, P. J. and Tinbergen, J. M. 2004. Fitness consequences of avian personalities in a fluctuating environment. - Proc. R. Soc. B Biol. Sci. 271: 847–852.

Gelman, A., Carlin, J. B., Stern, H. S., Dunson, D. B., Vehtari, A. and Rubin, D. B. 2013. Bayesian data analysis, third edition. Chapman and Hall/CRC.

Griffiths, R., Double, M. C., Orr, K. and Dawson, R. J. G. G. 1998. A DNA test to sex most birds. - Mol. Ecol. 7: 1071–1075.

Hall, M. E., Nasir, L., Daunt, F., Gault, E. A., Croxall, J. P., Wanless, S. and Monaghan, P. 2004. Telomere loss in relation to age and early environment in long-lived birds. - Proc. R. Soc. B Biol. Sci. 271: 1571–1576.

Hall, M. L., van Asten, T., Katsis, A. C., Dingemanse, N. J., Magrath, M. J. L. L. and Mulder, R. A. 2015. Animal personality and pace-of-life syndromes: Do fast-exploring fairy-wrens die young? - Front. Ecol. Evol. 3: 1–14.

Hamel, S., Côté, S. D., Gaillard, J. M. and Festa-Bianchet, M. 2009. Individual variation in reproductive costs of reproduction: High-quality females always do better. - J. Anim. Ecol. 78: 143–151.

Hauber, M. E. 2006. A future cost of misdirected parental care for brood parasitic young? - Folia Zool. Praha 55: 367–374.

Hauber, M. E., Yeh, P. J. and Roberts, J. O. L. 2004. Patterns and coevolutionary consequences of repeated brood parasitism. - Proc. R. Soc. B Biol. Sci. 271: 317–320.

Haussmann, M. F. and Marchetto, N. M. 2010. Telomeres: Linking stress and survival, ecology and evolution. - Curr. Zool. 56: 714–727.

Haussmann, M. F., Winkler, D. W. and Vleck, C. M. 2005. Longer telomeres associated with higher survival in birds. - Biol. Lett. 1: 212–4.

Heidinger, B. J., Blount, J. D., Boner, W., Griffiths, K., Metcalfe, N. B. and Monaghan, P. 2012. Telomere length in early life predicts lifespan. - PNAS 109: 1743–1748.

Hoover, J. P. 2003. Multiple effects of brood parasitism reduce the reproductive success of prothonotary warblers, Protonotaria citrea. - Anim. Behav. 65: 923–934.

Hoover, J. P. and Reetz, M. J. 2006. Brood parasitism increases provisioning rate, and reduces offspring recruitment and adult return rates, in a cowbird host. - Oecologia 149: 165–173.

Koleček, J., Jelínek, V., Požgayová, M., Trnka, A., Baslerová, P., Honza, M. and Procházka, P. 2015. Breeding success and brood parasitism affect return rate and dispersal distances in the great reed warbler. - Behav. Ecol. Sociobiol. 69: 1845–1853.

Krüger, O. 2007. Cuckoos, cowbirds and hosts: Adaptations, trade-offs and constraints. - Philos. Trans. R. Soc. B Biol. Sci. 362: 1873–1886.

Kullback, S. and Leibler, R. A. 1951. On Information and Sufficiency. - Ann. Math. Stat. 22: 79–86.

Lotem, A., Nakamura, H. and Zahavi, A. 1992. Rejection of cuckoo eggs in relation to host age: A possible evolutionary equilibrium. - Behav. Ecol. 3: 128–132.

Lotem, A., Nakamura, H. and Zahavi, A. 1995. Constraints on egg discrimination and cuckoo-host co-evolution. - Anim. Behav. 49: 1185–1209.

Mark, M. M. and Rubenstein, D. R. 2013. Physiological costs and carry-over effects of avian interspecific brood parasitism influence reproductive tradeoffs. - Horm. Behav. 63: 717–722.

Martin, T. E., Scott, J. and Menge, C. 2000. Nest predation increases with parental activity: Separating nest site and parental activity effects. - Proc. R. Soc. B Biol. Sci. 267: 2287–2293.

Martínez, J. G., Molina-Morales, M., Precioso, M. and Avilés, J. M. 2020. Age-related brood parasitism and egg rejection in magpie hosts. - Am. Nat. 195: 876–885.

McCulloch, R. E. 1989. Local model influence. - J. Am. Stat. Assoc. 84: 473–478.

Molina-Morales, M., Gabriel Martínez, J., Martín-Gálvez, D., A. Dawson, D., Rodríguez-Ruiz, J., Burke, T. and Avilés, J. M. 2013. Evidence of long-term structured cuckoo parasitism on individual magpie hosts. - J. Anim. Ecol. 82: 389–398.

Capítulo 4

Molina-Morales, M., Martínez, J. G., Martín-Gálvez, D., Dawson, D. A., Burke, T. and Avilés, J. M. 2014. Cuckoo hosts shift from accepting to rejecting parasitic eggs across their lifetime. - Evolution 68: 3020–3029.

Nussey, D. H., Kruuk, L. E. B. B., Donald, A., Fowlie, M. and Clutton-Brock, T. H. 2006. The rate of senescence in maternal performance increases with early-life fecundity in red deer. - Ecol. Lett. 9: 1342–1350.

Payne, R. B. and Payne, L. L. 1998. Brood parasitism by cowbirds: Risks and effects on reproductive success and survival in indigo buntings. - Behav. Ecol. 9: 64–73.

Precioso, M., Molina-Morales, M., Sánchez-Tójar, A., Avilés, J. M. and Martínez, J. G. 2020. Brood parasitism, provisioning rates and breeding phenology of male and female magpie hosts. - J. Avian Biol.: jav.02522.

Proaktor, G., Milner-Gulland, E. J. and Coulson, T. 2007. Age-related shapes of the cost of reproduction in vertebrates. - Biol. Lett. 3: 674–677.

R Core Team 2020. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. <https://www.r-project.org/>.

Réale, D., Reader, S. M., Sol, D., McDougall, P. T. and Dingemanse, N. J. 2007. Integrating animal temperament within ecology and evolution. - Biol. Rev. 82: 291–318.

Rebke, M., Coulson, T., Becker, P. H. and Vaupel, J. W. 2010. Reproductive improvement and senescence in a long-lived bird. - PNAS 107: 7841–7846.

Reed, T. E., Kruuk, L. E. B., Wanless, S., Frederiksen, M., Cunningham, E. J. A. and Harris, M. P. 2008. Reproductive senescence in a long-lived seabird: Rates of decline in late-life performance are associated with varying costs of early reproduction. - Am. Nat. 171: E89-E101.

Robertson, R. J. and Rendell, W. B. 2001. A long-term study of reproductive performance in tree swallows: the influence of age and senescence on output. - J. Anim. Ecol. 70: 1014–1031.

Rothstein, S. I. 1978. Mechanisms of avian egg-recognition: Additional evidence for learned components. - Anim. Behav. 26: 671–677.

Rothstein, S. I. 1990. A Model System for Coevolution: Avian Brood Parasitism. - Annu. Rev. Ecol. Syst. 21: 481–508.

Salomons, H. M., Mulder, G. A., Van De Zande, L., Haussmann, M. F., Linskens, M. H. K. K. and Verhulst, S. 2009. Telomere shortening and survival in free-living corvids. - Proc. R. Soc. B Biol. Sci. 276: 3157–3165.

Samaš, P., Grim, T., Jelínek, V., Abraham, M. M., Šulc, M. and Honza, M. 2019. No immediate or future extra costs of raising a virulent brood parasite chick. - Behav. Ecol. 30: 1020–1029.

Seeker, L. A., Ilska, J. J., Psifidi, A., Wilbourn, R. V., Underwood, S. L., Fairlie, J., Holland, R., Froy, H., Salvo-Chirnside, E., Bagnall, A., Whitelaw, B., Coffey, M. P., Nussey, D. H. and Banos, G. 2018. Bovine telomere dynamics and the association between telomere length and productive lifespan. - Sci. Rep. 8: 1–12.

Sih, A., Kats, L. B. and Maurer, E. F. 2003. Behavioural correlations across situations and the evolution of antipredator behaviour in a sunfish-salamander system. - Anim. Behav. 65: 29–44.

Smith, B. R. and Blumstein, D. T. 2008. Fitness consequences of personality: A meta-analysis. - Behav. Ecol. 19: 448–455.

Smith, J. N. M., Arcese, P. and McLean, I. G. 1984. Age, experience, and enemy recognition by wild song sparrows. - Behav. Ecol. Sociobiol. 14: 101–106.

Soler, M. and Møller, A. P. 1990. Duration of sympatry and coevolution between the great spotted cuckoo and its magpie host. - Nature 343: 748–750.

Soler, J. J., Soler, M., Møller, A. P. and Martínez, J. G. 1995. Does the great spotted cuckoo choose magpie hosts according to their parenting ability? - Behav. Ecol. Sociobiol. 36: 201–206.

Soler, M., Martînez, J. G. and Soler, J. J. 1996. Effects of brood parasitism by the great spotted cuckoo on the breeding success of the magpie host: an experimental study. - Ardeola 43: 87–96.

Spiegelhalter, D. J., Best, N. G., Carlin, B. P. and van der Linde, A. 2002. Bayesian measures of model complexity and fit. - J. R. Stat. Soc. Ser. B Statistical Methodol. 64: 583–639.

Spurgin, L. G., Bebbington, K., Fairfield, E. A., Hammers, M., Komdeur, J., Burke, T., Dugdale, H. L. and Richardson, D. S. 2018. Spatio-temporal variation in lifelong telomere dynamics in a long-term ecological study. - J. Anim. Ecol. 87: 187–198.

Stokke, B. G., Takasu, F., Moksnes, A. and Røskaft, E. 2007. The importance of clutch characteristics and learning for antiparasite adaptations in hosts of avian brood parasites. - Evolution 61: 2212–2228.

Tarwater, C. E. and Arcese, P. 2017. Young females pay higher costs of reproduction in a short-lived bird. - Behav. Ecol. Sociobiol. 71: 84.

Trnka, A. and Grim, T. 2014. Testing for correlations between behaviours in a cuckoo host: Why do host defences not covary? - Anim. Behav. 92: 185–193.

Trnka, A., Požgayová, M., Samaš, P. and Honza, M. 2013. Repeatability of Host Female and Male Aggression Towards a Brood Parasite. - Ethology 119: 907–917.

Venables, W. N. and Ripley, B. D. 2002. Modern Applied Statistics with S. - Springer.

Wilbourn, R. V., Moatt, J. P., Froy, H., Walling, C. A., Nussey, D. H. and Boonekamp, J. J. 2018. The relationship between telomere length and mortality risk in non-model vertebrate systems: A meta-analysis. - Philos. Trans. R. Soc. B Biol. Sci. 373: 20160447.

Capítulo 4

Young, A. J. 2018. The role of telomeres in the mechanisms and evolution of life-history trade-offs and ageing. - Philos. Trans. R. Soc. B Biol. Sci. 373: 20160452.

Supplementary material

Table S1: Results of model selection based on values of deviance information criterion (DIC) for the mortality function best fitting the complete dataset.

Function	DIC	ΔDIC
Gompertz	2612	0
Weibull	2891	279
Logistic	3039	427
Exponential	4030	1418

Table S2: Results of model selection based on values of deviance information criterion (DIC) for the mortality function best fitting the dataset that included the parasitism status across life as a covariate.

Function	DIC	ΔDIC
Weibull	548	0
Gompertz	552	3.58
Logistic	561	13.33
Exponential	610	61.91

Table S3: Results of model selection based on values of deviance information criterion (DIC) for the mortality function best fitting the dataset that included fledglings' RTL as a covariate.

Function	DIC	ΔDIC
Gompertz	875	0
Weibull	889	14
Logistic	924	49
Exponential	1209	335

Table S4: Results of model selection based on values of deviance information criterion (DIC) for the mortality function best fitting the dataset that included the parasitism status early in life as a covariate.

Function	DIC	ΔDIC
Gompertz	586	0
Weibull	593	6.15
Logistic	597	10.22
Exponential	673	86.17

Table S5: Results of model selection based on values of deviance information criterion (DIC) for the mortality function best fitting the dataset that included the parasitism status late in life as a covariate.

Function	DIC	ΔDIC
Gompertz	488	0
Weibull	545	56.8
Logistic	559	71.1
Exponential	887	398.7

Discusión general

Discusión General

A pesar de la extensa literatura que existe sobre la ecología y evolución de los parásitos de cría y sus hospedadores, recogida en varias revisiones recientes (por ejemplo (Feeney et al. 2014, Soler 2014, 2017), hay algunos aspectos que, aunque se han propuesto como fundamentales para entender las interacciones evolutivas entre ambos, han recibido menos atención (Krüger 2007, Avilés 2018). Esta tesis doctoral ha pretendido ahondar en algunos de esos aspectos, y más específicamente se ha centrado en estimar los costes potenciales que el parasitismo de cría puede suponer para sus hospedadores al margen de la pérdida de éxito reproductivo en las puestas parasitadas. Este tipo de costes potenciales se han denominado costes extra para distinguirlos de los costes en términos de éxito reproductivo cuantificados normalmente en los estudios de parasitismo de cría (por ejemplo Samaš et al. 2018) y se han propuesto como costes extra aquellos que de manera directa o indirecta influirían en la supervivencia y longevidad de los hospedadores (Hoover y Reetz 2006, Krüger 2007, Samaš et al. 2018). En esta tesis hemos pretendido cuantificar algunos de estos potenciales costes, y sus consecuencias sobre la supervivencia, longevidad y éxito reproductivo a lo largo de la vida de los hospedadores. Para ello ha sido necesario trabajar desde una perspectiva individual con animales marcados, en un estudio longitudinal y a largo plazo en el que se han monitorizado los eventos reproductivos de los individuos a lo largo de su vida, y que además se ha complementado con otras aproximaciones.

En primer lugar, se han estimado los costes o consecuencias a corto-medio plazo desde una aproximación comportamental y correlacional (Capítulo 1), y una aproximación molecular y experimental (Capítulo 2). La finalidad de estimar estos costes a corto plazo es poder integrarlos con los patrones de supervivencia y éxito reproductivo a lo largo de la vida de los hospedadores: la teoría de historias vitales predice que los costes reproductivos a corto plazo deben de tener consecuencias en la reproducción y supervivencia futuras (Capítulo 1, 2 y 4). En primer lugar se ha caracterizado si el esfuerzo reproductivo de los hospedadores, estimado como la inversión en cebas a los pollos (tasa de cebas en los nidos), es diferente en nidos parasitados y sin parasitar, y en qué sentido. Trabajos previos enfatizan que los posibles costes extra de parasitismo en términos de esfuerzo reproductivo de los hospedadores varían en función de las características del sistema parásito-hospedador, de forma que los costes pueden ser de cierta magnitud (Dearborn et al. 1998, Hoover y Reetz 2006, Grayson et al. 2013) o negligibles (Kilner et al. 1999, Samaš et al. 2019); incluso en algunos casos los costes de la reproducción en términos energéticos serían mayores en nidadas no parasitadas, es decir, no debería considerarse que el parasitismo tiene un coste en este contexto (Soler et al. 1995, Canestrari et al. 2014, Samaš et al. 2018). Además

se ha investigado si los costes reproductivos diferenciales existentes entre puestas parasitadas y no parasitadas podrían afectar también de forma distinta a la dinámica de los telómeros. El acortamiento de los telómeros es una de las consecuencias moleculares del esfuerzo reproductivo (Sudyka 2019), y se podría considerar como uno de los costes extra del parasitismo, si la dinámica telomérica variase entre individuos parasitados y no parasitados durante la reproducción. La dinámica telomérica es uno de los mecanismos propuestos como responsables del compromiso entre invertir en la reproducción en un momento dado o vivir más tiempo, al estar la longitud de los telómeros y/o su tasa de acortamiento relacionadas con la supervivencia y longevidad de los individuos en muchas especies (Wilbourn et al. 2018, Capítulo 2). Por esto motivo, también se ha explorado si la longitud telomérica al principio de la vida está relacionada con la esperanza de vida en nuestra especie de estudio (Capítulo 4). Para ello, y debido a la necesidad de trabajar con muestras obtenidas a lo largo de muchos años, en primer lugar se ha testado si existe un efecto a largo plazo del método de conservación de muestras que se había utilizado, y tras haber comprobado que existe dicho efecto, se ha cuantificado y se ha desarrollado un método para corregirlo (Capítulo 3).

Costes o consecuencias a corto/medio plazo del parasitismo

Para estimar la existencia de costes extra del parasitismo a corto/medio plazo en el sistema formado por críalos y urracas, se ha estudiado el esfuerzo reproductivo que supone el parasitismo de forma natural para los hospedadores en un evento reproductivo dado (comparado con criar nidadas no parasitadas) en términos de tasa de cebas en los nidos, y las posibles consecuencias sobre la supervivencia de los hospedadores al año siguiente (Capítulo 1). Los resultados muestran que el esfuerzo reproductivo necesario para criar nidadas parasitadas y no parasitadas de forma general depende del número de pollos que haya en el nido y no de la especie, es decir, el parasitismo *per se* no parece tener ningún efecto sobre la tasa de cebas de los adultos. Sin embargo, en la mayoría de nidos parasitados (que contienen sólo uno o dos pollos de críalo) el esfuerzo es menor comparado con el que conlleva criar una nidada no parasitada, ya que la mayoría de nidadas no parasitadas contienen más pollos (cuatro o más pollos de urraca). Por ello se puede deducir que para la mayoría de individuos parasitados el esfuerzo reproductivo, en términos de tasa de cebas a los pollos, es menor. Como sería esperable, debido a este menor esfuerzo, los datos muestran que el parasitismo no tiene un efecto sobre la supervivencia de sus hospedadores de un año para otro.

El Capítulo 2 corrobora con una aproximación diferente y novedosa la idea de que el parasitismo no representa costes a medio plazo superiores a los

asociados con criar nidadas no parasitadas. En este caso se abordan los costes de la reproducción y del parasitismo usando la dinámica telomérica como biomarcador de éstos costes. Parece demostrado que el acortamiento de los telómeros es un buen indicador de estrés fisiológico y costes reproductivos (Wilbourn et al. 2018, Chatelain et al. 2020) y además está asociado con la supervivencia, senescencia y longevidad de los individuos en muchas especies (Monaghan y Haussmann 2006). Hay que destacar que se ha utilizado una aproximación experimental, que complementa la aproximación correlacional del Capítulo 1, y que ha permitido eliminar el posible efecto de los parásitos eligiendo individuos con determinadas características. Los resultados muestran un efecto del tamaño de nidada criado por los individuos sobre el acortamiento de sus telómeros, en interacción con la longitud de los telómeros al comenzar el experimento. No hay pues tampoco en este caso un efecto significativo del parasitismo *per se*. Criar uno o varios pollos produce diferentes tasas de acortamiento de los telómeros, al menos en algunos individuos, lo que sugiere que el menor número de pollos de las nidadas parasitadas puede conllevar un menor esfuerzo reproductivo, de la misma forma que lo sugerían los resultados del Capítulo 1. La interacción con la longitud telomérica inicial sugiere que la edad de los individuos podría mediar las consecuencias del parasitismo sobre el acortamiento telomérico, ya que la longitud telomérica está asociada con la edad en muchas especies, y en concreto en la urraca como muestran los análisis presentados en el Capítulo 2. Así, los resultados sugieren que las diferencias en la tasa de acortamiento de los telómeros asociadas con distintos tamaños de nidada sólo son significativas en los individuos más jóvenes, de acuerdo con la idea de que los individuos jóvenes sufran mayores costes de reproducción, en particular cuando crían nidadas de mayor tamaño (Tarwater y Arcese 2017).

Finalmente, hay otras líneas argumentales en favor de una escasa magnitud de los costes extra del parasitismo sobre las urracas a corto/medio plazo. No se ha encontrado ningún efecto del parasitismo sobre el papel relativo de los sexos en los cuidados parentales, y se ha descartado que el parasitismo promueva diferentes estrategias entre hembras y machos en esta especie (Capítulo 1). Estos resultados están de acuerdo con la hipótesis que establece que en especies de vida larga y con vínculos de pareja de larga duración, como la urraca, los cambios en el valor reproductivo de las nidadas, como los que supondría el parasitismo en términos de números de pollos en el nido, provocarían una respuesta coordinada o similar en ambos sexos (Mariette et al. 2015). Los resultados de esta tesis sugieren además que podría ser debido a los pequeños costes que supone el parasitismo globalmente (Capítulo 4, ver abajo).

No obstante, sí que se ha encontrado en el estudio correlacional a corto plazo del Capítulo 1 un posible coste extra en términos fenológicos: hay un efecto

negativo del parasitismo en la fecha de puesta de los hospedadores al año siguiente de ser parasitados. Este retraso se puede interpretar como una consecuencia de costes extra durante otros periodos de los cuidados parentales no considerados en los análisis (como el cuidado de los volantones), aunque a la vista de los resultados globales de la tesis, y debido al pequeño tamaño de muestra de ese análisis, habría que tomar este resultado con cautela y en cualquier caso sería necesario investigarlo con más atención.

Costes o consecuencias a largo plazo

El Capítulo 2 sugiere una relación entre la tasa de acortamiento de los telómeros y la edad de los individuos, lo que implicaría que los costes y las consecuencias de la reproducción y del parasitismo podrían variar a lo largo de la vida de los hospedadores, y que no tendrá las mismas consecuencias ser parasitado al principio o al final de la vida. Este hecho no sería sorprendente, ya que los costes de la reproducción varían con la edad en diferentes especies de animales (Reid et al. 2003, Descamps et al. 2009, Tarwater y Arcese 2017), y el desempeño y éxito reproductivo están asociados frecuentemente a la edad de los individuos en muchas especies (Clutton-Brock 1988, Sæther 1990, Stearns 1992) porque los factores determinantes del éxito reproductivo, como la depredación o el parasitismo, muestran frecuentemente un patrón de dependencia con la edad, por ejemplo en aves (Martin 1995). Sin embargo, aunque se ha sugerido que la heterogeneidad en la susceptibilidad al parasitismo o sus consecuencia a lo largo de la vida de los hospedadores tendría un papel en la dinámica ecológica y evolutiva de los parásitos de cría y sus hospedadores (Krüger 2007), esta idea no está suficientemente explorada. Si los hospedadores no son igual de susceptibles al parasitismo o éste no tiene las mismas consecuencias en distintos momentos de la vida, es fundamental estudiar éstas a lo largo de toda la vida de los hospedadores para estimar la magnitud real de los costes del parasitismo y la presión selectiva que éste representa. En esta tesis se ha intentado estimar los costes a largo plazo del parasitismo de cría, tanto costes extra, relacionados con la supervivencia, como costes directos sobre el éxito reproductivo, a través de un estudio longitudinal de individuos seguidos a lo largo de su vida (Capítulo 4)

En su conjunto, el patrón observado a largo plazo es consistente con la estima de los costes reproductivos a corto plazo: el parasitismo no parece suponer un gran coste en términos de supervivencia. Los individuos que son parasitados al menos una vez a lo largo de su vida viven más tiempo debido a que sufren una menor tasa de mortalidad que los individuos que no son nunca parasitados. Hay 3 posibles factores que podrían explicar este patrón. a) Por un lado, los individuos que son parasitados sufren una menor mortalidad dependiente de la edad, lo que

puede ser una consecuencia del menor esfuerzo reproductivo que supone criar nidadas parasitadas (Capítulos 1 y 2), que se traduciría en un menor acortamiento telomérico debido a un menor estrés durante la reproducción en eventos de cría parasitados y que tendría un efecto acumulativo a lo largo de la vida (Capítulo 2). Es importante enfatizar que los análisis de trayectorias de supervivencia presentados en el Capítulo 4 no muestran una relación entre la longitud telomérica de los individuos al salir del nido y su esperanza de vida o longevidad, como ocurre en otras especies, por lo que los resultados de esta tesis sugieren que si existe una relación entre dinámica telomérica y supervivencia, en las urracas está mediada por el parasitismo. b) Por otro lado, los individuos parasitados también sufren una menor mortalidad basal, lo que podría estar relacionado con un menor riesgo de depredación de los adultos criando nidadas parasitadas que contienen un menor número de pollos. c) Finalmente, la menor tasa de mortalidad (basal o dependiente de la edad) podría estar asociada con características intrínsecas de los individuos que seleccionen lo parásitos y estén correlacionadas con la mortalidad, de forma que los individuos que son parasitados alguna vez a lo largo de su vida serían individuos que en cualquier caso vivirían más. La aproximación longitudinal y correlacional usada en esta tesis para estimar las trayectorias de supervivencia de los individuos no nos permite descartar esta posibilidad. No obstante, la aproximación experimental del Capítulo 2 elimina el efecto de selección de características de los hospedadores por parte de los parásitos, por lo que al menos uno de los factores que podría explicar la mayor longevidad de individuos parasitados algunas veces a lo largo de su vida (dinámica telomérica) no está relacionado con la calidad de los hospedadores sino que deriva de las consecuencias del parasitismo.

Otros resultados apuntan en la dirección de que la mayor longevidad en individuos parasitados podría derivarse del parasitismo. Uno de los análisis del Capítulo 4 sugiere, aunque indirectamente, que un esfuerzo diferencial en los primeros años de vida (ser o no parasitado) tiene consecuencias en la supervivencia de los individuos, y la mayoría de individuos que invierten un esfuerzo mayor en sus primeros años (no son parasitados) mueren más jóvenes. Eso sugiere que los costes reproductivos del parasitismo (no criar urracas) en los primeros años de vida se podrían compensar con mayores posibilidades de reproducción en el futuro, por lo que el parasitismo al principio de la vida estaría favoreciendo mayores longevidades. Sin embargo, este efecto parece desaparecer una vez que los individuos alcanzan cierta edad (Capítulo 4).

En cualquier caso, y aunque esclarecer los mecanismos concretos necesitaría de una aproximación experimental mucho más ambiciosa, estas diferencias en longevidad se traducen en que el éxito reproductivo a lo largo de la vida de los individuos que son parasitados algunas veces es similar al de los que no

son nunca parasitados (Capítulo 4). De nuevo este resultado apunta a unos costes del parasitismo menores de los esperables. Si los eventos de cría parasitados tienen siempre menos éxito reproductivo, los individuos parasitados a lo largo de su vida deberían tener menos éxito reproductivo total que los nunca parasitados, como sugerían de hecho análisis previos (Molina-Morales et al. 2013). Sin embargo, si los individuos parasitados viven más años que los no parasitados pueden compensar a lo largo de su vida las pérdidas reproductivas en eventos parasitados con más intentos de cría no parasitados. Esto es lo que los análisis de las trayectorias de supervivencia de los individuos de la población estudiada y de su éxito a lo largo de su vida sugieren. Este es probablemente el resultado más importante de esta tesis, ya que indica que los costes del parasitismo en la pérdida de éxito reproductivo de estos hospedadores, o al menos en nuestra población, son más pequeños de lo normalmente asumido. A pesar de que esta posibilidad se había anticipado teóricamente (Kruger 2007) e incluso mostrado en otro sistema parásito-hospedador (Brooker y Brooker 1996), se trata de un escenario no suficientemente bien explorado entre los hospedadores de parásitos de cría, quizás debido a que la mayoría de éstos son especies de vida corta.

Vivir muchos años en una población parasitada puede resultar beneficioso para las urracas por varios motivos. Aunque no hay un patrón claro de parasitismo asociado a la edad (Martínez et al. 2020), los costes del parasitismo podrían hacerse menores conforme los individuos envejecen debido a que el principal mecanismo de defensa frente al parasitismo, el rechazo de huevos extraños, es más probable en edades avanzadas. En la población de estudio se ha mostrado que el rechazo de huevos extraños por parte de las urracas comienza a edades tardías (aproximadamente a los 4 años en promedio, Martínez et al. 2020), lo que puede jugar un papel importante en la compensación del éxito reproductivo mencionado en el párrafo anterior de los animales más longevos. Si los individuos que viven más tiempo tienen mayor probabilidad de desarrollar las habilidades necesarias para el reconocimiento y rechazo de huevos parásitos (Molina-Morales et al. 2014, Martínez et al. 2020), podrían escapar del parasitismo a esas edades. Esto les permitiría y alcanzar el mismo éxito reproductivo que los individuos que no han sido nunca parasitados, siempre que éstos vivan menos años, como sugieren los análisis presentados en esta tesis. Incluso en ausencia de este patrón de defensa asociado a la edad, vivir más años podría aumentar las posibilidades de escapar del parasitismo con más frecuencia si el riesgo de parasitismo no fuese constante todos los años.

En la literatura sobre parasitismo de cría habitualmente se ha asumido que el parasitismo es extremadamente costoso para los hospedadores y por ello suponen una fuerte presión selectiva sobre la evolución de mecanismos de defensa en los hospedadores (Rothstein 1990). Sin embargo, los resultados de esta tesis

sugieren que la presión selectiva que ejerce el críalo sobre las urracas en nuestra población de estudio es pequeña. La zona donde se encuentra esta población es un área de colonización reciente por el críalo (desde aproximadamente los años 60 del siglo pasado; (Soler y Møller 1990)). En esta zona, se observó un aumento de la tasa de rechazo de huevos modelo miméticos con los huevos parásitos en un periodo de tiempo muy corto (de un 14 % a un 33% en 5 años, (Soler et al. 1994)), por lo que las defensas pueden evolucionar rápidamente en esta especie. Sin embargo, en nuestra población de estudio, la tasa de rechazo de huevos miméticos ha fluctuado en torno a un 30% durante los últimos 15 años. En un trabajo reciente se sugiere que este porcentaje está relacionado con la estructura de edad de las poblaciones (Martínez et al. 2020), y por lo tanto se corresponde con un escenario de equilibrio evolutivo (*sensu* Rohwer y Spaw 1988, Lotem et al. 1992, Avilés et al. 2005, Kruger et al. 2011). Los resultados de esta tesis apuntan en este sentido: la presión de parasitismo podría no ser lo suficientemente alta para mejorar las respuestas defensivas de los hospedadores, manteniéndose entonces una situación de equilibrio en la que los parásitos explotan a los hospedadores en base a limitaciones en sus capacidades defensivas (Martínez et al. 2020) y a que el parasitismo en sí mismo podría facilitar una respuesta compensatoria (mayor longevidad) que reduce los costes de éste a largo plazo.

Los resultados de esta tesis ponen de manifiesto la importancia de determinar los costes y las consecuencias del parasitismo a lo largo de la vida de los individuos, que es lo que va a determinar la verdadera presión que los parásitos ejercen sobre sus hospedadores, lo que nos va a permitir entender la evolución de las interacciones entre ambos, y además explorar nuevos mecanismos a través de los que la dinámica coevolutiva entre parásitos y hospedadores podría estar operando, como potenciales respuestas selectivas en la supervivencia y longevidad de los hospedadores. Estudios longitudinales combinados con aproximaciones experimentales serían necesarios para una mejor comprensión de este posible vínculo entre parasitismo y longevidad.

Referencias

Avilés, J. M. 2018. Can hosts tolerate avian brood parasites? An appraisal of mechanisms. - Behav. Ecol. 29: 509–519.

Avilés, J. M., Rutila, J. y Møller, A. P. 2005. Should the redstart Phoenicurus phoenicurus accept or reject cuckoo Cuculus canorus eggs? - Behav. Ecol. Sociobiol. 58: 608–617.

Brooker, M. y Brooker, L. 1996. Acceptance by the splendid fairy-wren of parasitism by Horsfield's bronze-cuckoo: further evidence for evolutionary equilibrium in brood parasitism. - Behav. Ecol. 7: 395–407.

Canestrari, D., Bolopo, D., Turlings, T. C. J., Roder, G., Marcos, J. M. y Baglione, V. 2014. From Parasitism to Mutualism: Unexpected Interactions Between a Cuckoo and Its Host. - Science 343: 1350–1352.

Chatelain, M., Drobniak, S. M. y Szulkin, M. 2020. The association between stressors and telomeres in non-human vertebrates: a meta-analysis. - Ecol. Lett. 23: 381–398.

Clutton-Brock, T. H. 1988. Reproductive Success: Studies of Individual Variation in Contrasting Breeding Systems. - University of Chicago Press.

Dearborn, D. C., Anders, A. D., Thompson, F. R. y Faaborg, J. 1998. Effects of Cowbird Parasitism on Parental Provisioning and Nestling Food Acquisition and Growth. - Condor 100: 326–334.

Descamps, S., Boutin, S., McAdam, A. G., Berteaux, D. y Gaillard, J. M. 2009. Survival costs of reproduction vary with age in North American red squirrels. - Proc. R. Soc. B Biol. Sci. 276: 1129–1135.

Feeney, W. E., Welbergen, J. A. y Langmore, N. E. 2014. Advances in the study of coevolution between avian brood parasites and their hosts. - Annu. Rev. Ecol. Evol. Syst. 45: 227–246.

Grayson, P., Glassey, B. y Forbes, S. 2013. Does Brood Parasitism Induce Paternal Care in a Polygynous Host? - Ethology 119: 489–495.

Hoover, J. P. y Reetz, M. J. 2006. Brood parasitism increases provisioning rate, and reduces offspring recruitment and adult return rates, in a cowbird host. - Oecologia 149: 165–173.

Kilner, R. M., Noble, D. G. y Davies, N. B. 1999. Signals of need in parent–offspring communication and their exploitation by the common cuckoo. - Nature 397: 667–672.

Krüger, O. 2011. Brood parasitism selects for no defence in a cuckoo host. - Proc. R. Soc. B Biol. Sci. 278: 2777–2783.

Krüger, O. 2007. Cuckoos, cowbirds and hosts: Adaptations, trade-offs and constraints. -

Philos. Trans. R. Soc. B Biol. Sci. 362: 1873–1886.

Lotem, A., Nakamura, H. y Zahavi, A. 1992. Rejection of cuckoo eggs in relation to host age: A possible evolutionary equilibrium. - Behav. Ecol. 3: 128–132.

Mariette, M. M., Griffith, S. C., Adkins-Regan, A. E. E. y Kalisz, E. S. 2015. The adaptive significance of provisioning and foraging coordination between breeding partners. - Am. Nat. 185: 270–280.

Martin, K. 1995. Patterns and mechanisms for age-dependent reproduction and survival in birds. - Integr. Comp. Biol. 35: 340–348.

Martínez, J. G., Molina-Morales, M., Precioso, M. y Avilés, J. M. 2020. Age-related brood parasitism and egg rejection in magpie hosts. - Am. Nat. 195: 876–885.

Molina-Morales, M., Gabriel Martínez, J., Martín-Gálvez, D., A. Dawson, D., Rodríguez-Ruiz, J., Burke, T. y Avilés, J. M. 2013. Evidence of long-term structured cuckoo parasitism on individual magpie hosts. - J. Anim. Ecol. 82: 389–398.

Molina-Morales, M., Martínez, J. G., Martín-Gálvez, D., Dawson, D. A., Burke, T. y Avilés, J. M. 2014. Cuckoo hosts shift from accepting to rejecting parasitic eggs across their lifetime. - Evolution 68: 3020–3029.

Monaghan, P. y Haussmann, M. F. 2006. Do telomere dynamics link lifestyle and lifespan? - Trends Ecol. Evol. 21: 47–53.

Reid, J. M., Bignal, E. M., Bignal, S., McCracken, D. I. y Monaghan, P. 2003. Age-specific reproductive performance in red-billed choughs Pyrrhocorax pyrrhocorax: Patterns and processes in a natural population. - J. Anim. Ecol. 72: 765–776.

Rohwer, S. y Spaw, C. D. 1988. Evolutionary lag versus bill-size constraints: a comparative study of the acceptance of cowbird eggs by old hosts. - Evol. Ecol. 2: 27–36.

Rothstein, S. I. 1990. A Model System for Coevolution: Avian Brood Parasitism. - Annu. Rev. Ecol. Syst. 21: 481–508.

Sæther, B. 1990. Age-specific variation in reproductive performance of birds. - Curr. Ornithol. 7: 251–283.

Samaš, P., Rutila, J., Honza, M., Kysučan, M., y Grim, T. 2018. Rearing a virulent common cuckoo is not extra costly for its only cavity-nesting host. - Proc. R. Soc. B Biol. Sci. 285: 20181710.

Samaš, P., Grim, T., Jelínek, V., Abraham, M. M., Šulc, M. y Honza, M. 2019. No immediate or future extra costs of raising a virulent brood parasite chick. - Behav. Ecol. 30: 1020–1029.

Soler, M. 2017. Avian Brood Parasitism (M. Soler, Ed.). - Springer International Publishing.

Soler, M. 2014. Long-term coevolution between avian brood parasites and their hosts. - Biol. Rev. 89: 688–704.

Soler, M. y Møller, A. P. 1990. Duration of sympatry and coevolution between the great spotted cuckoo and its magpie host. - Nature 343: 748–750.

Soler, M., Martínez, J. G., Soler, J. J. y Møller, A. P. 1994. Micro-evolutionary change in host response to a brood parasite. - Behav. Ecol. Sociobiol. 35: 295–301.

Soler, M., Martínez, J. G., Soler, J. J. y Møller, A. P. 1995. Preferential allocation of food by magpies Pica pica to great spotted cuckoo *Clamator glandarius* chicks. - Behav. Ecol. Sociobiol. 37: 7–13.

Stearns, S. C. 1992. The Evolution of Life Histories. - Oxford University Press.

Sudyka, J. 2019. Does Reproduction Shorten Telomeres? Towards Integrating Individual Quality with Life-History Strategies in Telomere Biology. - BioEssays 41: 1900095.

Tarwater, C. E. y Arcese, P. 2017. Young females pay higher costs of reproduction in a short-lived bird. - Behav. Ecol. Sociobiol. 71: 84.

Wilbourn, R. V., Moatt, J. P., Froy, H., Walling, C. A., Nussey, D. H. y Boonekamp, J. J. 2018. The relationship between telomere length and mortality risk in non-model vertebrate systems: A meta-analysis. - Philos. Trans. R. Soc. B Biol. Sci. 373: 20160447.

Conclusiones/Conclusions

Conclusiones

1. El esfuerzo reproductivo (medido como tasas de visitas al nido) que invierten los hospedadores es similar, por lo general, en nidadas parasitadas y no parasitadas. Sin embargo, para los tamaños de nidadas más comunes de la población, es menor en los nidos parasitados.

2. El parasitismo por parte del críalo no tiene un efecto sobre el papel de los sexos en los cuidados parentales en la urraca.

3. Los costes reproductivos del parasitismo en términos de acortamiento telomérico son, al menos para algunos individuos, menores que los asociados a criar una nidada de urracas normal y esto es debido al menor número de pollos de las nidadas parasitadas. Esto es así para individuos que tiene los telómeros más largos, y que son probablemente más jóvenes.

4. Los individuos que son parasitados al menos una vez a lo largo de su vida viven más tiempo que los individuos que no son nunca parasitados, debido a que sufren una menor mortalidad basal y mortalidad dependiente de la edad.

5. Este patrón podría venir determinado por diferencias en la cría en los primero años de vida, ya que las diferencias entre individuos parasitados y no parasitados parecen desaparecer al alcanzar cierta edad.

6. Esto, unido a la posible dependencia de la edad de las tasas de acortamiento telomérico, podría indicar que los costes de la reproducción, y las consecuencias del parasitismo, en relación a la supervivencia podrían variar con la edad en esta especie.

7. Los individuos que son parasitados al menos una vez en su vida tienen el mismo éxito reproductivo a lo largo de su vida que los individuos que no son nunca parasitados, lo cual indica que la presión que ejerce el parasitismo sobre los hospedadores en nuestra población de estudio es pequeña, y apoya la existencia de un equilibrio evolutivo entre parásitos y hospedadores.

8. La longitud telomérica al principio de la vida no está relacionada con la esperanza de vida en las urracas, pero la dinámica telomérica podría jugar un papel en determinarla.

9. Existe un patrón de degradación específica de secuencia telomérica durante los tres o cuatro primeros años de preservación de muestras de sangre en etanol a temperatura ambiente. Sin embargo, la existencia de un patrón claro permite corregir este efecto de la conservación, y destaca la necesidad de evaluar otros medios de conservación de muestras.

Conclusions

1. The reproductive effort (measured as nest visit rates) that hosts invest is, generally, similar in parasitized broods compared to non-parasitized broods. However, for the most common brood sizes in the populations it is smaller in the parasitized ones.

2. Brood parasitism has no effect on the relative role of the sexes in parental care.

3. The reproductive costs of parasitism in terms of telomere shortening are, at least for some individuals, smaller than those related to rear a normal host brood, due to the fewer number of chicks of the parasitized broods. This is so for the individuals that have longer telomeres and, thus, are probably younger.

4. Individuals that are parasitized at least once during their lifetime live longer than those that are never parasitized, because those individuals have both a smaller baseline mortality and a smaller age-dependent mortality.

5. This pattern could arise from differences in what they rear in their first years of life, given that the differences between parasitized and non-parasitized individuals seem to disappear at older ages.

6. This, together with the possible dependence of telomere shortening rates on age, could suggest that the costs of reproduction, and the consequences of parasitism, in relation to survival could vary with age in this species.

7. Those individuals that are parasitized at least once during their lifetime have the same lifetime reproductive success that those that are never parasitized, what suggests that the selective pressure due to parasitism in our study population is small, and supports the existence of an evolutionary equilibrium between parasites and hosts.

8. Early life telomere length is not related to magpies' lifespan, but telomere dynamics may play a role instead.

9. There is a specific pattern of telomere sequence degradation during the first three or four years in which blood samples are stored in ethanol at room temperature. However, the existence of a clear pattern enables to correct for this storage effect, and highlights the need to evaluate other sample storage buffers.

Agradecimientos

Agradecimientos

Me gustaría expresar mi agradecimiento a aquellas personas que, de una forma u otra, me han conducido a donde estoy ahora y han permitido que esta tesis llegue a su fin. Espero no olvidarme de ninguna.

En primer lugar me gustaría agradecer a mi familia, el apoyo que siempre me han dado y el interés que han mostrado por mi trabajo. Me gustaría agradecer especialmente a mi madre, que, aunque de vez en cuando me diga "Oye Marta, ¿pero lo que haces sirve para algo?", siempre ha tenido claro que éste era mi camino, incluso cuando yo he dudado de ello. Ella ha fomentado mi pasión por la naturaleza desde que era una enana y me ha apoyado. Me gustaría también agradecer a Pablo que la ilusión que ha despertado en él por la ciencia viendo a su tía, me llena de orgullo y me anima a seguir luchando por esta carrera.

A Rubén, por esa paciencia infinita que ha tenido con mi estrés, con mis idas y venidas por el trabajo, por todo el tiempo que he pasado fuera, por cuidar de nuestros bichines, por su ayuda en el campo, por cortar anillas,... ¡Por tantas cosas! Y por estar siempre ahí, incondicionalmente.

Esta tesis no habría sido posible sin mis directores, que desde que era alumna interna siempre me han trasmitido el entusiasmo por la ciencia, por lo que hacemos y que me han enseñado tanto. A Merche, por esas infinitas jornadas de campo en las que tanto he aprendido, por estar siempre disponible para lo que necesitara y por animarme tanto. A Juanga, por confiar en mí para esta tesis, por su dedicación y por todas las conversaciones sobre ecología y evolución. A los dos, por todos los momentos buenos que hemos pasado.

A Jesús, miembro fundamental de nuestro grupo de trabajo, por estar siempre dispuesto a explicarme cualquier cosa.

También tengo que agradecer a todas las personas que en algún momento han participado en el trabajo de campo conmigo y con las que siempre he aprendido mucho. A Mónica y Alfredo, con ellos aprendí, antes de empezar esta tesis, lo duro que puede ser el trabajo de campo, pero también lo gratificante (y por esos viajes locos acampando a -5 ºC). A mi querida Lola, que tantas horas ha pasado en el campo con el cuervo, la perdiz y conmigo, y que tanto me ha apoyado en casa. A Eva, por su esfuerzo y sacrificio. A los que fueron alumnos internos y me echaron una mano aunque hiciera mucho frío o viento, Rafa y Jorge.

A mis compañeros de la universidad, que tantos viajes de bicheo hemos hecho juntos y por el esfuerzo que ponen por seguir en contacto, aunque yo sea tan parca. A Fernando, María, Marisa, Juan, Antonio, Vito, Alfredo, Yedra, Manu y Sara. A mi

Jara, que siempre ha estado ahí, especialmente en los peores momentos, aun estando lejos.

No puedo olvidar a mi familia "adoptiva" de Granada, Eva, Jeremy, Jana, Carmen y Cristhian. Ellos siempre me han apoyado en los momentos más "flojos" y me han animado. A Jeremy, por esa gran ayuda de última hora con la portada, y por tantas conversaciones sobre ciencia.

A las personas del Departamento de Zoología, por su amabilidad y su ayuda cuando lo he necesitado, y porque siempre me han dado ánimos.

También tengo que agradecer a Roberto, del Departamento de Genética, y a Roca, de Ecología, el que me permitieran utilizar sus laboratorios al principio de la tesis.

Y por último me gustaría agradecer a las personas de la Universidad de Sheffield que, de una u otra forma, me han ayudado en mis estancias: Gavin, Debs, Celine, Natalie, Mirre, Aaron, Malika, y, por supuesto, Terry.

A todos, ¡gracias!