Defences against brood parasitism in the common blackbird (*Turdus merula*):

plasticity, physiology and evolution

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DEFENCES AGAINST BROOD PARASITISM IN THE COMMON BLACKBIRD (*TURDUS MERULA*): PLASTICITY, PHYSIOLOGY AND EVOLUTION

DEFENSAS FRENTE AL PARASITISMO DE CRÍA EN EL MIRLO COMÚN (*TURDUS MERULA*): PLASTICIDAD, FISIOLOGÍA Y EVOLUCIÓN

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Defences against brood parasitism in the common blackbird (*Turdus merula*): plasticity, physiology and evolution

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Granada, 25 de septiembre de 2017.

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A mis padres,

"If the old bird profited by this occasional habit through being enabled to migrate earlier or through any other cause; or if the young were made more vigorous by advantage being taken of the mistaken instinct of another species than when reared by their own mother, encumbered as she could hardly fail to be by having eggs and young of different ages at the same time; then the old birds or the fostered young would gain an advantage. And analogy would lead us to believe, that the young thus reared would be apt to follow by inheritance the occasional and aberrant habit of their mother, and in their turn would be apt to lay their eggs in other birds' nests, and thus be more successful in rearing their young. By a continued process of this nature, I believe that the strange instinct of our cuckoo has been generated."

Charles Darwin,
The Origin of Species, Chapter VIII

Contents

Summary

Antagonistic interactions between avian brood parasites and their hosts provide excellent examples of reciprocal evolution of host adaptations and parasite counter-adaptations. The high fitness cost imposes by brood parasitism selects for the evolution of host defences, which set the stage for a co-evolutionary arms race, in which brood parasites evolve adaptive counter-defences that select for improved host defences, further parasitic adaptations, and so on. Although these adaptations can be found in all stages of the breeding cycle, the main host defence is the rejection of parasitic eggs. Importantly, previous studies suggest that egg rejection is a complex process in which different stages can be differentiated: egg recognition, decision and the action of reject itself. Throughout this thesis, the study of egg rejection is approached according to this perspective, focusing on the factors that govern each of these stages.

Most studies on brood parasitism have focused on the last stage of the egg-rejection process (action itself), which can be carried out by deserting the parasitized nests, ejecting the foreign egg, or even burying it in the nest. However, as potential costs associated with each of these egg-rejection mechanisms vary, the choice of the egg-rejection mechanism by hosts should consequently be modulated by a balance between the costs and benefits of the response. In Chapter 1, we investigated whether nest desertion can be considered an egg-rejection mechanism in medium or large-sized hosts. We found that nest desertion cannot be seen as an unequivocal response to brood parasitism in the common blackbird *Turdus merula*, a medium-size potential host species. Thus, our findings suggest that future studies on egg rejection using similar species should cautiously consider nest desertion as a response to brood parasitism.

Importantly, each stage of the egg rejection process is likely to be impacted by different factors that determine the host response. In this thesis, different traits of parasitic eggs (egg mass, colour and size) are proven to affect particular stages of the egg-rejection process, including the decision to eject, which may lead to the acceptance of previously recognized eggs. Chapter 2 shows that hosts are not willing to assume the potential costs associated to the ejection of a slightly heavier egg (low motivation), which turned out in acceptance decisions,

therefore confirming that egg recognition is not necessarily followed by egg rejection. Chapter 3 goes one step further and studies how the different stages of the egg-rejection process can be independently impacted by different traits of the parasitic egg. In particular, this chapter describes how colour mimicry makes it difficult to recognize parasitic eggs (the first stage of the process), thus leading to egg acceptance. On the other hand, egg size does not seem to affect recognition, but it can impose mechanical restrictions on the action stage (the third stage) and delay the decision to eject (the second stage of the process). Importantly, the existence of acceptance decisions implies that the egg-rejection rate does not necessarily reflect the recognition abilities of hosts, so it is necessary to study other variables clearly attributable to egg recognition in order to fully understand the rejection process. In this thesis, the "egg-touching behaviour" exhibited by blackbirds is revealed as a reliable indicator of the egg-recognition abilities in this species, allowing a more detailed study of the relationships between recognition and rejection.

Given its importance in regulating animal behaviour, endocrine pathways are excellent candidates to continue unravelling the proximate mechanisms underlying the egg-rejection process. However, the detailed knowledge of behavioural responses to parasitic eggs contrasts with the limited information available on the hormonal processes that govern these behaviours. Chapter 4 therefore investigates how hosts adjust their hormonal state to deal with parasitic eggs, as well as the potential effects of these adjustments on the physical state of hosts. Our results show that a parasitic egg is, from a hormonal perspective, a stressful factor for adult hosts since parasitized individuals showed higher baseline corticosterone levels, and lower body condition, than non-parasitized individuals. In addition, unaffected levels of prolactin suggest that blackbirds tend to maintain parental care even when parasitized, which may explain some behaviours found in this species, such as the absence of nest desertion in response to experimental parasitism.

The important role of decision-making in the host response revealed by the previous chapters suggests that plastic responses in egg rejection could be common among host species. Consequently, Chapter 5 reviews the evidence of plastic defences against parasitic eggs, showing that phenotypic plasticity in egg rejection is less widespread than might be expected. This chapter discuss the factors that could favour the evolution of phenotypic plasticity and its importance in the co-evolutionary relationship between brood parasites and their hosts, where fixed responses seems to be more frequent than previously suspected, even in the absence of brood parasitism. Precisely, Chapter 6 provides experimental evidence on the evolutionary origin of the egg-rejection abilities exhibited by species not currently impacted by interspecific brood parasitism, as is the case of blackbirds. Although previous studies have suggested that egg rejection evolved in these species because of conspecific brood parasitism, our results indicate that the most probable origin of these abilities is a past exploitation by interspecific parasites. Likewise, current absence of interspecific brood parasitism in these species could be

due to their well-developed defences, which are maintained even after speciation events, probably due to the lack of costs for their maintenance.

Since it has been assumed that females play the leading role in the egg-rejection process, Chapter 7 tries to confirm such assumption and investigates whether there are sex differences in the host response to parasitic eggs. This question is particularly relevant in species, like blackbirds, in which the incubation depends exclusively on the female. Contrary to conventional theory, we found that blackbird males are able to recognize and eject parasitic eggs. Interestingly, recognition abilities of males are less developed than those of females, probably due to their looser association to the nest during the incubation stage. The evolution of egg recognition in non-incubating males is an intriguing fact that can have important implications in the evolution of anti-parasitic defences within host populations.

The results of previous chapters provide a detailed insight into the egg-rejection process. These findings in addition to the recent new theoretical framework in the field of animal decision making highlights the need to update some terms frequently used in egg-rejection studies, as well as the inclusion of new concepts to future studies. This thesis therefore concludes with the proposal, in Chapter 8, of an updated terminology advocating for a consistent use of terms in future works on egg rejection, which could contribute the advance of scientific knowledge in the field of brood parasitism.

Summarizing, this thesis addresses the study of egg rejection as a complex and potentially plastic process in which host decision-making plays a key role. The environmental context, clutch characteristics and cognitive abilities of hosts will determine the outcome of egg rejection by modulating the different stages of this process, which is probably mediated by endocrine pathways. This approach is complemented by an evolutionary perspective on the origin of egg rejection abilities in non-exploited potential hosts, as well as the importance of considering the role of males in egg rejection. This thesis therefore provides significant advances in our understanding of the co-evolutionary interactions between parasites and hosts, particularly in relation to one of the main anti-parasitic defences, egg rejection.

Resumen

Las interacciones antagonistas entre las aves parásitas de cría y sus hospedadores proporcionan excelentes ejemplos de evolución recíproca de adaptaciones por parte de los hospedadores y de contra-adaptaciones por parte del parásito. Los altos costes en fitness impuestos por el parasitismo de cría favorecen la evolución de defensas en los hospedadores, lo cual sienta las bases para una carrera de armamentos coevolutiva en la cual los parásitos de cría evolucionan contra-defensas, lo que a su vez favorece la evolución de mejores defensas por parte de los hospedadores, posteriores adaptaciones en los parásitos, y así sucesivamente. A pesar de que estas adaptaciones se encuentran en todas las etapas del ciclo reproductor, la principal defesa de los hospedadores es el rechazo de los huevos parásitos. Es importante destacar que estudios previos han sugerido que el rechazo de huevos es un proceso complejo en el que pueden diferenciarse distintas etapas: el reconocimiento del huevo, la decisión, y la propia acción de rechazo. Esta tesis aborda el estudio del rechazo de huevos bajo esta perspectiva, centrándose en los factores que rigen cada una de estas etapas.

La mayoría de los estudios sobre parasitismo de cría se han centrado en la última etapa del proceso de rechazo del huevo (la propia acción del rechazo), la cual se puede llevar a cabo mediante el abandono del nido parasitado, expulsando el huevo extraño, o incluso enterrándolo en el nido. Sin embargo, dado que los potenciales costes asociados con cada uno de estos mecanismos de rechazo varían, la elección del mecanismo por parte del hospedador debería, consecuentemente, ser modulada por un equilibrio entre los costes y los beneficios de la respuesta. En el Capítulo 1 se investigó si el abandono del nido puede considerarse un mecanismo de rechazo de huevos en hospedadores de tamaño medio o grande. Los resultados indican que el abandono del nido no puede ser visto como una respuesta inequívoca al parasitismo de cría en el mirlo común *Turdus merula*, una potencial especie hospedadora de tamaño mediano. Por lo tanto, los estudios futuros sobre el rechazo de huevos en especies similares deberían considerar con precaución el abandono del nido como una respuesta al parasitismo de cría.

Es importante destacar que cada etapa del proceso de rechazo de huevos puede verse afectada por diferentes factores que determinarán la respuesta del hospedador. Esta tesis pone de manifiesto como diferentes rasgos del huevo parásito (la masa del huevo, el color y el tamaño) pueden afectar a etapas concretas del proceso de rechazo de huevos, incluyendo la decisión de expulsar, lo cual puede conducir a la aceptación de huevos parásitos previamente reconocidos. En el Capítulo 2 se muestra como los hospedadores no están dispuestos a asumir los potenciales costes asociados con la expulsión de un huevo ligeramente más pesado (baja motivación), lo cual resultó en decisiones de aceptación, confirmando así que el reconocimiento de huevos no es necesariamente seguido por el rechazo del huevo parásito. El capítulo 3 va un paso más allá y estudia cómo las diferentes etapas del proceso de rechazo de huevos pueden ser afectadas independientemente por diferentes características del huevo parásito. En concreto, este capítulo describe cómo el mimetismo de color dificulta el reconocimiento de los huevos parásitos (la primera etapa del proceso), dando lugar a la aceptación del huevo. Por otro lado, el tamaño del huevo parásito no parece afectar al reconocimiento, pero puede imponer restricciones mecánicas en la etapa de acción (la tercera etapa) y retrasar de esta manera la decisión de expulsar (la segunda etapa del proceso). Es importante destacar que la existencia de decisiones de aceptación implica que la tasa de rechazo de huevos parásitos no refleja necesariamente las habilidades de reconocimiento de los hospedadores, lo cual hace necesario el estudio de otras variables claramente atribuibles al reconocimiento del huevo, para así comprender completamente el proceso de rechazo. En esta tesis, el "comportamiento de tocar el huevo" que muestran los mirlos es propuesto como un indicador fiable de las capacidades de reconocimiento de huevos parásitos en esta especie, lo cual permite un estudio más detallado de las relaciones entre reconocimiento y rechazo de huevos.

Debido a su importancia en la regulación del comportamiento animal, las vías endocrinas son excelentes candidatos para seguir desentrañando los mecanismos próximos que rigen el proceso de rechazo de huevos. Sin embargo, el detallado conocimiento que existe sobre las respuestas comportamentales frente a los huevos parásitos contrasta con la limitada información disponible sobre los procesos hormonales que regulan estos comportamientos. Por lo tanto, el capítulo 4 investiga cómo los anfitriones ajustan su estado hormonal para hacer frente a los huevos parásitos, así como los potenciales efectos que estos ajustes tienen en el estado físico de los huéspedes. Nuestros resultados muestran que un huevo parásito es, desde la perspectiva hormonal, un factor estresante para los hospedadores adultos, dado que los individuos parasitados mostraron niveles basales de corticosterona significativamente más altos, y una condición corporal inferior, que los individuos no parasitados. Además, el hecho de que los niveles de prolactina no se vieran afectados sugiere que los mirlos tienden a mantener el cuidado parental incluso cuando son parasitados, lo cual puede explicar algunos comportamientos encontrados anteriormente en esta especie, como la ausencia de abandono del nido en respuesta al parasitismo experimental.

El importante papel que juega la toma de decisiones en la respuesta del

hospedador, puesto de manifiesto en los capítulos anteriores, sugiere que las respuestas plásticas en el rechazo de huevos podrían ser comunes entre las especies hospedadoras. En consecuencia, el Capítulo 5 revisa las evidencias actuales de defensas plásticas frente los huevos parásitos, demostrando que la existencia de plasticidad fenotípica en el rechazo de huevos es menos generalizada de lo que cabría esperar. Este capítulo discute los factores que podrían favorecer la evolución de plasticidad fenotípica, así como su importancia en la relación coevolutiva entre los parásitos de cría y sus hospedadores, donde las respuestas fijas parecen ser más frecuentes de lo que se sospechaba previamente, incluso en ausencia de parasitismo de cría. Precisamente, el capítulo 6 proporciona evidencias experimentales sobre el origen evolutivo de las capacidades de rechazo de huevos mostradas por especies que actualmente no son explotadas por parásitos interespecíficos de cría, como es el caso de los mirlos. Aunque algunos estudios previos han sugerido que el rechazo de huevos evolucionó en estas especies como consecuencia del parasitismo de cría intraespecífico, nuestros resultados indican que el origen más probable de estas habilidades es una explotación por parásitos interespecíficos en el pasado. Del mismo modo, la ausencia actual de parasitismo de cría interespecífico en estas especies podría deberse a sus bien desarrolladas defensas, que son mantenidas incluso después de eventos de especiación, probablemente como consecuencia de la falta de costes asociados con su mantenimiento.

Dado que se ha asumido que las hembras juegan el papel principal en el proceso de rechazo de huevos, el Capítulo 7 trata de confirmar tal suposición e investiga si hay diferencias de sexo en la respuesta del hospedador a los huevos parásitos. Esta pregunta resulta especialmente relevante en especies, como los mirlos, en los que la incubación depende exclusivamente de la hembra. Contrariamente a la teoría convencional, encontramos que los machos de mirlo son capaces de reconocer y expulsar huevos parásitos. Curiosamente, las capacidades de reconocimiento de los machos están menos desarrolladas que las de las hembras, probablemente debido a un vínculo más débil con el nido durante la etapa de incubación. La evolución del reconocimiento de huevos en machos que no incuban es un hecho de naturaleza intrigante que puede tener importantes implicaciones en la evolución de las defensas antiparasitarias dentro de las poblaciones de hospedadores.

Los resultados de los capítulos anteriores proporcionan una visión detallada del proceso de rechazo de huevos. Estos hallazgos, además del nuevo marco teórico reciente en el campo de la toma de decisiones en animales, ponen de manifiesto la necesidad de actualizar algunos términos frecuentemente usados en estudios de rechazo de huevos, así como la inclusión de nuevos conceptos en estudios futuros. De esta manera, esta tesis concluye con la propuesta, en el Capítulo 8, de una terminología actualizada que aboga por un uso coherente de términos en futuros trabajos sobre el rechazo de huevos, lo que podría contribuir al avance del conocimiento científico en el campo del parasitismo de cría.

En resumen, esta tesis aborda el estudio del rechazo de huevos como un

proceso complejo y potencialmente plástico en el cual la toma de decisiones del hospedador juega un papel clave. El contexto ambiental, las características de la puesta y las capacidades cognitivas de los hospedadores determinarán el resultado del rechazo del huevo modulando las diferentes etapas de este proceso, lo cual es probablemente mediado por determinadas rutas endocrinas. Este enfoque se complementa con una perspectiva evolutiva sobre el origen de las capacidades de rechazo de huevos en potenciales huéspedes no explotados, así como la importancia de considerar el papel de los machos en el rechazo de huevos. Esta tesis, por lo tanto, proporciona avances significativos en nuestra comprensión de las interacciones coevolutivas entre los parásitos de cría y sus hospedadores, concretamente en relación con una de las principales defensas antiparasitarias, el rechazo del huevo.

General introduction

Brood parasitism as reproductive strategy

Parental care is widely spread among birds, where one or both parents exhibit traits originated and maintained to increase the probabilities of survival of their offspring, often with costs for their own survival or reproduction (Royle et al. 2012). This successful reproductive strategy is, however, susceptible of being exploited by other individuals: brood parasites, which benefit from the parental care that other birds provide to their offspring while reducing the potential costs associated with these behaviours (Roldán and Soler 2011). Some brood parasites lay their eggs in the nest of other conspecifics (conspecific brood parasitism), a strategy which has been documented in 234 species of birds, being especially common among precocial species or those breeding colonially (Yom-Tov 2001), such as grebes, grouse and allies, waterfowl, rails, estrildid finches, swallows, starlings, and weaverbirds (Lyon and Eadie 2008). However, the best-known examples of brood parasitism come from obligate interspecific brood parasites, which never care their offspring, but lay their eggs in the nest of other species (hosts), and entirely rely on unrelated foster parents to rear their parasitic young (Rothstein 1990, Davies 2000).

Obligate brood parasitism has independently evolved seven times in birds (Spottiswoode et al. 2012) and occurs in about one hundred of species (1% of bird species) distributed in five different families (Davies 2000). Cuckoos (family Cuculidae) are probably one of the best-known group regarding brood parasitism as they include 57 species of obligate brood parasites, 54 in the Old World and 3 in the New Word (Davies 2000). Two parasitic cuckoos live in Europe: the common cuckoo *Cuculus canorus* and the great spotted cuckoo *Clamator glandarius*, both migrants (Payne 1997). Parasitism by the common cuckoo has been described in more than 100 passerine species in Europe (Moksnes and Røskaft 1995), but its main hosts in this continent are reed warblers *Acrocephalus scirpaceus* in marshlands, dunnock *Prunella modularis* and European robins *Erithacus rubecula* in woodlands, meadow pipits *Anthus pratensis* in moorlands and pied wagtail *Motacilla alba* in open farmlands (Davies 2000). The eggs of the common cuckoo are highly variable in both background colours and spotting patterns, and individual females usually lay eggs tending to match the host´s egg appearance (Brooke and Davies 1988). Cuckoo females are therefore divided into host-specific races or "gentes" with distinctive eggs that, at least in the case of blue cuckoo eggs, have been found to be under maternal inheritance (Fossøy et al. 2016). The common cuckoo is an "evictor" parasite because the newly hatched parasitic young evicts all host offspring, imposing severe fitness costs on hosts (Davies 2000, Honza et al. 2007b). On the other hand, chicks of the great spotted cuckoo usually share the nest with young of the common magpie *Pica pica* or, less frequently, the carrion crow *Corvus corone* (its secondary host), so it is a "non-evictor" parasite (Davies 2000).

Parasitic lifestyle occurs in other bird families too. For instance, family Indicatoridae includes 17 species of small parasitic birds confined to Africa and Asia: the honeyguides. These brood parasites victimize mainly hole-nesting species such as woodpeckers, barbets or hoopoes, among others; but some species also parasitize open-nesting hosts. They lay white eggs, like those of their hosts, probably reflecting a hole-nesting ancestry rather than an adaptation to parasitic habits (Davies 2000). Honeyguides are virulent brood parasites since the parasitic chick usually kills all the host´s offspring, so their nestling is raised alone in the nest (Spottiswoode et al. 2012).

The family Viduidae comprises 19 species of *Vidua* finches which victimize Estrildid finches in Africa. These brood parasites lay white eggs, like those of their hosts, and the parasitic chick is raised in the nest together with the host chicks (Davies 2000). Parasitic and host nestlings share elaborate mouth markings that could have evolved through to competition between brood mates for parental resources (Hauber and Kilner 2007). This family also includes the cuckoo finch *Anomalospiza imberbis*, an African parasite who shows spectacular polymorphysms in egg colour and patterns, as also occurs in its host species (Spottiswoode and Stevens 2010).

In the New World, 5 members of the family Icteridae, known as cowbirds, exhibit parasitic habits. Some of them, such as the brown-headed cowbird *Molothrus ater*, the bronzed cowbird *Molothrus aeneus*, or the shiny cowbird *Molothrus bonariensis*, are generalist parasites exploiting tens of hosts in North, Central and South America, respectively. Generalist cowbirds typically lay eggs dissimilar to those of their host, whose nests can be parasitized by more than one female. But cowbirds can also be specialized in a reduced number of host species, as occurs in the giant cowbird *Molothrus oryzivorus* or the screaming cowbird *Molothrus rufoaxillaris* (Davies 2000). Finally, the black-headed duck *Heteronetta atricapilla* is the only obligate brood parasite in precocial birds and inhabits in South America, where parasitize several hosts including other duck, coots, gulls or ibises (Davies 2000).

Parasitic lifestyle is therefore a sophisticated strategy allowing some birds to achieve reproductive success while reducing the costs of reproduction. The astonishing reproductive habits exhibited by brood parasites are the consequence of both ecological pressures and host defences (Davies 2000). Thereby, the evolution of brood parasitism was likely preceded by changes in the ecology and life story of ancestral species with parental care, including the colonization of new habitats, increase in migratory habits and changes in diet. Later, more elaborate parasitic strategies emerged as a result of the co-evolution between brood parasites and their hosts (Krüger and Davies 2002).

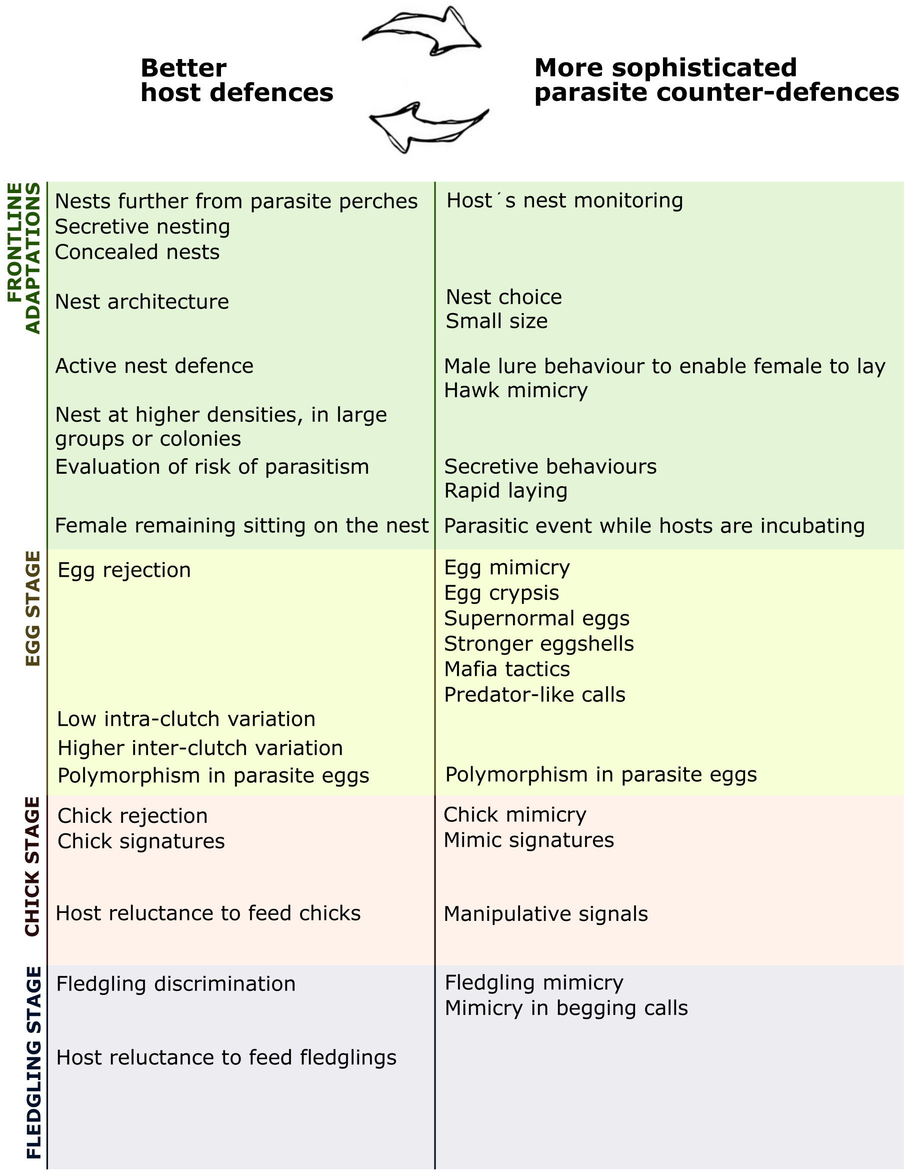
Co-evolution between brood parasites and their hosts

Brood parasitism usually imposes severe costs on hosts since the reproductive success of parasitized individuals is dramatically reduced (Davies 2000, 2011). These costs are especially important in host species exploited by highly virulent parasites such as honeyguides or cuckoos, whose parasitic young generally kill or evict the host offspring (Spottiswoode et al. 2012). It is therefore expected that this selective pressure favours the evolution of defences on hosts, such as active nest defence, egg rejection abilities or chick and fledglings recognition (Soler 2014). In turn, natural selection will select for counter-defences on parasites, which will favour the evolution of better host defences, further parasitic adaptations, and so on. The result is a "co-evolutionary arms race" in which the evolution of better host defences is followed by the evolution of more sophisticated counter-defences by parasites, leading to one of the clearest examples of co-evolution in nature (Rothstein 1990).

Host defences have traditionally been thought to be confined to the egg stage of the breeding cycle, mainly because many host species showing high egg-rejection abilities are unable to reject odd parasitic chicks (Rothstein 1990, Davies 2000). Today, it is widely accepted that host defences can operate in all stages of the breeding cycle (Figure 1), leading to the evolution of new counter-defences on the part of brood parasites (Krüger 2007, Davies 2011, Soler 2014). Some of the best-studied examples of adaptations and counter-adaptations within the framework of this co-evolutionary arms race come from cuckoos and their hosts. Successive lines of defences used by cuckoo hosts include strategies such as nest defence, egg rejection, chick recognition and fledgling abandonment (Davies 2011). On the other hand, cuckoo counter-defences comprises numerous adaptations to evade the successive lines of host defence previously described (Fig. 1), including secretive behaviours, hawk mimicry, rapid laying, egg mimicry, strong eggshells and chick mimicry, among others (Davies and Brooke 1988, Davies 2000, 2011, Krüger 2007, Soler 2014).

Undoubtedly, among the elaborate cuckoo counter-defences, egg mimicry has received most attention (e. g. (Brooke and Davies 1988, Brooker et al. 1990, Antonov et al. 2010, Spottiswoode and Stevens 2012)). Cuckoo eggs usually show an astonishing resemblance to the eggs of hosts in terms of colour, spotting patterns, shape and size. Currently, we know that many traits of parasitic eggs are the evolutionary consequence of host defences against brood parasitism (Davies 2000). Thus, experimental studies have revealed that rejection of dissimilar eggs by hosts is the selective pressure responsible for the evolution of egg mimicry in cuckoos, so host species showing more fine-tuned recognition abilities are parasitized with highly mimetic eggs, which make egg rejection harder (Brooke and Davies 1988). On the other hand, the lack of egg mimicry in cuckoo eggs usually reflects the absence of rejection abilities by host, and non-mimetic cuckoo eggs are found in the nest of species lacking rejection behaviour, such as the dunnock *Prunella modularis* (Brooke and Davies 1988). Under this coevolutionary arms race, hosts will gradually improve their rejection ability and brood parasites will improve their egg mimicry. It seems fair to think that the brood parasite will evolve such a fine degree of egg mimicry that the host will hardly detect the parasitic egg, which could lead to a stable end of the arms race (Takasu 1998; Davies 2000). However, hosts are able to adopt additional measures against the parasitic egg, such as high levels of inter-clutch egg variability, which would hinder brood parasites to mimic host eggs and facilitate recognition of parasitic eggs by hosts (Øien et al. 1995, Soler and Møller 1996, Stokke et al. 2007). This variation in the egg appearance of different host females can give rise to astonishing egg polymorphisms in both hosts and parasites (Spottiswoode and Stevens 2012). Alternatively, a reduction in intra-clutch variability of egg appearance will promote foreign egg recognition by hosts (Davies and Brooke 1989a). However, this is not so clear in all host-parasite systems and some studies have found that higher rather than low levels of intra-clutch variation may facilitate egg rejection both in great reed warblers *Acrocephalus arundinaceus* (Cherry et al. 2007) and common magpies *P*ica pica (Avilés et al. 2004), which suggest a species-specific effect of intra-clutch variation.

In addition to egg mimicry, other traits of the parasitic egg have also evolved as an adaptation to the parasitic lifestyle of cuckoos. Cuckoo eggs usually tend to match the host eggs in size, so parasitic cuckoos usually lay smaller eggs than do non-parasitic cuckoos of a similar size (Krüger and Davies 2004). Within parasitic cuckoos, evictor species are usually larger than their host species, so they lay even smaller eggs than non-evictor cuckoos, which parasitize hosts of a similar body size (Krüger and Davies 2004). Laying eggs of a similar size to those of the host is likely to improve incubation efficiency (Payne 1974, Davies and Brooke 1988) and to increase egg acceptance since some common cuckoo hosts, such as reed warblers, reject more frequently extremely large eggs than slightly large eggs (Davies and Brooke 1998, Davies 2000). Small-billed hosts will find difficult to reject large eggs, so they will use other ejection mechanisms such as puncture ejection, egg burial or nest desertion (Moksnes et al. 1991). In some host species that rely in discordance mechanisms, the relative size of eggs in the clutch is also used to modify the rejection decisions (Marchetti 2000).



	Better host defences	More sophisticated parasite counter-defences
FRONTLINE ADAPTATIONS	Nests further from parasite perches Secretive nesting Concealed nests	Host´s nest monitoring
	Nest architecture	Nest choice Small size
	Active nest defence	Male lure behaviour to enable female to lay Hawk mimicry
	Nest at higher densities, in large groups or colonies	
	Evaluation of risk of parasitism	Secretive behaviours Rapid laying
	Female remaining sitting on the nest	Parasitic event while hosts are incubating
EGG STAGE	Egg rejection	Egg mimicry Egg crypsis Supernormal eggs Stronger eggshells Mafia tactics Predator-like calls
	Low intra-clutch variation Higher inter-clutch variation Polymorphism in parasite eggs	Polymorphism in parasite eggs
CHICK STAGE	Chick rejection Chick signatures	Chick mimicry Mimic signatures
	Host reluctance to feed chicks	Manipulative signals
FLEDGLING STAGE	Fledgling discrimination	Fledgling mimicry Mimicry in begging calls
	Host reluctance to feed fledglings	

Figure 1: **Co-evolution of host defences and parasite counter-defences at different stages of the breeding cycle.**

Brood parasites have also evolved eggs with thicker shells than would be expected from their body size (Spaw and Rohwer 1987, Igic et al. 2011). Although a stronger eggshell may have evolved to prevent that parasite eggs are broken during laying (Davies 2000), the most accepted hypothesis suggests that these stronger eggshell make it more difficult for hosts to puncture and eject the parasitic egg, forcing smallest hosts to desert the nest (Moksnes et al. 1991). In addition, the more rounded shape of parasitic eggs has also been suggested to contribute to increase the puncture resistance of parasitic eggs (Picman 1989).

Finally, in addition to these detailed adaptations, there are some cuckoo strategies that are aimed at ensuring the success of parasitism but that do not necessarily lead to coevolution. These strategies comprises behaviour such as host egg removal, adequate host selection or predation of advanced stage host nests (Davies 2011).

Rejection of parasitic eggs

This thesis focuses on the rejection of parasitic eggs, which is the most widespread and effective defence used by hosts (Rothstein 1990, Davies 2000). Obviously, egg rejection implies the successful recognition of the foreign egg as the first step of a more complex process. But, how host identify the foreign egg? Two different mechanisms have been proposed to explain the recognition of parasitic eggs by hosts: (1) recognition by discordance, and (2) template-based recognition. The former, which is also the simplest mechanism for egg recognition, is based in the rejection of the less frequent (odd) egg. On the other hand, in template-based recognition, also called "true recognition", egg recognition is achieved by comparing visual features of parasitic eggs to a template of the host's own eggs, which can be innate or learned (Rothstein 1974, 1975, 1978). Once the parasitic egg has been recognized, there are two main different mechanisms by which hosts can reject the parasitic egg and elude the costs of brood parasitism: ejection of the parasitic egg and desertion of the parasitized nest. The former implies the removal of the parasitic egg from the nest, which can be done by grasping the parasitic egg with the bill (grasp-ejection) or, alternatively, the host can pierce the egg's shell, hold it by the hole (puncture-ejection) and remove it (Davies 2000).

But egg rejection is also costly given that highly mimetic parasitic eggs can make hosts eject their own eggs by mistake (recognition costs), or some own eggs may be accidentally broken during the ejection process (ejection costs)(Davies 2000). Moreover, nest desertion implies higher costs since it supposes the total loss of the current reproduction, and individuals must invest time and energy in a replacement clutch; therefore, host defences will emerge from a trade-off between costs and benefits of anti-parasitic behaviours (Krüger 2007). For instance, the relative size of the host and the parasitic egg is crucial for the election of the mechanism used by hosts to reject the parasitic egg (Rohwer and Spaw 1988, Davies and Brooke 1989b). Less costly strategies, such as egg ejection, are preferably used by medium or large-sized hosts instead of nest desertion, which is usually exhibited by small-sized hosts unable to eject the parasitic egg (Moksnes et al. 1991). Nest desertion is likely the only viable mechanism of egg rejection for small-sized hosts since their small bills limits their ability to grasp or puncture the parasitic egg (Antonov et al. 2006, 2009). Despite this, many studies on egg rejection include nest desertion as a response towards the parasitic egg even in medium or large-sized hosts, such as the common blackbird *Turdus merula* (Grim et al. 2011, Hauber et al. 2013). Given the high costs associated to nest desertion, it would be expected that medium or large-sized hosts, who could easily eject the parasitic egg, would use less costly mechanisms for egg rejection (i.e. egg ejection). In Chapter 1, the common blackbird is used as a model species to experimentally test whether nest desertion can be unequivocally considered an anti-parasitic strategy in medium-sized hosts since this response may be the result of other factors (i.e. predation risk).

When considering the important fitness costs associated to rearing a parasitic chick, specially those of high virulent brood parasites, lack of egg rejection exhibited by some host populations may result puzzling (Stokke et al. 2005). According to the “evolutionary-lag hypothesis” (Rothstein 1990, Davies 2000), acceptance of parasitic eggs might reflect an initial stage in the arms race between brood parasites and hosts as a result of a recent parasitism, so hosts would not have had enough time to evolve egg-rejection behaviours. In other cases, egg rejection may cease to be adaptive under certain circumstances, which would occur whenever costs of maintaining egg rejection are higher than costs of parasitism, as suggested by the “evolutionary-equilibrium hypothesis” (Lotem et al. 1992, 1995, Lotem and Nakamura 1998). However, egg acceptance found in some host populations may also be due to other factors such as gene flow of "acceptor alleles" from non-parasitized populations (Martínez et al. 1999, Soler et al. 1999a), successful frontline defences blocking the evolution of other defensive behaviours (Britton et al. 2007), or the existence of cryptic parasitic eggs preventing hosts to evolve egg recognition (Brooker et al. 1990, Langmore et al. 2009).

Contrary to what traditional theory assumes, absence of egg rejection might not reflect recognition failures but acceptance decisions. In some cases, certain traits of the parasitic egg can lead to the acceptance of previously recognized eggs as the result of a trade-off between benefits and costs of egg rejection (Antonov et al. 2009, Soler et al. 2012a). Thus, under certain environmental contexts, hosts might decide to accept the parasitic egg, placing host decision-making as a keystone in the egg-rejection process (Stokke et al. 2005; Moskát and Hauber 2007; Soler et al. 2012a). Egg rejection could therefore be understood as a complex process in which different stages are differentiated, namely: recognition, decision and action itself (Soler et al. 2012a). Importantly, each stage would be susceptible of being affected by specific factors such as clutch characteristics, recognition abilities of hosts, presence of conditional stimuli, or the host genotype (Stokke et al. 2005). As some characteristics of parasitic eggs could also determine the result of the egg rejection process, Chapter 2 and Chapter 3 address how specific traits of parasitic eggs, such as egg mass, size and background colour, affect the outcome of egg rejection by impacting different stages of this complex process. Importantly, some of these traits could modify the rejection decisions without affecting egg recognition. This could be the case of egg mass, which does not seem to be important for egg recognition since it is strongly correlated with egg size (Christians 2002). The study of single characteristics of parasitic eggs impacting different stages of the egg rejection process will allow us to improve our understanding on the relationship between egg recognition and egg rejection. Therefore, the first part of the present thesis (Chapters 1-3) focuses on the main factors governing host defences during the egg stage as well as, more specifically, how some characteristics of parasitic eggs may affect the outcome of the egg-rejection process.

Brood parasitism and hormones

Behavioural and morphological consequences of brood parasitism on host species are well known, and many experimental studies have focused on how parasitized individuals modulate their behaviour to respond to brood parasitism (see above). However, despite the importance of endocrine pathways in regulating both parental care and individual responses to environmental perturbations, very little is known about the endocrine mechanisms underlying the host responses to brood parasitism (Ibáñez-Álamo et al. 2012). According to previous studies, brood parasitism seems to be a hormonal stressor under certain circumstances. For example, sharing the nest with a parasitic chick induces an increase in the corticosterone levels of host´s offspring (Ibáñez-Álamo et al. 2012). Adult individuals are also affected by brood parasitism as they suffer physiological costs during the fledgling stage (Mark and Rubenstein 2013) or modify the levels of maternal androgens in their egg's yolk (Hahn et al. 2017). Surprisingly, very little is known about the impact of brood parasitism on the endocrine and physical state of hosts during incubation, as well as its effect on the hormonal responses of hosts to stress. Thus, Chapter 4 addresses these questions by assessing changes in the hormonal profiles of hosts in response to experimental egg parasitism.

The study of the proximate mechanisms behind egg rejection, including the potential role of certain hormones, will allow us to better understand host responses toward parasitic eggs. Egg rejection abilities rely on a genetic basis, as revealed by both theoretical models (Rothstein 1990, Takasu 1998a, Robert et al. 1999, Servedio and Lande 2003) and empirical evidence (Martín-Gálvez et al. 2006, 2007), but some hosts modify their responses according to the current conditions of parasitism too (see above). Sometimes, a low risk of parasitism may lead hosts to accept parasitic eggs in order to avoid the potential costs of egg rejection (Lindholm 2000, Soler et al. 2012b, Thorogood and Davies 2013). In addition, retaliation by parasites (mafia tactics) may force hosts to accept parasitic eggs (Soler et al. 1995). Therefore, under certain circumstances, costs associated to anti-parasitic defences might favour the emergence of plastic defences in host populations. The role of phenotypic plasticity in egg rejection behaviours is addressed in Chapter 5, which investigate the current evidence and evolutionary consequences of flexible host responses towards parasitic eggs.

As mentioned, the complex nature of the egg rejection process indicates that hosts recognize more eggs than will ultimately be rejected, which implies that decision-making plays a key role in the outcome of the egg rejection process. Some important concepts related to animal decision-making in several domains of behavioural ecology, such as matting, foraging and habitat selection has been reviewed and updated under the framework of cognitive phenotypes (Mendelson et al. 2016). Thus, once studied the mechanisms that underlie the egg-rejection process, some terms commonly used in egg-rejection studies will be reviewed, at the end of this thesis (Chapter 8), for their consistent use in future studies on brood parasitism according to the new findings in the field of animal decisions.

Long-term outcome of coevolutionary relationships between brood parasites and their hosts

According to the coevolutionary theory, coevolutionary cycles should be the most common outcome of the relationships between brood parasites and their hosts, involving a dynamic change in which brood parasites select the currently least defended host population (Davies and Brooke 1989a, b, Rothstein 1990, Marchetti 1992). This idea is supported by the fact that ecological interactions between brood parasites and their hosts show geographical variation resulting in the emergence of coevolutionary hotspots, populations where both the intensity of interactions and the selection of coevolved traits are more intense, as geographic mosaic models of coevolution predicts (Thompson 2005). In populations where intensity of interactions is low, costs linked to anti-parasitic defences would favour selection against the maintenance of egg recognition, facilitating the appearance of cycles. Although there are evidence supporting the existence of coevolutionary cycles, this coevolutionary outcome is absent in most brood parasite-host systems (Soler 2014). In fact, it has been found that successful resistance is a very common outcome of brood parasite-hosts relationship, which implies retention of anti-parasitic defences in absence of parasitism over long time periods (Soler 2014). Retention of defences could therefore explain why some potential host species exhibit egg rejection abilities even though they are not currently exploited by interspecific brood parasites, as is the case of Turdus thrushes. But egg rejection abilities found in blackbirds have also been proposed to be a consequence of conspecific brood parasitism (Samas et al. 2014). In Chapter 6, we experimentally test these alternative hypotheses and discuss the evolutionary origin of egg rejection abilities in the common blackbird Turdus merula.

Despite the type of brood parasitism (inter- or conspecific), the evolution of host defences could also be determined by host-associated factors, like the role played by each sex in different anti-parasitic responses. Males and females could show different cognitive abilities, which will importantly determine their roles in the host response against parasitic eggs, and ultimately affect the coevolutionary relationship between parasites and hosts (Sealy and Neudorf 1995). This issue has received little attention since it has been traditionally assumed an exclusive role of females in egg rejection in those species where only females are involved in egg incubation; on the other hand, in those host species where both sexes incubate, both females and males are expected to show rejection abilities (Soler et al. 2002). But these assumptions are mainly based in egg ejection experiments (refs), without considering egg recognition independently. Therefore, Chapter 7 investigates the sex roles in the host response of blackbirds to parasitic eggs, a species in which only females incubate (Ibáñez-Álamo and Soler 2012).

Objectives

General objectives

1. To advance the understanding of the egg-rejection process and the main (ultimate and proximate) factors that mediate in their different stages.
2. To better comprehend the endocrine mechanisms mediating anti-parasitic host responses.
3. To assess the role of phenotypic plasticity in the hosts responses against brood parasitism.
4. To elucidate the evolutionary origin of anti-parasitic defenses exhibited by potential host species that are not currently impacted by interspecific brood parasites.
5. To determine the sex roles in the hosts´ response towards parasitic eggs.

Specific objectives

1. To test whether nest desertion can be unequivocally considered a response to brood parasitism in medium-sized hosts (Chapter 1).
2. To investigate whether an egg trait that does not impact egg recognition (egg mass) can lead to the acceptance of recognized parasitic eggs (Chapter 2).
3. To explore whether specific traits of the parasitic egg, such as mimicry in size and colour, can impact independently different stages of the egg rejection process (Chapter 3).
4. To determine how the presence of a parasitic egg modifies the hormonal state of hosts, as well as its potential impact on the physical state of parasitized individuals (Chapter 4).

5. To explore the role of phenotypic plasticity in the coevolutionary relationship between brood parasites and their hosts, particularly regarding egg rejection (Chapter 5).
6. To test whether egg-rejection abilities exhibited by blackbirds are a by-product of conspecific parasitism, or whether they are the result of past coevolutionary interactions with interspecific brood parasites (Chapter 6)
7. To investigate the role played by incubating females and non-incubating males in egg recognition and ejection (Chapter 7).
8. To propose an updated terminology for its consistent use in egg-rejection studies, reflecting the complex nature of the egg rejection process as well as the recent advances in the study of animal decision (Chapter 8).

Objetivos

Objetivos generales

1. Avanzar en la comprensión del proceso de rechazo de huevos y los principales factores (próximos y últimos) que median en sus diferentes etapas.
2. Comprender mejor los mecanismos endocrinos que median las respuestas antiparasitarias de los hospedadores.
3. Evaluar el papel de la plasticidad fenotípica en las respuestas de los hospedadores frente al parasitismo de cría.
4. Dilucidar el origen evolutivo de las defensas frente al parasitismo presentes en potenciales especies hospedadoras que actualmente no son explotadas por parásitos de cría interespecíficos.
5. Determinar el papel de cada sexo en las respuestas de los hospedadores frente a los huevos parásitos.

Objetivos específicos

1. Comprobar si el abandono del nido puede considerarse una respuesta inequívoca al parasitismo de cría en hospedadores de mediano tamaño (Capitulo 1).
2. Investigar si un rasgo del huevo que no afecta al reconocimiento (la masa del huevo) puede dar lugar a la aceptación de huevos parásitos previamente reconocidos (Capítulo 2).
3. Explorar si características específicas del huevo parásito, como el mimetismo en tamaño y color, pueden influir en el rechazo del huevo afectando a diferentes etapas del proceso (Capítulo 3).

4. Determinar como la presencia de un huevo parásito modifica el estado hormonal del hospedador, así como su potencial impacto en el estado físico de los individuos parasitados (Capítulo 4).

5. Explorar el papel de la plasticidad fenotípica en las relaciones coevolutivas entre los parásitos de cría y sus hospedadores, particularmente en lo que respecta al rechazo de huevos (Capítulo 5).

6. Comprobar si las habilidades de rechazo de huevos presentes en los mirlos son un subproducto derivado del parasitismo conespecífico, o si por el contrario son el resultado de una pasada interacción coevolutiva con parásitos de cría interespecíficos (Capítulo 6).

7. Investigar el papel de las hembras incubadoras y de los machos no-incubadores en el reconocimiento y la expulsión de huevos parásitos (Capítulo 7).

8. Proponer una terminología actualizada para su consecuente uso en estudios de rechazo de huevos, reflejando la compleja naturaleza del proceso de rechazo de huevos, así como los recientes avances en el estudio de la toma de decisiones en animales (Capitulo 8).

General methodology

Study system

The common blackbird *Turdus merula* is a medium-sized passerine that belong to the family Turdidae, including 9 subspecies widely distributed throughout Europe, North Africa, and some areas of the Near East and China (Collar 2005). It is also present in Australia and New Zealand, where it was introduced in the late 19^{th} century (Thomson 1922). Blackbirds are relatively common birds, with sedentary, partially migratory or migratory habits, depending on latitude (mainly sedentary in Central and Western Europe), and occupy a wide range of habitats ranging from mountainous areas to urban habitats, although its main habitat is broadleaf, mixed and deciduous forests (Collar 2005). They are 24-29 centimetres in length, weigh 85-105 grams, and feed mainly on earthworms, insects and their larvae, as well as on fruits and seeds (Collar 2005). Males exhibit black plumage, brown legs, orange-yellow bill and a distinctive yellow eye-ring. Females are brownish in colour, with a yellowish-brown beak, lacking in the characteristic yellow eye-ring of males, and occasionally show a lighter mottling on the throat and chest (Figure 2).

In Europe, the breeding period can begin at the end of February and may last until August, varying with latitude. It is a fundamentally monogamous species, although some divorce rates have been described between seasons (19%) and within seasons (5%)(Collar 2005). Their nests, which usually show a large cup formed of dry grass and cemented with mud, are constructed by females in trees and bushes, at a variable height above ground (0.5-15 m). Blackbirds lay 2-6, usually 3-4, pale greenish-blue with pale reddish-brown spots eggs that are incubated by females for 12-15 days. Both parents feed their young during the nestling period that lasts 13-14 days. Fledglings stay with parents for 20 days after leaving the nest (Collar 2005).

Some life history traits of the common blackbird, such as its habitat, its medium body size, its open nests, and its short nestling period, make blackbirds potential hosts of the common cuckoo (Soler et al. 1999b). In fact, blackbirds has been

Figure 2: **Blackbird adults.** Blackbird male (above) visiting the nest while female (below) incubates the eggs.

described as a "potential" (Moksnes and Røskaft 1995, Martín-Vivaldi et al. 2012) or "suitable but rare" (Davies and Brooke 1989b) host of the common cuckoo Cuculus canorus in Europe. Although rarely exploited by the cuckoo, blackbirds exhibit high egg-rejection abilities (Moskát et al. 2003, Hale and Briskie 2007, Samas et al. 2011, Martín-Vivaldi et al. 2012), are aggressive against cuckoo dummies (Grim et al. 2011) and usually refuse to feed cuckoo chicks (Grim 2006, Grim et al. 2011). Given these anti-parasitic behaviours, it seems likely that blackbirds were victimized by the common cuckoo in the past but probably were victors in their coevolutionary relationship with this brood parasite, as is probably the case with other hosts who are currently not parasitized (Soler 2014). However, it has also been suggested that egg-rejection abilities of blackbirds might be a by-product of conspecific brood parasitism (Grim et al. 2011, Samas et al. 2014).

All the experimental studies included in this thesis have been carried out in a natural population of common blackbird located in the Valle de Lecrín, Granada (southern Spain: 37° 17′ N, 3° 59′ W, 580 m a.s.l.). The study site is a rural area located in the mesomediterranean belt, with high temperatures in summer and scarce annual rainfall. It is dominated by orange (Citrus sinensis) and lemon (Citrus limon) grooves, dotted with centuries-old olive (Olea europaea) trees (Figure 3). Blackbirds usually make their nests in orange and lemon trees, and exceptionally in olive trees. In the study population, the first nests are built by females in early March, and the last nestlings usually leave the nest at the end of July. In the study population, females lay 2.8 ± 0.12 eggs on average (range 2-4 eggs; Figure 4), that are incubated exclusively by females for a mean of 13.49 ± 0.29 days (Ibáñez-Álamo and Soler 2010). After hatching, blackbird nestlings stay in the nest 11.80 ± 0.32 days on average (Ibáñez-Álamo and Soler 2010). Breeding density on our study population is 2.9 pairs/ha (Ibáñez-Álamo 2010).

General field procedure

The data used in this thesis were obtained during the field seasons corresponding to the years 2012-2015. Each year, the blackbird population was monitored following a general field procedure established in 2007 (Ibáñez-Álamo and Soler 2010). The monitoring of the blackbird population was initiated a few weeks before the beginning of spring in order to locate the first nests of the season. Blackbird nests were found by an active search of the study area systematically checking all trees in an area of about 158 hectares through a systematic review of the trees present in the study area. Once a nest was found, its exact location was marked with a Global Positioning System device (Garmin Gecko 201). Blackbird nests found at the laying stage, incubation and nesting stage were tracked by periodic visits (every two days) to obtain the most important breeding parameters: laying date, clutch size, hatching success and fledgling production.

Figure 3: **Study site.** Orange trees in which blackbirds usually nest in the Valley of Lecrín, Granada (Spain).

Egg-rejection experiments

The experimental studies carried out in this thesis consisted mainly in egg rejection experiments in which blackbird nests were artificially parasitized with experimental model eggs (see Fig. 1 in Chapter 2). The nature of the experimental eggs varied depending on the question posed and the needs of the experimental design: real blackbird eggs, collected from deserted nests, were used in Chapters 1, 2, 3, 6 and 7; real house sparrow *Passer domesticus* eggs from deserted nests were used to simulate cuckoo parasitism in Chapter 6; and real common quail *Coturnix coturnix* eggs were used in Chapters 1, 3, 4 and 7 to achieve parasitic eggs slightly larger than blackbird eggs. Some of these eggs were painted with acrylic paints to obtain both mimetic and non-mimetic model eggs (see Fig. 1 in Chapter 1). Once the parasitic egg was introduced in the blackbird nest, parasitized nests were daily checked for five days to determine the ejection time for each egg-rejection test. This five-day period is widely employed in other egg-rejection experiments conducted in thrushes (Honza et al. 2005, 2007a, Grim et al. 2011, Samas et al. 2011). In each visit, both blackbird and experimental eggs were inspected to look for possible cracks or broken eggs (ejection costs) or mistakenly ejected blackbird eggs (recognition costs). Experimental eggs were considered accepted when they remained intact at the nest for five days after the experimental parasitism. Finally, nests were considered deserted when the clutch, including the experimental egg, remained cold in the nest for at least three consecutive visits (6 days). In such case, we collected all blackbird eggs and kept them in a refrigerator at 5°C for their use in subsequent tests. Each experimental egg was used only once (in only one trial) and discarded afterwards.

Video monitoring of blackbird nests

This thesis approaches the study of egg recognition by analysing several variables related to the specific behaviour of blackbirds in the presence of the parasitic egg. Unlike traditional studies that rely on rejection rates to infer the recognition abilities of hosts, the experiments included in this thesis use the touches of blackbirds to the eggs as an indicator of recognition, which has been employed in previous studies on egg recognition (Lindholm 2000, Soler et al. 2012a). Since hosts can recognize more eggs than they eventually eject (Lindholm 2000, Antonov et al. 2009, Soler et al. 2012a), the use of rejection rates as a proxy of egg-recognition may underestimate host recognition abilities. Thus, experimental and control blackbird nests were video-monitored to assess both the nest attendance and the response of parasitized individuals towards parasitic eggs. To do this, we followed the standard method used to study blackbird behaviour at the nest (Ibáñez-Álamo and Soler 2012, 2017). Just after the introduction of the parasitic egg, we placed a video camera (Panasonic HDC-SD40) near the nest (2-3 m), appropriately camouflaged between the leaves of the trees and attempting to film the entire contents of the nest. The average recording time of each nest was 2 hours. Chapter 6 includes a variant of the video-monitoring methodology since, in addition to the post-parasitism nest monitoring, the blackbird behaviour was filmed for 1.5 h before egg introduction (previous session).

For the purpose of this thesis, approximately 682 hours were filmed and processed to extract information regarding to the behaviour of both sexes in the nest, as well as their response to the parasitic egg.

Figure 4: **Blackbird clutch.** Blackbird eggs and a newly hatched chick.

Adult capture and the standardized stress protocol

Throughout Chapter 4, we investigated the effect of brood parasitism on the hormonal status of the host. For that, blackbird females were captured by using a mist net for the subsequent blood sampling. All individuals were ringed to ensure that each female was used only once. Females were released near the nest within 5-15 minutes after the second blood sampling, and all the nests were checked in the following days to make sure that our manipulation did not provoke the abandonment of nests.

In order to obtain both baseline and stress-induced hormonal levels, we collected the blood samples by following the standardize stress protocol (Wingfield 1994, Angelier et al. 2013). Within 3 min after capture, an initial blood sample was collected from the brachial vein to quantify the baseline hormonal levels. Afterwards, females were kept in an individual cloth bag and suspended off the ground. After 30 min, a second blood sample was collected from the other wing to assess the stress-induced hormonal levels. Blood samples were kept cold and were centrifuged at 4500 RCF for 3 min as soon as possible (maximum 5 hours after collection). Plasma was separated and stored at 20 °C until the hormonal assay.

Body condition

In Chapter 4, the body condition of all the females was analysed to determine the effect of the brood parasitism on the physical state of hosts, as well as the possible relation of the latter with the hormonal profile of individuals. After the blood sampling, we extracted body measurements of the females referring to body mass and tarsus length (Figure 5) to subsequently calculate the "scaled mass index" (SMI)(Peig and Green 2009, 2010) for each individual *i*:

$$SMI_i = M_i \cdot (\frac{L_0}{L_i})^b$$

M_i and L_i are the body mass and the tarsus length of individual *i*, respectively; L_0, the arithmetic mean value of tarsus length in our study population and *b*, the slope of a standardised major axis (SMA) regression of log body mass on log tarsus length. The reason for using this body condition index was that, unlike other widely used indices such as residual index, the scaled mass index considers allometry by estimating a scaling exponent, allowing to estimate the structural effects of growth on mass-length relationships (Peig and Green 2009, 2010).

Figure 5: **Body measurements.** Measure of the tarsus length of a blackbird female.

Risk parasitism manipulation

In Chapter 6, the risk of parasitism was also manipulated by placing plaster dummies (with wooden pieces) near the nests. Immediately after the experimental egg was introduced in the nest, we placed either a common cuckoo or a blackbird dummy to simulate a risk of interspecific or conspecific parasitism, respectively. In addition, as a control, some females were exposed to common turtle dove *Streptopelia turtur* dummies. Dummies, including wooden dummies, have been frequently used to simulate the risk of parasitism (Welbergen and Davies 2008, 2009). Host responses towards wooden dummies show a strong correlation with those found to taxidermic mounts (Welbergen and Davies 2009), which are similar to the host responses to life cuckoos (Duckworth 1991). The response of blackbirds towards dummies was observed for 5 min to note the potential aggressive behaviour of blackbirds against an intruder.

Hormonal assays

The impact of brood parasitism on the hormonal status of blackbird females was evaluated, in Chapter 4, by studying two different hormones: corticosterone and prolactin. Corticosterone is a hormone of crucial importance in the individual response to environmental perturbations since it is involved in the reallocation of resources from reproduction to self-maintenance (Wingfield et al. 1998, Breuner et al. 2008). On the other hand, prolactin plays a key role in the regulation of parental care in birds (Buntin 1996), and its decrease is linked to the disruption of parental care and abandonment of the nest (Sockman et al. 2006, Angelier and Chastel 2009, Ouyang et al. 2011). Corticosterone concentration were determined by radio-immunoassay (RIA) after diethyl-ether extraction (Lormée et al. 2003). To determine the plasma concentrations of prolactin, it was also used a heterologous RIA assay following the method described by (Cherel et al. 1994) and validated for blackbirds (Préault et al. 2005). Duplicate samples were run in one assay for both hormones. Hormonal assays were performed at the Centre d'Études Biologiques de Chizé (CEBC-CNRS, Villiers en Bois, France).

Chapter 1

Nest desertion cannot be considered an egg-rejection mechanism in a medium-sized host: an experimental study with the common blackbird (*Turdus merula*)

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Abstract

Two main mechanisms of egg rejection, the main defence of hosts against brood parasites, have been described: ejection and desertion. Desertion of the parasitized nest is much more costly and is usually exhibited by small-sized host species unable to remove the parasitic egg. However, nest desertion is frequently assumed to be an anti-parasite strategy even in medium or large-sized host species. This assumption should be considered with caution because: (1) large-sized hosts able to eject the parasitic egg should eject it rather than desert the nest, and (2) breeding birds may desert their nests in response to different disturbances other than brood parasitism. This problem is especially important in the common blackbird (*Turdus merula*) because this is a medium-sized species, potential host of the common cuckoo (*Cuculus canorus*), in which desertion has been frequently reported as a response to cuckoo egg models. Here, we seek to determine whether nest desertion can be considered a response unequivocally directed to the parasitic egg in medium-sized hosts using the blackbird as the study species. In an experimental study in which we have manipulated levels of mimicry and size of experimental eggs, we have

found that both colour (mimetic and non-mimetic; at least for human vision) and size (small, medium, and large) significantly affected ejection rates but not nest desertion rates. In fact, although large eggs disproportionally provoked nest desertion more frequently than did small or medium-sized eggs, cuckoo-sized parasitic eggs were not deserted allowing us to conclude that desertion is unlikely to be an adaptive response to brood parasitism at least for this species.

Introduction

Coevolutionary arms races have driven the evolution of adaptations and counter-adaptations by brood parasites and their host species. Host defences can work at any stage of the breeding cycle (pre-laying, laying, nestling, and fledging; Soler 2014). However, the most common and effective host defence against brood parasitism in order to overcome its negative effect on host fitness is the rejection of parasitic eggs laid in host nests (Johnsgard 1997, Davies 2000).

Two main egg-rejection mechanisms have been described: ejection of the parasitic egg and desertion of the parasitized nest. Egg ejection, implying the removal of the parasitic egg from the nest, can be done in two main ways: the host can grasp the parasitic egg with its bill (grasp-ejection) or, alternatively, the host can pierce the egg's shell, hold it by the hole (puncture-ejection) and remove it (Davies 2000). Egg ejection has low costs and is usually limited to medium or large-sized host species (Davies and Brooke 1989, Soler and Møller 1990, Moksnes et al. 1991, Briskie et al. 1992, Langmore et al. 2005). Desertion of the parasitized nest is a much more costly strategy because it requires the hosts looking and competing for a new suitable nest site, building a new nest, laying a replacement clutch and raising their chicks later in the season (Hoover 2003, Servedio and Hauber 2006). For this reason, nest desertion is usually exhibited mainly by small-sized host species unable to remove the parasitic egg (Rohwer and Spaw 1988, Moksnes et al. 1991, 1993, Røskaft et al. 1993, Hosoi and Rothstein 2000, Servedio and Hauber 2006, Martín-Vivaldi et al. 2002, Stokke et al. 2010, Soler et al. 2012).

Given that desertion is thought to be more costly, it could be predicted that large-sized hosts able to grasp eject or puncture the parasitic egg should eject it instead of deserting the nest. There is some empirical evidence supporting this prediction: medium and large-sized species do not desert experimentally parasitized nests (Lorenzana and Sealy 2001, Soler 1990, Underwood et al. 2004, but see Begum et al. 2012) or desert them at a low rate (Underwood et al. 2004, Peer and Rothstein 2010). Furthermore, as Peer and Rothstein (2010) found working with a large sample of common grackle (*Quiscalus quiscula*) nests, when a group of nests without experimental parasitism is left as a control for desertions, the desertion rate of experimental nests does not differ from that of control nests. This should not be surprising because, although egg ejection is considered an unambiguously specific response against brood parasitism, nest desertion has not necessarily evolved as a specific anti-parasite defence. Breeding birds may desert their nests in response to different disturbances, especially in a situation of clutch reduction (i.e. loss of one or more eggs, (Hill and Sealy 1994)), high risk of predation or partial nest predation (Székely et al. 1996, Hosoi and Rothstein 2000).

This ambiguous response to brood parasitism is not restricted to large-sized hosts, as it has also been demonstrated that desertion rate in experimentally parasitized nests is similar to desertion rates in control nests in some small-sized hosts (Hill and Sealy 1994, Kosciuch et al. 2006, Vikan et al. 2010, Soler et al. 2011, but see Rothstein 1976). Furthermore, desertions sometimes occur after successful ejections of the parasitic eggs. These post-ejection nest desertions frequently occur in small clutches, but rarely in large clutches, suggesting that they are a response to small clutches (Moskát et al. 2011). Thus, caution is needed when considering desertion as a genuine response to brood parasitism. However, although in some egg-recognition experiments cases of desertion are not considered a response to experimental parasitism (Vikan et al. 2010, Samaš et al. 2011, Soler et al. 2011, Hanley et al. 2014) or data are analysed with and without desertion cases (Underwood et al. 2004, Samaš et al. 2014), in most studies it is assumed that both egg ejection and nest desertion are anti-parasite strategies even in medium or large-sized host species that could easily eject the parasitic egg (Lotem et al. 1995, Peer and Sealy 2004, Grim et al. 2011, Begum et al. 2012, Hauber et al. 2013).

Desertion rates in egg-recognition experiments are usually low, and therefore whether or not desertions are counted as rejections may have little or no effect on a study's conclusions. However, in thrushes (*Turdus* spp.) and specifically in the common blackbird (*Turdus merula*, hereafter "blackbird"), a group of medium-sized passerine species potential hosts of the common cuckoo (*Cuculus canorus*, hereafter "cuckoo") the problem is especially important. This is because this group has frequently been used as model species in egg-recognition studies, and in most egg-recognition experiments, thrushes in general and blackbirds in particular, use both egg-ejection and nest-desertion mechanisms to reject parasitic eggs (Davies and Brooke 1989, Hale and Briskie 2007, Polačiková and Grim 2010, Grim et al. 2011, Hauber et al. 2013, Samaš et al. 2014).

Whether to consider nest-desertion as egg rejection or not is a critical point that remains controversial in understanding the function of egg-rejection behaviour in thrushes and it may bear important evolutionary implications. In this group, the desertion rate can comprise a high proportion of rejection. For instance, in blackbirds the proportion of rejections that involve desertion reaches values of up to 15% in Davies and Brooke (1989), 20-25% in Samaš et al. (2014), 32% in Moskát et al. (2003), 40% in Polačiková and Grim (2010), 67% in Hale and Briskie (2007; mimetic European cuckoo eggs in Table 1), and 80% in Hauber et al. (2013).

In most of the about a dozen different studies published on egg rejection in blackbirds, nest desertion was considered a specific response to the experimentally introduced egg (but see Samaš et al. 2011). This decision was justified because of two facts: first, nests parasitized with non-mimetic eggs have been reported to be deserted more frequently than nests parasitized with mimetic eggs (Davies and Brooke 1989); and second, control nests are deserted less frequently than experimentally parasitized ones (Grim and Honza 2001, Bártol et al. 2002). However, these facts apply mainly to small-sized host species, those having difficulties to eject the experimental egg. The study by Grim and Honza (2001) is the one usually cited to justify the inclusion of nest desertion by blackbirds as a response to parasitic eggs; however, differences between desertion rate of experimental (1 out of 6) and control nests (0 out of 17) are not significant (χ^2 = 2.96, df = 1, p = 0.09). Moskát et al. (2003) also considered a control group in their blackbird study and they found that 11 out of 43 experimentally parasitized nests with non-mimetic model eggs were deserted, while three out of 30 control nests were deserted. Differences among the two groups are not significant, either (χ^2 = 2.77, df = 1, p = 0.10).

Given the results reported in the literature, it is difficult to accept nest desertion as a frequently used mechanism of egg rejection in a large-sized potential host species because desertion is extremely costly in terms of both time and energy. Thus, whether desertion by medium or large-sized host species can be considered a response against brood parasitism needs to be resolved. This is the aim of this paper. Using the blackbird as the study species, we seek to determine, first, whether nest desertion can be considered a response unequivocally directed to the parasitic cuckoo-sized egg; and, second, whether a parasitic egg which is larger and thus more difficult to eject would provoke nest desertion more frequently than cuckoo-sized eggs.

We designed an experimental study in which we filmed egg-rejection behaviour in parasitized nests and also a control group in which we installed the camera but did not introduce any experimental egg. In the experimental groups, we manipulated the two characteristics affecting nest desertion: the level of mimicry (at least for human vision), as Davies and Brooke (1989) found, blackbirds should desert more frequently nests parasitized with non-mimetic than nests parasitized with mimetic eggs (Prediction 1); and egg size (Marchetti et al. 2000, Guigueno et al. 2014), given that large eggs would be more difficult and costly to eject and so that they would be more prone to provoke nest desertion (Prediction 2). Our experimental manipulations enabled us to test another three more specific predictions. On the one hand, in the event that one nest is parasitized by a highly mimetic egg, if the host decides to reject because of unusually high egg variability, but cannot be sure which egg is parasitic, and if the cost of renesting is low, it might be optimal for the host to desert the nest instead of ejecting an egg. Given that the cost of renesting is low in our blackbird population (low clutch size and up to 3 clutches per season; Ibáñez-Alamo and Soler 2010), we predict that the desertion rate in nests parasitized with mimetic medium-sized eggs should be higher than in control nests (Prediction 3). On the other hand, considering the above discussion on the effect of experimental egg size on rejection behaviour, we predict that cuckoo-sized eggs should be easily ejected and thus desertion rate should not be higher than in control nests (Prediction 4). Finally, in a nest parasitized by a large non-mimetic egg, if the host attempts to reject but cannot because of the large size of the experimental egg or if ejection attempts would involve excessive time with a high likelihood of breaking host eggs, it would be more advantageous to desert the nest. Thus, we predict that large non-mimetic eggs should be deserted more frequently than control nests (Prediction 5).

Methods

Study species

The blackbird is a potential host species of the common cuckoo that is extremely rarely parasitized (Grim et al. 2011), probably because it presents a number of effective defences that prevent common cuckoo parasitism. Specifically, blackbirds are highly aggressive against adult cuckoos, present a high rejection rate of alien eggs and are reluctant to feed even lone cuckoo nestlings experimentally introduced in their nests (Grim 2006, Grim et al. 2011).

The blackbird has frequently been used as a model species in egg-recognition experiments, and detailed information has been compiled on the response of this potential host species to parasitic eggs experimentally introduced in their nests. The rejection rate of non-mimetic eggs (usually completely painted blue) has been reported in about a dozen different studies and it is highly variable, ranging between 53.3% and 73% (Polačiková and Grim 2010, Grim et al. 2011, Samaš et al. 2011, 2014). In New Zealand, where blackbirds were introduced in the 19^{th} century, a high rejection rate of non-mimetic eggs has also been reported (62%, Samaš et al. 2014, 83.9%, Hale and Briskie 2007). However, a rejection rate of 100% of non-mimetic eggs has been reported in two European areas (Moskát et al. 2003, Martín-Vivaldi et al. 2012). The rejection rate of mimetic eggs has been studied in fewer publications, but it has also been shown to be highly variable, ranging between 0% and 71% (Moskát et al. 2003, Hale and Briskie 2007, Grim et al. 2011, Samaš et al. 2011, 2014, Martín-Vivaldi et al. 2012). Nevertheless, this is not surprising given that the level of mimetism of experimental eggs has also been highly variable (Grim 2005): spotted eggs (Moskát et al. 2003, Grim et al. 2011, Martín-Vivaldi et al. 2012) or conspecific egg models (Moskát et al. 2003, Hale and Briskie 2007, Samaš et al. 2011).

Study site and general methods

We carried out this study in the Valley of Lecrín (southern Spain 36° 56′ N, 3° 33′ W; 580 m.a.s.l.), from mid-March to early June 2012. The study area is dominated by orange orchards, in which blackbirds usually nest. See Ibáñez-Álamo and Soler (2010) for a detailed description of the population and the study area.

We actively searched for blackbird nests throughout the breeding season from early March to the end of June. Once a nest was located, we checked it using a mirror mounted on a pole to determine the contents. If the nest was at the building stage, we visited it every three days until the first eggs appeared.

Experimental eggs were introduced into the nests during the laying stage (with a minimum of two eggs laid) or during the incubation period. We used nests in both stages because previous studies have shown that blackbirds reject experimental eggs at similar rates in both laying and incubation stages (Davies and Brooke 1989, Honza et al. 2005, Polačiková and Grim 2010, Grim et al. 2011). In a few nests the eggs hatched before the five-day time interval considered to calculate ejection rate (see below) and they were not included in the analyses.

Experimental design

We created six different treatments by varying two features in the experimental eggs: degree of mimicry (two levels) and size (three levels) (Fig. 1). We used mimetic and non-mimetic (at least for human vision; see Fig. 1) experimental eggs of three different sizes in comparison with blackbird egg size: (i) small: house sparrow (*Passer domesticus*) eggs (20.8 ± 0.4 x 15.4 ± 0.2 mm; N = 10); (ii) medium: blackbird eggs (30.3 ± 0.9 x 21.0 ± 0.2 mm; N = 10); and (iii) large: common quail (*Coturnix coturnix*) eggs (32.8 ± 0.4 x 25.7 ± 0.2 mm; N = 10). The size of sparrow eggs is similar to that of real cuckoo eggs (Martín-Vivaldi et al. 2002), but the size of common quail eggs is considerably larger. We included such a large egg (width 18% or 37% longer than host or real cuckoo eggs, respectively [see Martín-Vivaldi et al. 2002]) in order to confront blackbirds with eggs that are difficult to grasp or eject, a situation similar to that occurring in small host species. We created a seventh group (a control group) for which we followed the same experimental procedure as for the others (visit frequency, nest checking, filming procedure, etc.; see below) except for the fact that no egg was introduced into their nests. Each nest was assigned randomly to one of these seven groups. Mimetic eggs were painted with two different acrylic paints: blue-green (background) and light brown (spots). We used several eggs taken from abandoned nests of the same population as models to reproduce the exact colour of both paints. For this, a specialized company (Copingra Pinturas) used a laser scanner to determine the ground colour of the eggs and automatically produce exactly that colour for us to paint experimental eggs. Non-mimetic eggs were painted with a very different (red) acrylic paint to be sure they do not at all resemble to blackbird eggs. Non-mimetic eggs of a red colour have been frequently used in egg-recognition experiments in which it has been demonstrated that red eggs are good non-mimetic egg models (e.g. Soler and Møller 1990, Soler et al. 1999, Avilés et al. 2004, Martín-Vivaldi et al. 2012).

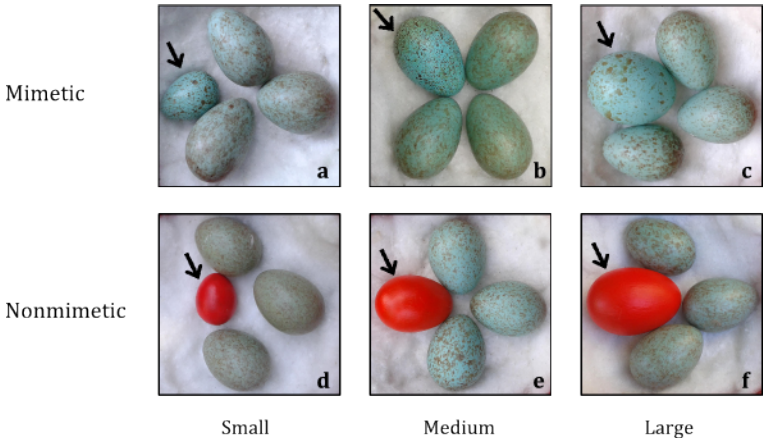

Figure 1: **Pictures of the six experimental treatments used in our study showing the variation in colour (mimetic or non-mimetic) and size (small, medium, and large) of the introduced eggs.** The figure also shows the natural variation in egg traits for the blackbird population studied. The arrow marks the model egg.

We used natural and fresh eggs, which exclude the possibility that unsuccessful attempts to puncture hard model eggs could increase the costs of rejection and/or provoke clutch desertion (Martín-Vivaldi et al. 2002). House sparrow and blackbird eggs were collected from previously failed clutches and kept in a refrigerator, but never for more than one week. House sparrow eggs were obtained from a captive population kept in a large outdoor aviary located at the Faculty of Sciences of the University of Granada (detailed information about this population and captivity conditions can be found in Soler et al. 2011). Fresh quail eggs were bought in the market. In all cases fresh eggs were painted the day before being placed in the experimental nest in order to decrease the potential effect of paint's odour on egg rejection (Soler et al. in press). Just after the introduction of the experimental egg, we filmed the response of blackbirds for the following two hours by using a Panasonic HDC-SD40 video camera placed near the nest (1.5 - 2.5 m) following a standardized procedure previously used in other studies of the incubation behaviour of this species (e.g. Ibáñez-Álamo and Soler 2012).

Data collection and statistical analyses

If the experimental egg was still in the nest after the first two recording hours, we checked it after 24 hours and continued this procedure for the following five days. At each nest visit, including control nests, we carefully inspected all eggs (introduced or not) looking for cracks in order to quantify ejection costs (number of broken eggs while trying to eject the experimental egg) and examining host eggs in order to determine whether any blackbird egg had mistakenly been broken (rejection costs) or ejected (recognition errors).

We considered the experimental egg to have been accepted when it remained intact and warm in the nest for five days. In this case we removed the experimental egg from the nest and finished the trial. We used five days as a good time interval for our trials because it is within the time interval considered in other egg-rejection experiments performed in thrushes (between 4 and 6; Honza et al. 2005, 2007, Hale and Briskie 2007, Polačiková and Grim 2010, Grim et al. 2011, Samaš et al. 2011). If the experimental egg disappeared within this five-day time interval while the other eggs were intact and warm in the nest, we assumed that the parents had ejected the egg, ending the trial. Finally, we considered a nest deserted when the clutch, including the experimental egg, remained cold in the nest for at least three consecutive days. In this case, we collected all eggs and kept them in a refrigerator at 5ºC until their use on the subsequent days. Each experimental egg was used only once (in one trial) and discarded afterwards.

The recordings were viewed using a KM Player 3.5 Plus in order to determine whether the experimental egg was ejected by grasping or by puncturing it.

We used Log-Linear Analysis of Frequency Tables to determine the effect of the colour of introduced eggs, egg size, and our treatment (interaction between degree of mimicry and egg size) in desertion and ejection rates. We originally tried to use a Generalized Linear Model (GLZ) to control for other potential variables such as clutch size, but this proved unfeasible given the heterogeneity in the response variables among groups (some being uni-modal; see Fig. 2). Nevertheless, date could influence desertion or ejection rates in species such as the blackbird that can re-nest relatively easily (see above). Log-linear analyses accept only discrete variables; therefore, date, which is a continuous variable, could not be included in our log-linear models. Thus we ran two independent GLZ fitted to a binomial distribution in order to explore the potential effect of date on desertion and ejection rates. Date did not affect ejection (GLZ binomial W = 0.000003, df = 1, p = 0.99) or desertion (GLZ binomial W = 0.45, df = 1, p = 0.50). All analyses were made using Statistica 7.0 (Statsoft). Values reported are means ± SE.

Ethical notes

The filming of adult behaviour with respect to the experimental egg did not exert any negative effect on blackbird egg hatchability relative to control nests. None of the 73 nests in which the birds were confronted with an experimental egg and none of the 21 control nests were abandoned because of camera installation (i.e. during or soon after the two hours of filming). This experimental study complies with the current laws of Spain. Research was conducted according to relevant national (Real

Decreto 1201/2005, de 10 de Octubre) and regional (permissions provided yearly by la Consejería de Agricultura, Pesca y Medio Ambiente de la Junta de Andalucía) guidelines.

Results

We completed our experimental treatments in 94 blackbird nests. We found significant differences in relation to the ejection rate among our treatments (χ^2 = 27.44, df = 5, p < 0.0001; Fig. 2). We also found a significant effect of colour in ejection rate (χ^2 = 6.58, df = 1, p = 0.01) indicating that non-mimetic eggs were ejected more frequently than mimetic eggs. The size of introduced eggs had a significant effect by itself (χ^2 = 23.12, df = 2, p < 0.0001) in that the larger the egg the lower probability of ejection.

None of the eggs (introduced or not) presented cracks (i.e. ejection costs = 0) and none of the host eggs had been mistakenly ejected (i.e. recognition costs = 0). In all filmed ejection events (N = 14) blackbirds ejected the experimental egg by grasping it in their bills.

We found no significant differences among our treatments for desertion rate (χ^2 = 6.98, df = 6, p = 0.32; Fig. 2). This implies that blackbirds did not desert their nests in any of the experimental groups significantly more frequently than those of the control group. Considered as a whole, experimental nests were not deserted more frequently than control nests were (χ^2 = 0.62, df = 1, p = 0.43). Furthermore, neither of the two general predictions derived from the idea that nest desertion is a response to experimental parasitism in blackbirds was supported. First, non-mimetic eggs were not more frequently deserted than mimetic ones (χ^2 = 0.002, df = 1, p = 0.97), and in fact in the medium-sized egg group the trend was the opposite (Fig. 2). Second, size did not affect the desertion rate, indicating that large eggs were not more frequently deserted than medium or small eggs were (χ^2 = 3.34, df = 2, p = 0.19).

Regarding the other three predictions, Prediction 3 was not supported, as experimental nests parasitized with a medium-sized mimetic egg (thus decreasing the probability of recognition of the parasitic egg) were not more deserted than control nests (χ^2 = 2.08, df = 1, p = 0.15). On the other hand, the desertion rate was no higher in experimental nests with small eggs than in control nests, fitting Prediction 4 (χ^2 = 0.63, df = 1, p = 0.43). Finally, the rationale behind Prediction 5 regarding large eggs was partially supported by the marginally significant differences found in the desertion rate between nests with large non-mimetic eggs and controls (χ^2 = 3.09, df = 1, p = 0.08).

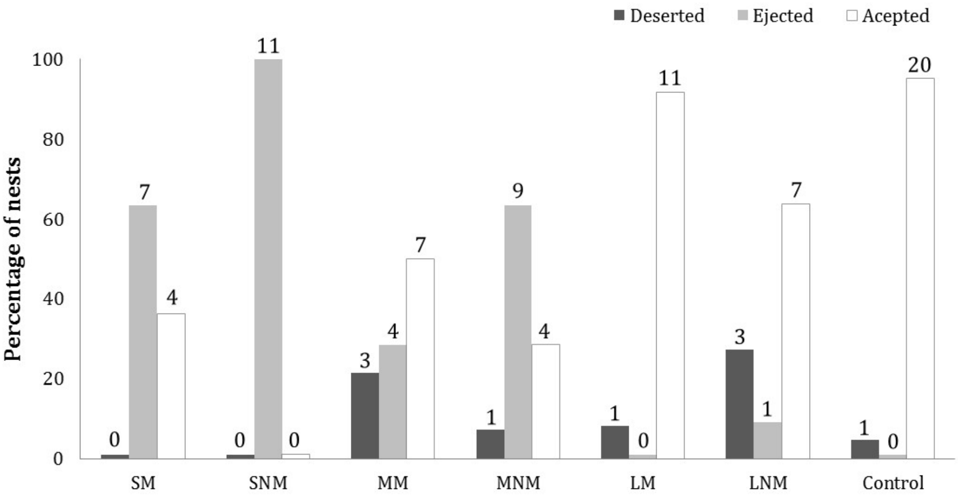

Figure 2: **Desertion, ejection and acceptance rates for each experimental treatment:** small mimetic (SM), small non-mimetic (SNM), medium mimetic (MM), medium non-mimetic (MNM), large mimetic (LM), large non-mimetic (LNM) and control. Sample sizes for each treatment are shown on the top of each column.

Discussion

Egg ejection

Previously published rejection rates in blackbirds are highly variable (see values and references above). Ejection rates found in our egg-recognition experiments are also highly variable, ranging between 0% and 100%, depending on the experimental treatment (Fig. 2). Both colour (i.e. mimetic and non-mimetic) and size (i.e. small, medium, and large) significantly affected ejection rate. We have previously found that non-mimetic eggs are more frequently ejected than mimetic ones, in agreement with previously published information for blackbirds (Martín-Vivaldi et al. 2002, Hale and Briskie 2007, Samaš et al. 2011) and for most host species in general (Davies 2000). Small eggs were also more frequently ejected than medium or large eggs. Blackbirds are grasp-ejectors (Davies and Brooke 1989, Moksnes et al. 1991, Soler et al. 2002, Honza et al. 2005, this study), and thus this result is likely related to the difficulty of grasp-ejecting the experimental egg, which is considerably more difficult in the case of large eggs (see Video 1 in Supplementary Material), than in the cases of small or medium eggs (see Videos 2 and 3 in Supplementary Material respectively). However, despite the difficulty of grasp ejection, the cost of ejecting experimental eggs was zero, in agreement with what is expected in larger hosts (Reeve 1989, Martín-Vivaldi et al. 2012).

Nest desertion

Traditionally it is assumed that nest desertion is only a response to parasitism by small-sized host species unable to eject the large parasitic egg, while large species are more likely to eject it (Rohwer and Spaw 1988, Moksnes et al. 1991, Røskaft et al. 1993, Hosoi and Rothstein 2000, Martín-Vivaldi et al. 2002, Peer and Sealy 2004, Soler et al. 2012). Desertion by small-sized hosts is assumed to be triggered by the fact that a small bill prevents egg ejection by grasping the parasitic egg and/or by piercing the eggshell (Rohwer and Spaw 1988, Servedio and Hauber 2006). This is true of most host species in which desertion has frequently been reported, such as meadow pipits (*Anthus pratensis*; Moksnes et al. 1993), *Phylloscopus* warblers (Moksnes et al. 1991, Stokke et al. 2010, Martín-Vivaldi et al. 2012), and yellow warblers (*Dendroica petechia*; Guigueno and Sealy 2010). Cases of desertion can be especially frequent when puncturing host species are tested in egg-recognition experiments using artificial egg models that are difficult or impossible to be pierced (Martín-Vivaldi et al. 2002, Antonov et al. 2006). However, the blackbird cannot be considered a small-sized host of the common cuckoo, and, although experiments were made using impenetrable egg models in some of the published studies, blackbirds, like most thrushes species, are grasp ejectors (see above) and should be able to easily eject the experimental cuckoo-sized model egg given that such eggs are small relative to the blackbird's bill (Martín-Vivaldi et al. 2002).

In agreement with this, in our study, experimental nests were not more frequently deserted than control nests, and our treatments did not affect the desertion rate (Fig. 2). Furthermore, neither of the two general predictions derived from the idea that nest desertion is a response to experimental parasitism in blackbirds were supported. These results suggest that nest desertion cannot be considered a response to brood parasitism in egg-recognition experiments in blackbirds, and probably other medium or large-sized hosts. This is also true for some small-sized species. For instance, in clay-coloured sparrows (*Spizella pallida*), which often desert nests parasitized by parasitic brown-headed cowbirds (*Molothrus ater*), it was demonstrated that desertion was the consequence of clutch reduction provoked by female cowbirds rather than to brood parasitism itself (Hill and Sealy 1994). However, in cedar waxwings (*Bombycilla cedrorum*), another host species of brown-headed cowbirds, it was demonstrated that desertion was a response to the experimental treatment, because experimentally parasitized nests were deserted at a higher rate than control nests (Rothstein 1976).

Our treatment concerning egg colour did not provoke any significant effect on desertion rate (Predictions 1 and 3). This result agrees with that found in marsh warblers (*Acrocephalus palustris*), where highly mimetic eggs (painted conspecific, and conspecific) were never deserted (Antonov et al. 2006). However, recently, Samaš et al. (2014) have found that nest desertion rate in blackbirds was significantly higher in experimental nests parasitized with conspecific eggs than in control nests, while in nests parasitized with non-mimetic eggs this difference between desertion rates did not increase.

Our results concerning the relationship between egg size and nest desertion (Predictions 2, 4 and 5), have to be considered with caution. Our results indicate that egg size, in general, does not affect desertion rate, and this is clear for small and medium-sized eggs. Thus, we can conclude that cuckoo-sized parasitic eggs are not deserted as a response to brood parasitism. Notably, desertion rates of zero in blackbird egg-recognition experiments have been reported invariably in cases in which experimental eggs were smaller than host eggs (Grim and Honza 2001, Hale and Briskie 2007, Martín-Vivaldi et al. 2012). All these results considered together suggest that blackbird nests experimentally parasitized with small eggs are more rarely deserted than those parasitized with large eggs.

When considering larger eggs, it is remarkable that small eggs, either mimetic or non-mimetic, were in no case deserted (0%), while large eggs were deserted in 17.4% of the nests. The difference between these percentages is almost significant (small vs. large eggs: $\chi^2 = 3.39$, df = 1, p = 0.065). Furthermore, nests parasitized with large non-mimetic eggs were marginally significantly more deserted than control nests, and, if we consider only rejections, three of four rejections of large non-mimetic eggs were by desertion as opposed to zero of 11 of small non-mimetic eggs, a significant difference (Fisher exact test two tailed test, p = 0.043). Thus, we can conclude that large eggs provoke nest desertion more frequently than do small (i.e. cuckoo-sized eggs) or medium-sized eggs. This higher desertion rate of nests parasitized with large eggs could be explained if large eggs are more difficult and more costly to eject than small eggs. This possibility is supported because large-sized eggs seemed more difficult to eject than small or medium-sized eggs (see videos in Supplementary Material) and the fact that the only large egg that was ejected was smaller (31.61 x 24.54 mm) than the average (32.8 x 25.7 mm). We found no broken eggs in experimentally parasitized nests, but perhaps a high probability of ejection attempts resulting in broken host eggs make renesting less costly than ejection, at least in blackbirds (see above). Antonov et al. (2006) also found that marsh warblers only deserted nests experimentally parasitized with eggs more difficult to eject (i.e. artificial models and cuckoo eggs).

However, even considering the potential effect of large eggs, the tendency for blackbirds to desert large eggs more frequently than small eggs cannot be considered as an unequivocal response to experimental brood parasitism for two reasons: first, control nests are also deserted to some extent (Bártol et al. 2002, Moskát et al. 2003, Samaš et al. 2011, this study); and second, nests experimentally parasitized with small or medium-sized eggs (those used in previous studies), which can be easily grasp-ejected, are sometimes also deserted (Grim et al. 2011).

Therefore, an important question is why blackbirds sometimes desert their nests when they are experimentally parasitized with eggs that can be easily grasp-ejected. In host species of parasitic cowbirds (*Molothrus* spp.), it has been suggested that they are more prone to desert experimentally parasitized nests because egg rejection requires egg discrimination while nest desertion only requires suspecting that the nest has been parasitized (Rothstein 1975, Sealy and Lorenzana 1998, Hosoi and Rothstein 2000). This is not true of the blackbird, given that this species has the ability to clearly discriminate foreign non-mimetic eggs. Another response to this question is based on the fact that it is well known that nest desertion is not a specific response evolved against brood parasitism. Life-history theory predicts that offspring desertion would be adaptive for parents when investment in the current brood implies major costs on parent's future fitness compared to expected benefits (Winkler 1991, Székely et al. 1996). In fact, nest desertion in birds has been reported to be a response to disturbance, the sight of predators near the nest, and partial egg predation (Hill and Sealy 1994, 1996, Székely et al. 1996, Ackerman and Eadie 2003). Brood parasites are also commonly nest predators (Hill and Sealy 1994, 1996, Soler et al. 1995, Davies 2000, Hoover and Robinson 2007) and common cuckoos usually remove one or two host eggs at laying (Wyllie 1981, Davies 2000). Thus, nest desertion in current or historical hosts of brood parasites could be the response to any of these reasons. Indeed, in several host species it has been found that desertion can be triggered by egg loss (Sealy 1995, Hill and Sealy 1996, Moskat and Honza 2002, Antonov et al. 2006, Kosciuch et al. 2006).

Furthermore, desertion frequency may be influenced also by some host life-history traits, for instance when clutch size is small and when cost of re-nesting is relatively low (Servedio and Hauber 2006). It bears noting that the blackbird is a species that suffers very high predation risk (Tomialojc 1995, Hatchwell et al. 1996, Ibáñez-Álamo and Soler 2010, Samaš et al. 2013), lays a small clutch , and has a high re-nesting rate (Cramp 1988). All these three characteristics could predispose blackbirds to nest desertion under different situations (i.e. brood parasitism, nest predation risk or researchers' disturbance). In conclusion, blackbird desertion of their nests when parasitized with cuckoo-sized experimental eggs cannot be attributed exclusively to brood parasitism and should not be considered as such in egg-recognition experiments in general. This behaviour seems to be the response to a number of stimuli acting additively, which are favoured by some life-history traits of this species. In light of our results, we strongly recommend that egg-recognition experiments, especially in medium- or large-sized hosts, should include a control group in order to determine whether or not desertion is a genuine response to experimental parasitism for that particular species.

Supplementary material

Video 1: A blackbird female looking at the eggs (a large-sized experimental egg). She touches the egg several times but finally she does not eject it.
Video 2: Ejection of a small-sized egg
Video 3: Ejection of a medium-sized egg

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Chapter 2

Could a factor that does not affect egg recognition influence the decision of rejection?

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Abstract

Rejection of the parasitic egg is the most important defence of hosts against brood parasites. However, this response is variable among and within species, and egg discrimination is not always followed by egg rejection. Low risk of parasitism and high risk of rejection costs may lead to the acceptance of the parasitic egg even if it has been previously recognized. The main aim of this paper is to answer a relevant question: can a single egg trait provoke the acceptance of an experimental egg previously recognized as foreign? Increased egg mass should hamper the ejection of an egg that has been discriminated because ejection of a heavy egg may imply higher rejection costs for hosts. We have tested this prediction by experimentally parasitizing natural nests of Common Blackbirds (*Turdus merula*) with non-mimetic model eggs of different mass (heavy, normal-weight, and light) while controlling for potential confounding factors such as egg size and colour. Our results showed that blackbirds more frequently accepted heavy eggs, even when previously recognized. This differential acceptance may be related to insufficient motivation to assume the higher costs that the ejection of a heavy egg could impose.

Introduction

Avian brood parasites lay their eggs in the nest of other species (hosts), thereby imposing high fitness costs on parasitized individuals because the newly hatched parasitic chick is usually better at competing for food or can directly eliminate competition by ejecting or killing the host offspring (Rothstein 1990, Davies 2000). Accordingly, there is strong selective pressure on hosts to evolve defences in response to parasitism, such as nest defence or egg discrimination and rejection (Davies and Brooke 1988, Moksnes and Røskaft 1989). In response to this, brood parasites have evolved counter-defences to defeat host strategies, such as secretive laying or egg mimicry, resulting in a coevolutionary arms race between brood parasites and hosts (Soler 2014), which makes the brood parasite-host system one of the clearest examples of coevolution found in nature (Rothstein 1990, Davies 2000).

Rejection of parasitic eggs is the most widespread defence among hosts (Rothstein 1990, Davies 2000, Soler and Soler 2000) and consequently it has been the focus of many studies in the field of avian host-brood parasite interactions. Egg-recognition experiments have led to a breakthrough in our understanding of the mechanisms involved in egg recognition and rejection and their evolutionary implications, e.g. (Brooke and Davies 1988, Davies and Brooke 1988, Moksnes and Røskaft 1989, Soler and Møller 1990, Spottiswoode and Stevens 2010, 2011, Avilés et al. 2011, Soler et al. 2011, 2012). Ejection of the parasitic egg and desertion of the parasitized nest are the two main mechanisms of egg rejection used by hosts. Egg ejection implies removing the parasitic egg from the nest, either by directly grasping the egg with the bill (grasp ejection) or, alternatively, by making a hole in the egg's shell, gripping it and flying away with the egg (puncture ejection) (Davies 2000).

Most previous studies in brood parasitism have focused in the action component of the rejection process and most of them do not distinguish between recognition and rejection components. However, the rejection process is a complex behaviour in which the previous discrimination of the parasitic egg by hosts is the first necessary step for the process to result in successful ejection (Soler et al. 2012). Egg recognition is closely linked to the evolutionary background of hosts and their adaptations to brood parasitism (Langmore et al. 2005), but hosts also need to have the genetic basis and the cognitive abilities necessary to be able to "decide" whether to reject the parasitic egg or not (Martín-Gálvez et al. 2006). In terms of colour and design, it is well known that hosts are more likely to reject non-mimetic eggs than mimetic ones (Brooke and Davies 1988, Davies and Brooke 1988, Stokke et al. 1999, Lahti and Lahti 2002, Soler et al. 2015), which selects for egg mimicry (Davies and Brooke 1988) provoking many cuckoo species to lay eggs with such successful mimicry that the human eye is unable to distinguish them from the host eggs (Cherry and Bennett 2001, Stoddard and Stevens 2011).

Although the rejection of foreign eggs is the most widespread defence among actual and potential hosts of the common cuckoo (*Cuculus canorus*), there is a considerable variation in the response to parasitic eggs both among and within species (Davies and Brooke 1989, Moksnes et al. 1990, Álvarez 1996, Moskát and Hauber 2007, Stokke et al. 2008). Intra-specific variation in this respect indicates that genetic support and cognitive abilities involved in the recognition process are not the only factors responsible for the rejection of parasite eggs. Indeed, several studies have shown that egg discrimination is not necessarily followed by egg rejection (Lyon 2003, Underwood and Sealy 2006, Moskát and Hauber 2007, Antonov et al. 2008, 2009, Soler et al. 2012), signifying that rejection is a plastic response, as previously suggested by theoretical models as well as empirical data (Davies et al. 1996, Hauber et al. 2006, Stokke et al. 2008, Svennungsen and Holen 2010, Soler et al. 2012).

Given that during rejection events hosts may accidentally break their own eggs (ejection costs) or eject a host egg by mistake (recognition costs) (Davies and Brooke 1988), the decision of whether to reject or not depends on a trade-off between costs and motivation, so that greater incentive is needed to assume higher costs (Soler et al. 2012). Previous studies have suggested that the acceptance of recognized eggs is due to the impossibility of puncturing the parasitic egg by small-sized hosts, indicating that some egg traits (i.e. eggshell strength) may critically influence the acceptance of a previously recognized egg (Antonov et al. 2009). However, very little is known about the role of other egg traits that could affect the egg-rejection decision. In this regard, egg mass is one of these poorly studied traits. Egg mass and egg size are highly correlated (normally $r^2 > 0.8$; Christians 2002), so the importance of egg mass by itself in the ejection of the parasitic egg is not usually considered. It is possible that an egg with greater mass will be more difficult for grasp ejectors to take away from the nest due to greater difficulty in egg handling. While egg mimicry and egg size relative to the host- egg size can affect egg recognition, it is likely that egg mass alone cannot. The high degree of correlation between size and mass (see above) leads recognition to rely on egg size instead of the mass. Furthermore, open-nesting hosts (such as blackbirds) normally rely on visual cues to discriminate against parasitic eggs instead of tactile cues, which are preferably used by some dome-nesting hosts where the lower light availability make it harder to visually identify the alien egg (Langmore et al. 2005).

The main aim of this study was to experimentally determine whether a single egg trait (i.e. egg mass), which does not affect egg recognition, can influence egg rejection decisions in the Common Blackbird (*Turdus merula*), a grasp-ejector species. For this purpose, we designed an experimental study in which blackbird nests were parasitized with non-mimetic blackbird eggs with exactly the same colour and size, but different weight, thus ensuring that introduced eggs were easily recognized (i.e. non-mimetic eggs). We predict that (1) non-mimetic model eggs will be equally recognized by blackbirds regardless of egg mass; (2) differences in egg mass will influence the acceptance of model eggs previously recognized:

blackbirds will find it harder to eject heavy eggs and, as a result, they will be more likely to be accepted than lighter eggs; and (3) the ejection of a heavy egg will imply ejection costs due to greater handling difficulties.

Methods

Study site and species

We conducted our study in the Valley of Lecrín (southern Spain, 36º56′ N, 3º 33′ W; 580 m a.s.l.) during May-June 2013. The study area is dominated by orange groves, in which blackbirds usually nest. For a detailed description of the population, see (Ibáñez-Álamo and Soler 2010).

The common blackbird is a medium-sized passerine frequently used as a model species in egg-recognition experiments, e.g. (Polačiková and Grim 2010, Grim et al. 2011, Samas et al. 2011, 2014, Martín-Vivaldi et al. 2012, Soler et al. 2015), which is a potential host of the common cuckoo but is not actually parasitized. However, blackbirds are able to recognize and eject alien eggs artificially introduced in their nests (Soler et al. 2002, 2015, Grim et al. 2011, this study). We actively searched for blackbird nests in the study area throughout the breeding season from early May to the end of June. Once a nest was located, we checked it to determine its content and status, and visited it every two days to record laying date and clutch size.

Model eggs

Non-mimetic model eggs used in our study were similar in size and colour but not in weight. We used natural eggs (from naturally deserted nests) whose weight was modified by adding a mixture of sand and silicone. Collected eggs were emptied through a small hole (< 3 mm) made in the eggshell using a needle and then filled with the sand-silicone mixture. We varied the sand-silicone ratio in the egg to achieve different weights in completely filled eggs. Finally, the small hole was sealed with silicone. Three different treatments were created in terms of egg mass: (i) light eggs (mean ± SE; 3.0 ± 0.1 g; N = 14): on average 55% lighter than natural blackbird eggs, similar to egg mass of Common Cuckoos that parasitize rufous-tailed scrub robins, *Cercotrichas galactotes* (Álvarez 2003); (ii) normal-weight eggs: the same weight as the blackbird eggs (mean ± SE; 6.7 ± 0.2 g; N = 14.); and (iii) heavy eggs: on average 49% heavier than natural blackbirds eggs (mean ± SE; 10.0 ± 0.3 g; N = 16). This increase in egg mass was not disproportionately large in comparison with that of our population (mean ± SE; 6.95 ± 0.05 g; range min = 5.5 g, max = 7.8 g; N = 154) or other populations of this species (7.58 ± 0.03 g; N = 772; range not provided; Magrath 1992). In fact, similar differences are found in egg mass for some host-parasite systems in which the parasitic egg can be 48.5%

heavier than that of host egg (Payne 1977). We created an additional group of nests (hereafter natural group) for which we followed the same procedure in relation to visits, checking, and filming but which were not experimentally parasitized.

Model eggs were coloured red with acrylic paint the day before being placed in the experimental nest. We used non-mimetic eggs to ensure that model eggs were easily recognized and to standardize the colour of all manipulated eggs. Model eggs painted red have been frequently used in egg-recognition experiments in which it has been demonstrated that they are good non-mimetic model eggs (Soler and Møller 1990, Soler et al. 1999, 2015, Avilés et al. 2004, Martín-Vivaldi et al. 2012). Each model egg was used only in one trial and discarded afterwards.

Experimental design

Blackbird nests were experimentally parasitized by using non-mimetic model eggs of different weights (see above). Model eggs were introduced into the nests during the laying (minimum of two eggs laid) or incubation stages (Fig. 1). Previous studies have shown that blackbirds reject experimental eggs at similar rates in both the laying and the incubation stages, e.g. (Davies and Brooke 1989, Honza et al. 2005, Polačiková and Grim 2010, Grim et al. 2011). Each nest was assigned randomly to one of the three egg mass treatments (light, normal-weight or heavy eggs; see above).

A video camera (Panasonic HDC-SD40) was placed near the nest (1.5 – 2.5 m) just after the introduction of the model egg in order to film the response of blackbirds to the model egg for the following two hours. We used a standardized procedure previously used in other studies of the incubation behaviour of this species e.g. (Ibáñez-Álamo and Soler 2012, Soler et al. 2015). For this species, the placement of a camera near the nest does not affect their behaviour in relation to egg-introduction experiments (Soler et al. 2015).

We checked for the introduced egg in the nest after the two recording hours. If the model egg was in the nest, we checked it again after 24 h and continued this procedure for the following five days. This (and the analysis of recordings) enabled us to determine the ejection time of each experimental egg. When the model egg disappeared, we assigned the ejection time considering that the ejection occurred between the last two visits adding 12 hours to the time (in hours) of the last visit in which the introduced model egg was still present. If the egg remained intact in the nest for five days after its introduction, we considered it to have been accepted. In each visit, we inspected all eggs (host and experimental), looking for ejection costs (cracks or broken eggs) and recognition costs (one or more blackbird eggs mistakenly ejected). We used five days for our trials following the procedure used in other egg-rejection experiments conducted in thrushes, e.g. (Honza et al. 2005, 2007, Polačiková and Grim 2010, Grim et al. 2011, Samas et al. 2011, Soler et al. 2015).

Figure 1: **Photo of a blackbird nest containing three natural eggs parasitized with a normal-weight non-mimetic egg.**

The recordings were visualized using the KM Player 3.5 Plus software. We compiled information on egg-recognition behaviour by analysing different variables of recordings: (1) first contact touches (number of times the female touched the eggs from their arrival to the nest until she sat on the nest for the first visit), (2) first contact touches per visit (similar to the previous variable but for the complete filmed period -2 hours-, corrected by the number of visits) and (3) incubation touches (number of times the female touched the eggs while incubating per hour). Female touches have been used in several studies of parasitism as a measure of recognition of parasitic egg, e.g. (Antonov et al. 2009, Soler et al. 2012, 2015). In this study, we used differences in female touches between non-mimetic and natural eggs as an indicator of recognition.

Statistical procedures

We differentiated between two different ejection variables: (i) immediate ejection: female response against the parasitic egg within the two hours after parasitism and (ii) long-term ejection: female response to the parasitic eggs considering the whole experimental period (five days). To assess the effect of our treatment for each of these two types of ejection variables, we used generalized linear models (binomial error and logit link function). Recognition variables were analysed using generalized linear models (poisson error and log link function). Differences between levels were compared using the *multcomp* R package for generalized linear models. All analyses were performed using R version 3.1.1.

Ethical Note

The filming of adults did not negatively affect blackbird egg hatchability relative to natural nests and none of the nests used in this study was deserted. This research was conducted according to national (Real Decreto 1201/2005, de 10 de Octubre) and regional (permissions provided yearly by Consejería de Medio Ambiente de la Junta de Andalucía) guidelines.

Results

We performed our experiment in 58 blackbird nests. Four of them could not be used in our long-term ejection analyses because they were predated before the end of the trial (the fifth day after the egg introduction).

Regarding egg recognition, we found no significant differences among the tree non-mimetic model eggs for first contact touches (χ^2 = 0.91, df = 2, p = 0.6, N = 44; Table 1) or first contact touches per visit (χ^2 = 0.06, df = 2, p = 1.0, N = 44; Table 1). Furthermore, the weight of the model egg did not significantly influence the number of touches during the incubation (χ^2 = 3.03, df = 2, p = 0.2, N = 44; Table 1). These results allow us to assume that the three types of model eggs were equally recognized regardless of weight. Model eggs (heavy, light, and normal-weight eggs) taken together were significantly more pecked by females than their own blackbird eggs (natural eggs) for the first contact after being away from the nest both during the first visit (z = -4.485, p < 0.001, N = 58; Table 1) and considering all visits together (number of touches per visit, z = -4.055, p < 0.001, N = 58; Table 1). We also found differences for female touches during incubation between model and natural eggs (z = 12.38, p < 0.001, N = 58; Table 1) indicating that natural eggs were more touched during the incubation than model eggs.

Table 1: **Mean ± SE of female recognition touches for the first visit, touches per visit, and during incubation in the three non-mimetic model eggs (light, normal-weight, and heavy eggs) and natural blackbird eggs.**

Treatment	IBP N	First contact touches	First contact touches per visit	Incubation touches
Light	14	2.57 ± 0.58	2.71 ± 0.45	33.00 ± 10.18
Normal-weight	14	3.14 ± 0.98	2.57 ± 0.82	33.01 ± 7.66
Heavy	16	2.69 ± 0.63	2.69 ± 0.67	29.99 ± 5.01
Natural	14	0.43 ± 0.29	0.57 ± 0.23	55.48 ± 23.66

Considering the immediate ejection rate, we found a significant effect of our treatment in the immediate ejection rate (χ^2 = 9.58, df = 2p = 0.01, N = 44). None of the heavy eggs was ejected during the filming period (within the first two hours). In contrast, 36.5% of light eggs and 28.6% of normal-weight eggs were ejected during this period. These data seem to indicate that heavy eggs are harder to eject from the outset.

We found significant differences among our three treatments of parasitized nests for long-term ejection rate (χ^2 = 10.41, df = 2, p = 0.01, N = 40; Fig. 2). As we predicted, the main effect of our treatment in the ejection rate was due to heavy eggs. Thus, the ejection rate was significantly lower when blackbird nests were parasitized with heavy eggs than in those cases containing normal-weight (Tukey HSD: p = 0.046) or light ones (Tukey HSD: p = 0.02). No differences in long-term ejection rate were found between normal-weight nests and those parasitized with light eggs (Tukey HSD: p = 0.9).

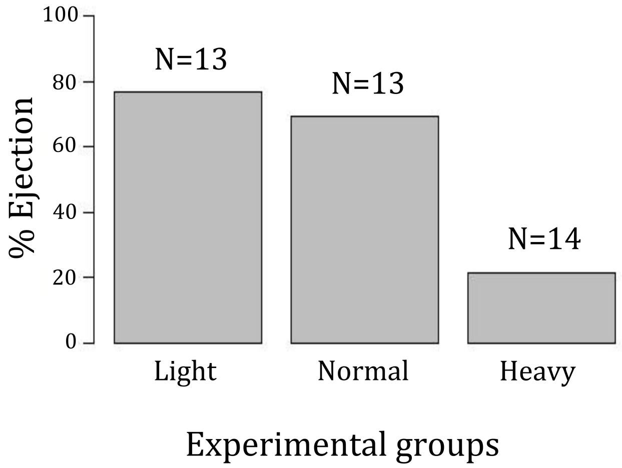

Figure 2: **Ejection rate for each experimental treatment: light, normal-weight, and heavy eggs.** Sample sizes for each treatment are shown at the top of each column.

Filmed ejection events (N = 9) showed that blackbirds ejected the experimental egg by grasping it with their bills in all cases. None of the eggs (experimental or natural) presented cracks and none of the host eggs were mistakenly ejected, indicating that there were no recognition or ejection costs.

Discussion

Our results show that experimental eggs introduced into the blackbird nests were recognized by the blackbirds as foreign eggs and this recognition was not affected by the egg mass (Table 1). Despite this, heavy eggs were more frequently accepted than the other two experimental egg types (Fig. 2), indicating that egg mass critically affects egg rejection. This work supports previous studies highlighting that hosts may recognize more eggs than they finally reject (Lyon 2003, 2007, Underwood and Sealy 2006, Moskát and Hauber 2007, Antonov et al. 2008, 2009, Soler et al. 2012).

Egg rejection is a complex process in which three phases can be considered: recognition, decision whether to reject or not, and action, (see Fig. 6 in (Soler et al. 2012)). If egg recognition does not occur the outcome of parasitism is acceptance of the parasitic egg. Red model eggs used in our study seem to be easily recognized, as indicated by the fact that blackbirds pecked non-mimetic model eggs more frequently than their own (Table 1) in accordance with previous results (Soler et al. 2015). Incubation touches differed between models and natural eggs, thus model eggs were pecked less frequently during incubation than natural eggs. Likely, this variable is not a good proxy to quantify egg recognition because incubation touches are related to egg turning and, thus, inherently related to the incubation process (New 1957, Deeming 1989, Finger 1992). According to our results, model eggs were recognized from the first visit to the nest and they were less turned than natural eggs during incubation. Furthermore, first contact touches for the first visit and per visit of the experimental red egg did not differ among all the three experimental groups (light, normal-weight, and heavy), suggesting that experimental eggs were equally recognized, regardless of their weight. As expected, these results confirm that other cues (mainly visual cues such as colour patterns) rather than egg mass are critical for hosts to differentiate parasitic eggs, especially in open nests (Brooke and Davies 1988, Davies and Brooke 1988, Stokke et al. 1999, Lahti and Lahti 2002, Langmore et al. 2005). However, although model eggs were recognized at similar rates, light and normal-weight eggs were ejected at higher rates than were heavy eggs (Fig. 2), this constituting the first experimental demonstration that a trait which does not affect recognition (eggs mass) may lead to the decision of acceptance instead of ejection.

Several studies have shown that egg discrimination is not always followed by egg rejection (see references above). Antonov et al. (2009) suggested that the acceptance of previously pecked eggs in eastern olivaceuos warblers *Hippolais pallida* was due to the impossibility of puncturing the parasitic egg by this small-sized host. However, other studies have suggested that egg rejection has to be considered a conditional process in which, once the alien egg has been recognized, the individual host response against the parasitic egg may be modulated by the perceived risk of parasitism and potential rejection costs (Brooke et al. 1998, Soler et al. 1999, 2012, Lindholm and Thomas 2000, Stokke et al. 2008). Soler et al. (2012) demonstrated that the frequent cases of discrimination without ejection of experimental eggs found in the rufous-tailed scrub robin, a common host of the common cuckoo in southern Spain, were not due to the impossibility of ejecting the experimental egg. That study showed that when scrub robins were parasitized with soft eggs (real house sparrow eggs), 80% of the pecked eggs were not ejected, either. Thus, discrimination without ejection was not the consequence of the physical inability of birds to puncture-eject them, but the consequence of a decision against ejecting due to low motivation.

Clearly, in the case of blackbirds confronted to heavy model eggs, discriminated eggs that were not ejected was not because of the impossibility of ejection because the eggs were of the same size as the ones of the other two experimental groups and so the mechanical difficulty of grasping them was the same. Although a greater egg mass makes it difficult to handle the experimental egg, blackbirds are capable of ejecting heavy eggs (Fig. 2). Furthermore, recordings did not show failed ejection attempts (i.e. eggs that fall from the female bill while trying to eject them). Instead, blackbirds immediately recognize the introduced model egg and they pecked it softly before starting the incubation. Just as previously suggested by Soler et al. (2012), higher motivation to eject is required for the host to take on higher costs and continue the rejection process. In the scrub robin, soft pecking was suggested to constitute tentative testing behaviour and that it was not part of the recognition process because only the experimental egg was pecked, indicating recognition. Presumably, touching the eggs by blackbirds has the same function once the egg is recognized.

Blackbirds had no problem ejecting either normal-weight or light eggs by grasping them with their bills. In fact, 36.5% of light and 28.6% of normal-weight eggs were ejected during the first two hours after parasitism but no heavy egg was ejected during this period. These trends were confirmed regarding the long-term ejection rates as only 21.4% of heavy eggs were ejected. One explanation for these results could be that a greater mass limits the handling of the egg and makes it more difficult (but still possible) to be removed from the nest. Accordingly, hard work would be needed to eject a heavy egg from the nest and this may imply a higher risk of breaking one or more of their own eggs, so more motivation would be required to assume these higher costs of ejecting a heavier egg. In our study, none of the blackbird eggs presented cracks or disappeared from the nest. As shown in the recordings, this may be due to females slightly checking the parasitic egg with their bill before attempting to eject it. These soft checks were in no case failed attempts to expel the egg, which indicates that acceptance of a heavy egg is due to a decision not to assume the potential cost of ejecting a heavy egg. This may indicate that females that decide to eject the heavy egg are those in good physical condition and thus capable of ejecting the egg without breaking any of their own eggs. The females that decide not to eject the heavy egg would be the ones that, after testing (i.e. touching and moving) the egg, regard the risk of destroying one or more of their own eggs to be high and therefore decide against rejection.

In conclusion, blackbirds accepted some previously recognized heavy eggs, probably because their motivation was not high enough to assume the possible higher ejection costs of ejecting a heavy egg. These results highlight the importance of distinguishing recognition and rejection in brood-parasitism studies and considering the conditional component of the host response.

Supplementary material

S1 Table. Summary of female responses to experimental parasitism (S1A Table) and number of touches used to determine recognition of experimental eggs (S1B Table).

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Chapter 3

Relationships between egg recognition and egg ejection in a grasp ejector species

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Abstract

Brood parasitism frequently leads to a total loss of host fitness, which selects for the evolution of defensive traits in host species. Experimental studies have demonstrated that recognition and rejection of the parasite egg is the most common and efficient defence used by host species. Egg-recognition experiments have advanced our knowledge of the evolutionary and coevolutionary implications of egg recognition and rejection. However, our understanding of the proximate mechanisms underlying both processes remains poor. Egg rejection is a complex behavioural process consisting of three stages: egg recognition, the decision whether or not to reject the putative parasitic egg and the act of ejection itself. We have used the blackbird (*Turdus merula*) as a model species to explore the relationship between egg recognition and the act of egg ejection. We have manipulated the two main characteristics of parasitic eggs affecting egg ejection in this grasp-ejector species: the degree of colour mimicry (mimetic and non-mimetic, which mainly affects the egg-recognition stage of the egg-rejection process) and egg size (small, medium and large, which affects the decision to eject), while maintaining a control group of non-parasitized nests. The behaviour of the female when confronted with an experimental egg was filmed using a video camera. Our results show that egg touching is an indication of egg recognition and demonstrate that blackbirds recognized (i.e., touched) non-mimetic experimental eggs significantly more than mimetic eggs. However, twenty per cent of the experimental eggs were touched but not subsequently ejected, which confirms that egg recognition does not necessarily mean egg ejection and that accepting parasitic eggs, at least sometimes, is the consequence of acceptance decisions. Regarding proximate mechanisms, our results show that the delay in egg ejection is not only due to recognition problems as usually suggested, given that experimental eggs are not touched significantly more often. Thus, the delay in egg ejection is mainly the consequence of a delay in the decision to eject, probably triggered by mechanical constraints imposed by eggs that are harder to eject (i.e. larger). Our results offer important information on the relationships between recognition and ejection and contribute to a better understanding of host defences against brood parasites.

Introduction

Avian brood parasites impose strong selection for evolution of defensive traits on their hosts because brood parasitism frequently leads to a total loss of host fitness. This is the case in the common cuckoo (*Cuculus canorus*, hereafter "cuckoo") given that the cuckoo chick usually ejects all host offspring from the nest (Honza et al. 2007a). Therefore, many hosts have evolved defences, which can function at any stage of the breeding cycle (pre-laying, laying, nestling and fledging; (Soler 2014)). The most common and efficient defence used by hosts is recognition and ejection of the parasitic egg (Rothstein 1990, Davies 2000, Soler and Soler 2000). Many experimental egg-recognition studies have led to major advances in the study of co-evolution (Brooke and Davies 1988, Davies and Brooke 1988, 1989a, b, Moksnes and Røskaft 1989, Soler and Møller 1990, Soler et al. 2001).

Egg recognition experiments are the most efficient tool used in the study of the relationships between brood parasites and their hosts. Pioneer naturalists began to study egg discrimination by hosts by introducing alien eggs into their nests as early as the beginning of the nineteenth century (Sealy and Underwood 2012). In the 1970s the first properly designed experiments (Rothstein 1970, in (Sealy and Underwood 2012)) provided the foundation for future studies (Alvarez et al. 1976, Davies and Brooke 1988, 1989a, Higuchi 1989, Moksnes and Røskaft 1989, Cruz and Wiley 1989, Soler 1990, Sealy 1992). Numerous studies involving egg recognition experiments have been performed since then (see Appendix in (Soler 2014)). Aided by technological discoveries, these have enormously advanced our knowledge on host responses to brood parasitism. These advances have elucidated not only the evolutionary and coevolutionary implications of egg recognition and rejection, but also the mechanisms involved in the evolution of this ability that many host species display.

Most studies of egg-recognition behaviour have only dealt with the act of rejection (Moskát and Hauber 2007). However, egg rejection is a complex behavioural process (Hauber and Sherman 2001, Soler et al. 2012a) consisting of at least three stages: first, the host has to recognize the parasitic egg; second, it has to decide whether or not to reject the putative parasitic egg, and third, the act of ejection itself must take place (Soler et al. 2012a). Very little is yet known regarding the cognitive processes leading to egg rejection, although the use of cameras in egg-recognition experiments at the beginning of the present century (Soler et al. 2002) has provided key information that has expanded our knowledge of the proximate mechanisms responsible for egg recognition and rejection decisions (Soler et al. 2002, 2012a, Underwood and Sealy 2006, Moskát and Hauber 2007, Antonov et al. 2008a, 2009).

The timing of egg ejection is highly variable both among and within host species. Some individuals may eject a foreign egg the same day that it is introduced into the nest (even immediately, within minutes or even seconds), while others delay their response for several days (Rothstein 1976, Sealy and Neudorf 1995, Soler et al. 2002, Honza et al. 2005, 2007b, Antonov et al. 2008a, Požgayová et al. 2011). Information on rejection latencies could provide important insights for understanding the proximate mechanisms responsible for egg recognition and rejection decisions. For instance, delayed ejection of the parasitic egg can be provoked by perceptual problems affecting the recognition and decision making processes (Antonov et al. 2008a, Požgayová et al. 2011). On the other hand, delayed rejection is not always caused by recognition problems. Although several studies have provided evidence demonstrating that recognition precedes rejection (Soler et al. 2002, Underwood and Sealy 2006, Moskát and Hauber 2007, Antonov et al. 2008a), in certain circumstances hosts may decide not to eject the parasitic egg, even if it has been recognized (Antonov et al. 2009, Guigueno and Sealy 2012, Soler et al. 2012a). Recently, it has been shown that the acceptance of parasitic eggs is not exclusively linked to recognition errors, but egg rejection can also be interrupted at later stages of the process such as during the ejection process itself (Ruiz-Raya et al. 2015).

The blackbird (*Turdus merula*) has been frequently used as a model species in egg-recognition experiments (Hale and Briskie 2007, Polačiková and Grim 2010, Grim et al. 2011, Samas et al. 2011, Martín-Vivaldi et al. 2013, Ruiz-Raya et al. 2015, Soler et al. 2015). We have detailed knowledge of the response of blackbirds to experimentally introduced parasitic eggs, but not regarding the proximate mechanisms and cognitive processes associated with egg recognition and rejection decisions. For instance, the ejection latency, the interval before egg ejection, has previously been studied in this species (2 and 2.5 days for non-mimetic and mimetic eggs, respectively), showing that the degree of mimicry did not have a significant effect on the timing of rejection decisions (Grim et al. 2011). Recently, Ruiz-Raya et al. (2015) showed in this same species that a trait that does not affect egg recognition (egg mass) hampered the ejection of the parasitic egg suggesting that the act of ejection may be limited by mechanical constraints. Thus, an understanding how different egg traits can influence the host response, including the latency of that response, by affecting different stages of the rejection process is essential given that these may have important implications for the evolutionary relationships between brood parasites and their hosts.

Here we expand previous findings on the acceptance of parasitic eggs (see above) by performing an experimental study using blackbirds as the model species. The blackbird is a grasp-ejector, using the beak to hold and remove eggs. We manipulated two characteristics of the parasitic egg that can affect specific components of the egg rejection process: (a) the degree of colour mimicry, related mainly to the first (recognition) stage; and (b) egg size, which should affect mainly the last stage (the act) of the egg rejection process in grasp ejectors (such as blackbirds, (Davies and Brooke 1989a, Soler et al. 2002, 2015)). We video-recorded female behaviour in both experimental and control nests in order to quantify different parameters of the female response to experimentally introduced eggs.

We predicted that: (1) the latency to ejection will be longer for larger eggs because, even if they are recognized, egg volume should be critical for grasp ejectors, given that they have to pick the egg up with their beak. This prediction contrasts with the situation in puncture-ejector species that pierce eggs to hold them, in which the time to ejection is mainly determined by shell thickness rather than egg volume (Antonov et al. 2008a, Požgayová et al. 2011). (2) Latency to ejection will be longer for mimetic eggs in comparison with non-mimetic eggs. Usually, non-mimetic eggs are ejected sooner than mimetic ((Lotem et al. 1995, Underwood and Sealy 2002, Honza et al. 2004, 2005, Antonov et al. 2008a), but see (Grim et al. 2011)), probably because of the greater recognition difficulties involving mimetic eggs (Rodríguez-Gironés and Lotem 1999, Antonov et al. 2008a). (3a) Experimentally introduced eggs that are recognized should be ejected. This prediction is based on two pieces of evidence. First, grasp-ejection is a nearly cost free method of rejection (Davies and Brooke 1989a, Soler et al. 2015); and second, consistency in egg-rejection behaviour in blackbirds is very high (Grim et al. 2011). Alternatively, (3b) some experimental eggs that are recognized will be accepted. This prediction is based on the the recent demonstration that blackbirds sometimes recognize experimental eggs but do not eject them (Ruiz-Raya et al. 2015). For this last prediction, we assumed that egg touching can be considered a good proxy of egg recognition (see Material and Methods). Pecking or "touching" of foreign eggs is a frequent behaviour shown by hosts when confronted with an experimental egg, and it has been considered to indicate recognition, even if ejection does not occur

(Soler et al. 2002, 2012a, Underwood and Sealy 2006, Antonov et al. 2008a, 2009, Ruiz-Raya et al. 2015).

Methods

Ethics Statement

Research has been conducted according to relevant Spanish national (Real Decreto 1201/2005, de 10 de Octubre) and regional guidelines. All necessary permits were obtained from the "Consejería de Medio Ambiente y Ordenación del Territorio de la Junta de Andalucía", Spain. Approval for this study was not required according to Spanish law since it is not a laboratory study in which experimental animals have to be surgically manipulated and/or euthanized. Our study area is unprotected private land, whose owners allowed us to work on their properties. This study did not involve endangered or protected species.

Experimental design and data collection

We conducted this study in the Lecrín valley (Southern Spain 36° 56′ N, 3° 33′ W). The study area is dominated by orange groves. See (Ibáñez-Álamo and Soler 2010) for a detailed description of the blackbird population.

We actively searched for blackbird nests in the study area throughout the breeding season from early March to the end of June 2012. Once a nest was located, we checked it to determine its contents. If the nest was found at the building stage we visited it every three days until the first egg appeared.

Our experiment consisted of introducing an experimental egg into nests during the laying stage (after at least two eggs were laid) or during the incubation period. We chose to use both stages because several studies indicate that breeding stage does not affect egg rejection rate in this species (Davies and Brooke 1989a, Honza et al. 2005, Polačiková and Grim 2010, Grim et al. 2011).

We created six different treatments by combining two features in the model eggs, which allowed us to affect two stages of the egg-rejection process directly (see predictions). In particular, we manipulated (a) the degree of mimicry in terms of colour and (b) the egg size (see Fig. 1 in (Soler et al. 2015)). We used natural fresh eggs painted to be mimetic or non-mimetic and of three different sizes relative to blackbird egg size: (i) small: house sparrow (*Passer domesticus*) eggs, (ii) medium: blackbird eggs and (iii) large: common quail (*Coturnix coturnix*) eggs. Sparrow eggs are similar in size to cuckoo eggs (Martín-Vivaldi et al. 2002), whereas common quail eggs are considerably bigger (Soler et al. 2015) and allowed us to examine the ejection behaviour of blackbirds when confronted with eggs that were very difficult to grasp-eject, a situation similar to that encountered by small-sized host species. See (Ruiz-Raya et al. 2015) for detailed information on the masses of the three egg types. We created a seventh group (control) for which we followed the same experimental procedure as for the others (visit frequency, clutch manipulation, filming procedure, etc.; see below) except that no egg was introduced into the nests. Each nest was assigned randomly to one of these seven groups. More detailed information regarding the experimental design, egg painting and the egg models used can be found in (Soler et al. 2015).

We placed a Panasonic HDC-SD40 video camera near the nest (1.5–2.5 m) to film female activity at the nest for the two hours immediately following the introduction of the experimental egg. During the egg stage, blackbird behaviour is not influenced by placing a camera near the nest (Ibáñez-Álamo and Soler 2012, Ruiz-Raya et al. 2015, Soler et al. 2015). We successfully filmed the behaviour of blackbird females in a total of 106 nests.

After the two recording hours, we checked for the presence of the introduced egg in the nest in order to determine the "immediate ejection rate". If the model egg was still in the nest after the first two recording hours, we checked again after 24 hours and daily for the following five days to determine ejection latency: the interval until ejection, and the long-term ejection rate or egg acceptance. The results for the long-term ejection rate have been previously published (Soler et al. 2015) and are not included in the Results section of this paper; however, they have been used in the "Time to ejection" and "Relationships between recognition and ejection" subsections, topics that were not studied in the earlier paper (Soler et al. 2015). We considered the experimental egg to have been accepted when it remained in the nest for five days. If it disappeared during this five-day interval (see (Soler et al. 2015) for more details about the choice of this five-day period) while the remaining eggs were intact and warm, we assumed the parents had ejected the egg, finishing the trial. In such cases, we assigned an ejection interval considering that ejection had occurred between the last two visits, adding 12 hours to the time (in hours) of the last visit in which the introduced egg was still present. Eggs from deserted nests were collected and kept in a refrigerator at 5ºC for use on subsequent days. Each experimental egg was only used once and then discarded.

The recordings were viewed using a 3.5 Plus KM Player. Each recording was carefully examined to extract information on the following variables for each nest: (1) first-contact touches during the first visit (the number of times the female touches the eggs before settling on the nest during her first return to the nest during the experimental period), (2) first-contact touches per visit (similar to variable 1 but taking all visits together and corrected by the number of visits), (3) incubation touches (the number of times the incubating female touches the eggs corrected by the time spent in the nest), and (4) the egg inspection time for all visits combined.

Relationships between egg touches and egg recognition

Weak pecking or touching of foreign eggs by hosts is probably a tactile method that allows birds to gather information about the identity of an egg (own or foreign), its state of incubation and/or the potential costs of ejecting it (Soler et al. 2012a). Blackbird females "touch" experimental eggs so weakly (see S1 Video) that it is very difficult to distinguish this behaviour from touches performed to move their own eggs during incubation, unless the beak is clearly visible when touching the eggs. A recent study has found that sometimes eggs in control nests are also "touched" (Ruiz-Raya et al. 2015). For this reason we have called this behaviour in this species egg-touching instead of egg-pecking. However, we consider that egg-touching is also an indication of recognition in blackbirds because egg-touching is much more frequent in experimental than in control nests (Ruiz-Raya et al. 2015), as also found in the present study, (2.57 ± 0.45 touches per visit in experimental nests; 0.71 ± 0.23 touches per visit in control nests). Also, another blackbird study found that the number of touches in a nest did not vary after the introduction of a conspecific (i.e. highly mimetic) egg, whereas they increased significantly following the introduction of a non-mimetic egg (Ruiz-Raya et al. 2016). Thus, to study the relationships between recognition and ejection we have assumed that egg-touching indicates recognition and, to be conservative, we considered an experimental egg to have been recognized (even if it was not ejected) when it was touched more frequently than in 95% of control nests (i.e. percentile 95).

Statistical analyses

We used generalized linear models (GLM) in order to test the effect of our treatment, i.e. the interaction between degree of colour mimicry and egg size, on immediate ejection rate (binomial error and logit link function) and the interval to ejection (Gamma error and log link function). We built our maximal model by including the following predictors: egg colour, egg size, clutch size, their interactions and date. During model simplification, non-significant terms were dropped and models were fitted by using different link functions. Akaike's Information Criterion (AIC) was used to evaluate the resulting models. Following Zuur et al. (2009), we performed Zero-affected negative binomial models (ZANB or hurdle) by using *pscl* (R package v.1.4.9 (Jackman 2015)) in order to cope with zero-inflation and overdispersion in our egg recognition variables. For these variables, the significance of effects was assessed from likelihood ratio tests (LRT) for nested models by using *lmtest* (R package v.0.9-34 (Zeileis and Hothorn 2002)). Differences in first-contact touches between the first and last visits were assessed by means of generalized linear mixed models (GLMM) including female identity as the random factor while considering zero-inflation. For that, we used the *glmmADMB* package (v. 0.8.3.3 (Skaug et al. 2016)) and then Wald tests were generated by using the *car* package (Fox and Weisberg 2011). Post-hoc analyses of interactions were performed by using the *phia* package (v. 0.2-1 (De Rosario-Martinez 2015)). We did not include large eggs in the analysis of ejection latency since there was no variation in their response (only one large non-mimetic egg was ejected). We also performed correlation analyses in order to identify some possible relationships between ejection latency and three different variables: inspection time, first-contact touches on first visit and first-contact touches per visit. Values provided are means ± SE. All analyses were performed using R version 3.2.3 (R Core Team 2015). Data used in this paper are included in S1 Table.

Results

Immediate ejection

We managed to film female behaviour when confronted with an experimental egg in 85 experimental and 21 control blackbird nests. In 13 of the filmed nests the experimental egg was ejected, always by the female, during the two hours of filming (immediate ejection). Colour mimicry had a significant effect on the immediate ejection rate (χ^2 = 14.84, df = 1; $p < 0.001$). Thus, non-mimetic experimental eggs were ejected significantly more often during the two first hours than mimetic experimental eggs. Size also had a significant effect on immediate ejection (χ^2 = 7.37; df = 2; p = 0.03) as small experimental eggs were ejected significantly more often than large eggs (z = 2.15, p = 0.03). No differences were found between medium and large (z = 1.28, p = 0.20) or between medium and small experimental eggs (z = 1.24, p = 0.22). Clutch size, date and all interactions between predictors did not significantly affect the immediate ejection rate (all p-values > 0.1).

Ejection latency

The size of model eggs affected the ejection latency ($F_{1,27}$ = 12.9, p = 0.001). Small model eggs were ejected sooner than medium-sized eggs (12.62 ± 3.79 h and 53.66 ± 13.87 h, respectively; Fig. 1a), which is in agreement with Prediction 1. Furthermore, we detected a significant effect of colour on ejection interval ($F_{1,26}$ = 8.9, p = 0.006), non-mimetic eggs being ejected sooner (18.69 ± 8.11 h) than mimetic eggs (43.74 ± 10.73 h; Fig. 1b), which supports Prediction 2. This effect of colour on ejection latency is also supported by the fact that 63.2% of the ejections of non-mimetic eggs, but only of 9.1% of mimetic eggs, occurred within the first two hours after their introduction, i.e. during the filming period. However, we did not find any effect of the interaction between colour and size ($F_{1,26}$ = 1.06, p = 0.31).

Finally, it is worth noting that the ejection latency is positively correlated to inspection time (Spearman R = 0.43, p = 0.02; N = 29).

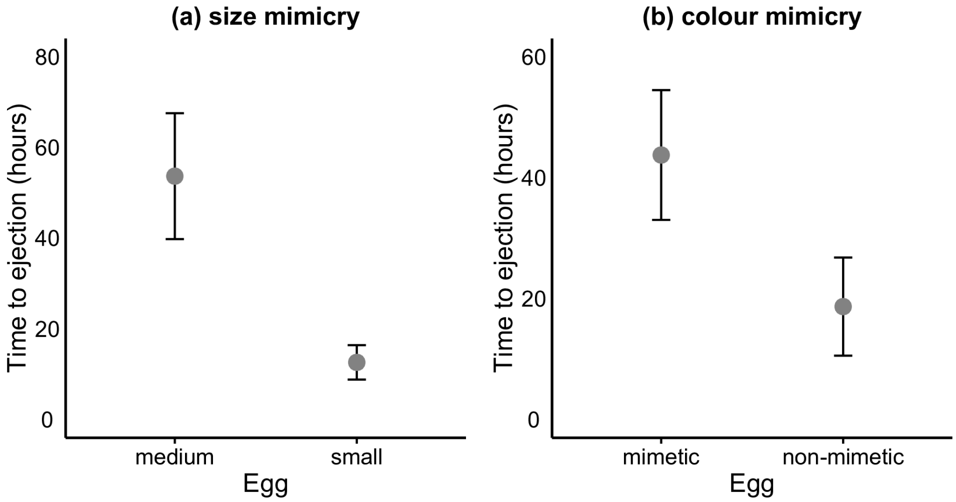

Figure 1: **Time needed by female blackbirds to eject an introduced egg with respect to (a) size mimicry and (b) colour mimicry.**

The relationship between egg touching and recognition

Blackbird females did not modify their number of first-contact touches during their first visit on account of egg size (χ^2 = 2.63; p = 0.62; Fig. 2a). However, colour significantly affected this variable (χ^2 = 14.6; df = 1; p = 0.001) showing that non-mimetic eggs were touched significantly more often (5.54 ± 0.89 touches) than mimetic eggs (2.38 ± 0.65 touches) by females during their first visit (Fig. 2c). Interestingly, we found an important effect of clutch size on the number of touches during the first visit (χ^2 = 12.6; df = 1; p = 0.002) showing that females gave significantly more touches in clutches of two (5.89 ± 1.03 touches) than three eggs (2.00 ± 0.39 touches). Furthermore, during the first visit, blackbird females touched non-mimetic eggs more often that eggs of control nests (5.54 ± 0.89 and 0.52 ± 0.3 female touches during the first visit, respectively; z = 4.50, p < 0.001; Fig. 2c). The interaction between colour and size did not show a significant effect for female touches during their first visit (χ^2 = 4.68; df = 1; p = 0.32).

In relation to the number of touches per visit (considering the complete filming period), there was again no size-related effect (χ^2 = 4.47; df = 1; p = 0.35; Fig. 2b). However, we found a significant effect of egg colour (χ^2 = 16.46; df = 1; p < 0.001) as females touched non-mimetic eggs significantly more often (4.12 ± 0.82 touches/visit) than the mimetic ones (1.30 ± 0.39 touches/visit; Fig. 2d). Similarly, as for the first visit, there was no significant interaction between colour and size (χ^2 = 2.73; df = 1; p = 0.60). Interestingly, eggs in nests parasitized with non-mimetic experimental eggs were touched significantly more often (per visit) than those in control nests (4.12 ± 0.82 and 0.71 ± 0.23 touches/visit, respectively; z = 2.47, p = 0.01; Fig. 2d). We did not find significant differences for this variable between the group with medium-sized mimetic eggs (1.06 ± 0.68 touches/visit) and controls (0.71 ± 0.23 touches/visit; z = -0.82, p = 0.41). Finally, neither colour mimicry, size nor their interaction significantly affected the number of incubation touches (all p-values > 0.11).

Blackbirds touched the eggs less frequently during their last visit in comparison with their first visit (χ^2 = 16.94; df = 1; p < 0.0001). However, this effect was more pronounced with non-mimetic eggs (χ^2 = 7.24; df = 1; p = 0.007) and we did not find any effect of colour on first-contact touches for the last visit (χ^2 = 3.57; df =1, p = 0.18). Egg size also had an important effect on the frequency of touches between the first and last visit (χ^2 = 22.31; df = 2; p < 0.0001) as blackbirds reduced their number of touches of large (χ^2 = 8.66; df = 1; p = 0.01) and small eggs (χ^2 = 8.46; df = 1; p = 0.01) but not medium-sized eggs (χ^2 = 1.64; df = 1; p = 0.64) during the last visit. The significant three-way interaction between colour, size and visit (χ^2 = 7.04; df = 2; p = 0.029) indicates that our treatment affected the reduction in touches between the first and last visits. Thus, we found no differences in touches between these two visits for both non-mimetic medium-sized eggs (χ^2 = 0.19; df = 1; p = 0.66) and mimetic large eggs (χ^2 = 4.23; df = 1; p = 0.12).

Relationships between recognition and ejection

Nests fall into four categories according to the relationships between egg recognition (first visit) and egg ejection: those in which (a) the egg was touched (more frequently than in 95% of control nests) and ejected (25.4%), (b) the egg was not touched but was ejected (22.2%), (c) the egg was touched and accepted (17.5%), and (d) the egg was not touched and not accepted (34.9%). The rejection rate was therefore 48% and blackbirds recognized (i.e. touched) the eggs but decided not to eject them in 17.5% of nests. This result contradicts Prediction 3a, but supports the alternative Prediction 3b. The number of first-contact touches per visit varied significantly between these groups of nests (χ^2 = 47.92; df = 1; p < 0.0001) but, more importantly, there is no difference between the number of first contact touches per visit in nests in which the egg is ejected (3.74 ± 0.9 touches/visit) and those in which it is not ejected (2.39 ± 0.73 touches/visit; χ^2 = 2.04; df = 1; p = 0.36). We obtained similar results when considering only the first visits (nests in which the egg is ejected = 5.17 ± 1.07 touches, and those in which it is not ejected = 3.67 ± 0.89 touches (χ^2 = 1.04; df = 1; p = 0.59)).

We also found a nearly significant negative correlation between female touches and ejection latency (First visit: Spearman R = -0.36, p = 0.058; per visit: Spearman R = -0.61, p < 0.001; N = 29). Hence, the more the female touched the eggs the sooner they were ejected.

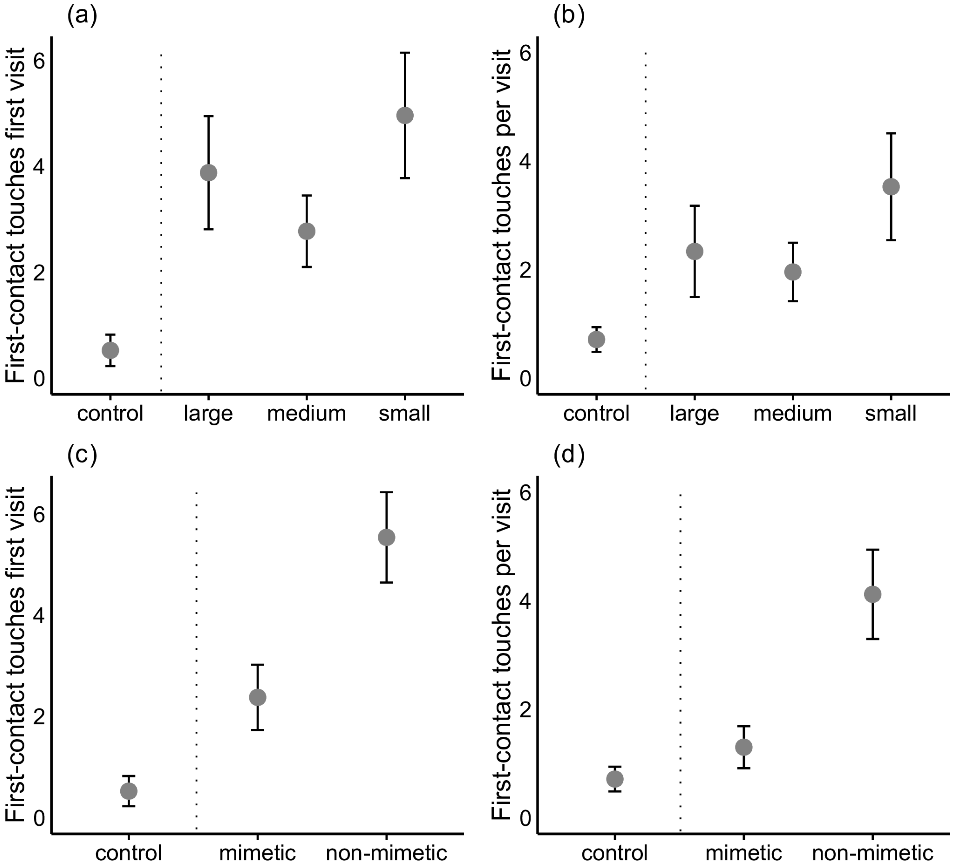

Figure 2: **Recognition of the parasitic egg.** First-contact touches during the first visit with respect to (a) size mimicry and (c) colour mimicry of the parasitic egg; and first-contact touches per visit with respect to (b) size mimicry and (d) colour mimicry of the parasitic egg. The figure also includes information from control nests (the dashed line separates control nests and those containing experimental eggs).

Discussion

Immediate ejection and ejection latency

We found that both the degree of mimicry and the size of the parasitic egg have a significant effect on the immediate ejection rate. Thus, non-mimetic model eggs were ejected more frequently at the onset (i. e. within two hours after parasitism). Many studies have documented that the egg rejection latency both within and among host species is highly variable (Soler et al. 2002, Honza et al. 2007b, Antonov et al. 2008a, Požgayová et al. 2011). However, the reasons for such variation are poorly understood. Our first prediction stated that the time taken to eject large parasitic eggs will be longer than for smaller eggs because it is more difficult for the female to pick up a large egg with her beak. Although we were unable to test this prediction since only one large egg was ejected, thus preventing the inclusion of this group in this analysis, we have found that the ejection interval was longer for medium-sized than for small experimental eggs, partly supporting our first prediction.

The effect of egg size on ejection latency in the case of medium-sized eggs could be due to two different reasons: (i) they are harder to recognize because of their better mimicry (in size) than small eggs, or (ii) they are harder to be eject because they are more difficult to pick up. Our results related to egg-touching behaviour suggest that egg size is not an important factor in the recognition of the parasitic egg, so that the delay in ejecting medium-sized eggs is probably because their larger size hinders the act of ejection. These results are important from a theoretical point of view because they imply that difficulty in egg ejection is an important factor that affects ejection latency, which can be related to some physical characteristic of the parasitic eggs that are not necessarily linked to egg recognition. These findings are supported by a recent experimental study that reported that heavier (but same-sized) eggs were ejected less frequently by blackbird females than normal-weight or light eggs (Ruiz-Raya et al. 2015).

In agreement with our second prediction, we have found that non-mimetic eggs were ejected sooner than mimetic ones. This result accords with previous findings reported for several species (Lotem et al. 1995, Underwood and Sealy 2002, Honza et al. 2004, 2005, Antonov et al. 2008a), which suggests that difficulty in egg recognition is the principal factor affecting ejection latency. However, our results show that difficulty in egg ejection also seems to be an important factor, affecting not only ejection latency (this study), but also the decision to eject (Ruiz-Raya et al. 2015).

Clutch inspection is another important factor affecting ejection latency given that parasitized hosts need time to process the visual characteristics of the eggs because of recognition problems (Antonov et al. 2008a, Požgayová et al. 2011). Indeed, females of three egg-puncturing ejector species that looked at their parasitized clutches for longer periods ejected the experimental egg sooner than females that inspected them for a shorter time (Antonov et al. 2008a, Požgayová et al. 2011, Soler et al. 2012a). Surprisingly, we have found the opposite result: ejection latency was positively correlated with time looking at the eggs in the grasp-ejector blackbird. Moreover, we have also found that the more the female touched the eggs the sooner they were ejected. These findings have important theoretical implications for the proximate mechanisms driving egg-rejection behaviour (see below).

Relationships between egg-touching and egg-recognition

Weak pecking by hosts when confronted with an experimental egg has been reported several times but this behaviour has traditionally been interpreted as trials to puncture the experimental egg (Lotem et al. 1995, Soler et al. 2002, Antonov et al. 2006, 2008a, 2009, Honza and Moskát 2008). Currently, egg-pecking behaviour is considered evidence of egg recognition even if ejection does not occur (Soler et al. 2002, 2012a, Underwood and Sealy 2006, Antonov et al. 2008a, 2009, Ruiz-Raya et al. 2015). In several species egg pecking is a clear behaviour directed only against an experimental (foreign) egg and is thus considered an unambiguous demonstration of egg recognition (Antonov et al. 2009, Soler et al. 2012a). This is not the case in the blackbird, perhaps because it is a grasp-ejector species. In the blackbird the experimental egg is not clearly pecked, but touched (some eggs in control nests are also touched). However, two pieces of evidence show that egg touching is a clear indication of egg recognition in this species. First, we have found that blackbird females touched the eggs in experimental nests more frequently than those in control nests. Second, clutches with non-mimetic eggs were touched significantly more often than those with mimetic eggs (Fig. 1b in this study, (Ruiz-Raya et al. 2015)). Evidence that egg-touching (or egg-pecking) implies egg recognition has been reported in a nest-deserter species (Underwood and Sealy 2006) and in two egg-puncturing species (Antonov et al. 2009, Soler et al. 2012a). The blackbird, a grasp-ejector, can now be added to the list of species in which egg touching is considered an indicator of egg recognition ((Ruiz-Raya et al. 2015), this study).

Egg recognition without ejection

In nearly 18% of the experimental nests in which the eggs were touched, the experimental eggs were not subsequently ejected. This contradicts our Prediction 3a, but supports the alternative prediction 3b, which confirms that host species, at least sometimes, recognize more eggs than they reject (Antonov et al. 2009, Guigueno and Sealy 2012, Soler et al. 2012a, Ruiz-Raya et al. 2015). Recognition without rejection has been experimentally demonstrated in three other species. Repeated parasitism of yellow warblers *(Setophaga petechia*) nests revealed that after recognizing a parasitic egg, individuals may either accept it or desert the nest (Guigueno and Sealy 2012). Antonov et al. (2009) showed that eastern olivaceous warblers (*Hippolais pallida*) pecked the experimental egg very often, but only half of such eggs were finally ejected. Finally, female rufous-tailed scrub robins, which were able to eject the experimental egg easily by grasping it, also frequently (55% of cases) pecked the experimental egg but did not eject it (Soler et al. 2012a). The fact that sometimes mimetic and larger experimental eggs are recognized but accepted implies that motivation is crucial to reaching the threshold needed to decide whether or not to eject, as previously suggested (Rodríguez-Gironés and Lotem 1999, Antonov et al. 2008a, Soler et al. 2012a, Ruiz-Raya et al. 2015).

Egg-recognition studies traditionally assume that rejection implies recognition whereas acceptance implies absence of (Rothstein 1990, Davies 2000, Soler 2014). However, the fact that hosts often recognize the parasitic egg but do not reject it (as demonstrated in the four host species tested so far, including the blackbird) implies that the recognition rate is higher than rejection rate, i.e. that rejection is not always the result of recognition. In fact, our results show that accepting parasitic eggs is not always the consequence of recognition failure. Instead they demonstrate that, at least sometimes, it is the consequence of acceptance decisions, a finding that should be taken into account in future studies on egg recognition.

Proximate mechanisms

We have found that blackbird females eject non-mimetic eggs sooner than mimetic experimental eggs, which is in agreement with previous findings (Antonov et al. 2008a, 2009, Soler et al. 2012a). This result supports the idea that ejection of mimetic eggs should need a longer time given that they are more difficult to recognize, making recognition errors more possible (Davies et al. 1996, Rodríguez-Gironés and Lotem 1999). However, our finding that blackbird females that inspected their clutches for longer periods of time ejected the parasitic eggs later than females that inspected their clutches for a shorter period of time is not in agreement with previously published results for three different species (Antonov et al. 2008a, Požgayová et al. 2011, Soler et al. 2012a). Two of these three species are puncture ejectors, and another rejects by nest-desertion, but the cognitive mechanisms involved in the egg rejection process (Moskát and Hauber 2007, Moskát et al. 2010, Soler et al. 2012a) should not be different depending on the rejection method (desertion, puncturing or grasping). An important difference between those three species and the blackbird, that could explain the above mentioned difference, is that the former are current cuckoo hosts with intermediate ejection rates (see Appendix in (Soler 2014)), while the latter is not currently used as a host by cuckoos and presents a high rejection rate of non-mimetic eggs (Grim et al. 2011). The perception of risk of parasitism by hosts increases in the presence of brood parasites, which consequently increases ejection rates (Moksnes and Røskaft 1989, Soler et al. 2012b). Thus, absence of parasitism implies absence of stimuli related to activity of brood parasites near host nests, which would promote rejection. Such absence would reduce motivation to eject because the threshold needed to decide ejection would be higher. This implies that the motivation to eject is lower in species that are not currently used as hosts and so the decision to eject an experimental egg will involve more time spent inspecting the eggs.

Blackbird females touched the eggs more frequently during their first visit to the nest after the introduction of the experimental egg than during the last filmed visit. Moreover, the difference in the number of touches between these two visits is significantly higher for non-mimetic (either in colour or size) than for mimetic eggs. This means that when an experimental egg is recognized this occurs immediately after the females' return to the nest. Thus, our results show that the delay in egg ejection is mostly the consequence of a delay in the decision to reject (i.e. the second step in the egg-rejection process). This decision needs an increase in motivation, and so takes longer (Soler et al. 2012a).

The time spent on each of the three stages of the egg-rejection process (recognition, decision and ejection (Soler et al. 2012a)) probably depends on the costs associated with each of them. Where the risk of making recognition errors is high (hosts could reject their own oddly coloured eggs (Davies and Brooke 1988, 1989b, Marchetti 1992, Davies et al. 1996, Rodríguez-Gironés and Lotem 1999)), hosts will inspect their clutches for longer until they are confident that the odd egg is different enough to be considered foreign. This would be the case with a foreign mimetic egg and it has in fact been suggested that the recognition problem is the main factor affecting ejection latency (Lotem et al. 1995, Underwood and Sealy 2002, Honza et al. 2004, 2005, Antonov et al. 2008a). However, ejection costs (hosts could damage their own eggs while trying to eject the parasitic egg (Davies and Brooke 1988, Rohwer and Spaw 1988, Marchetti 1992, Roskaft et al. 1993) are also known to be potentially important in affecting ejection latency because eggshell strength may impede egg-puncturing ejection (Antonov et al. 2008b, Spottiswoode 2010). Furthermore, the size and weight of the foreign egg are also known to impede ejection in both egg-puncturing and egg-grasping ejector species (Antonov et al. 2008a, Ruiz-Raya et al. 2015, Soler et al. 2015). Thus, a delayed decision to reject could be due not only to recognition problems as usually assumed (see references above), but also to potential difficulties in ejection. Therefore, the interval between recognition and the act of ejection (decision phase) is devoted to assessing (always in relation to the risk of parasitism) both whether or not the putative foreign egg really is foreign, and the potential costs of egg ejection. In fact, a recent blackbird study has found that heavy eggs previously recognized as foreign were more frequently accepted than light or control eggs, the consequence of motivation not being high enough to enable blackbirds to assume the higher costs that the ejection of a heavy egg could impose (Ruiz-Raya et al. 2015). Thus, the key element deciding the fate of the experimental egg throughout the egg rejection process is the motivation to reject together with a flexible rejection threshold based on phenotypic plasticity and risk of parasitism (Soler et al. 2012a).

In conclusion, our results with blackbirds show that (1) egg touching is a reliable indication of recognition even in the absence of egg ejection, (2) the delay in egg ejection does not seem to be due to recognition problems but is mainly the consequence of a delay in the decision to reject, (3) accepting parasitic eggs, at least sometimes, is the consequence of acceptance decisions provoked by the recognition constraints imposed by highly mimetic eggs or by mechanical constraints imposed by larger (i.e. harder to eject) eggs, and (4) given the potential costs associated with the rejection process, motivation is essential to deciding whether or not to eject, needing to be strong enough to reach the threshold needed to trigger the act of ejection. More egg-recognition experiments that film host behaviour when confronted with a foreign egg are needed to fully understand the proximate mechanisms driving the egg recognition and egg rejection processes.

Supplementary material

S1 Video *Blackbird females touching and ejecting an experimental non-mimetic egg.*
S1 Table *Data used in this paper.*

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Authors' contributions

Conceptualization: MS. Data curation: FR JDI. Formal analysis: FR JDI. Funding acquisition: MS. Investigation: JDI FR GR. Methodology: MS JDI. Project administration: MS. Resources: MS JDI. Supervision: MS JDI. Visualization: FR JDI. Writing ± original draft: MS FR JDI. Writing ± review and editing: MS FR JDI GR.

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Chapter 4

Effects of parasitic eggs on the hormonal state of hosts: a study in the common blackbird *Turdus merula*

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Abstract

Many host species have evolved sophisticated defences to mitigate the high fitness costs imposed by brood parasitism. Even though the physiological mechanisms behind such defences can offer important insights into the evolutionary relationship between brood parasites and hosts, they remain poorly known. Hormones play a critical role in the regulation of bird reproduction, making of them excellent candidates to investigate the physiological effects of brood parasitism on hosts. Here we experimentally parasitized blackbird (*Turdus merula*) nests with non-mimetic eggs to study the impact of brood parasitism on the hormonal levels of adult hosts during the egg stage, as well as the magnitude of the response to the standardised-stress protocol in parasitized and non-parasitized individuals. Parasitized females had higher corticosterone levels and showed a poorer body condition than non-parasitized birds, while we found no differences for prolactin levels. Both parasitized and non-parasitized females responded to the standardised-stress protocol with a marked increase in corticosterone levels. However, the decrease in prolactin after the standardised-stress protocol was more pronounced in parasitized individuals. Our results suggest that the presence of a non-mimetic parasitic egg involves a stressful situation for hosts, negatively affecting the physical state of parasitized females. Unaffected prolactin levels of parasitized individuals could explain the absence of nest desertion found in this species in response to parasitism. Finally, both hormones were not correlated in blackbirds, confirming that their combined study provides valuable pieces of information on the endocrine mechanisms underlying behavioural responses in animals, including hosts of brood parasites.

Introduction

Interspecific avian brood parasites exploit the parental care that other species provide to their offspring and impose high fitness costs on hosts since the parasitic chick is usually a better competitor for food or evicts all host offspring (Davies 2000, Roldán and Soler 2011, Soler 2014). In response, some host species have evolved defences that enable them to avoid such costs, egg rejection being the most widespread and effective of anti-parasitic behaviours (Davies 2000, Soler 2014). As occurs with other environmental stressors, brood parasitism induces individuals to modulate their behaviour to optimize their responses. Therefore, given the importance of the endocrine pathways on the behavioural adjustments of birds, hormones are excellent candidates to explore the poorly known physiological mechanisms underlying these anti-parasitic behavioural responses. To our knowledge only three studies have investigated these endocrine mechanisms so far. Ibáñez-Álamo et al. (2012) showed that great spotted cuckoo (*Clamator glandarius* Linnaeus) parasitic chicks induced an elevation of corticosterone levels in the nestlings of its main hosts, the magpie (*Pica pica* Linnaeus), especially during periods of high food requirements. Afterwards, it has been found that adults parasitized by the Central American striped cuckoo (*Tapera naevia* Linnaeus) had higher stress-induced corticosterone levels than non-parasitized parents during the fledgling stage, which involved important fitness costs in subsequent breeding seasons (Mark and Rubenstein 2013). Finally, a recent study including three host species of the brown-headed cowbird (*Molothrus ater* Boddaert) has highlighted the potential role of maternal androgens in response to brood parasitism and competition in both hosts and parasites, respectively (Hahn et al. 2017). These studies have provided crucial information to understand the endocrine mechanisms associated to brood parasitism; however, how parasitic eggs impact the host physiology during incubation, where the most important host defence (i.e. egg rejection) has evolved, remains unknown.

From a general perspective, environmental stressors induce the allocation of available resources from reproduction to behaviours enhancing survival (concept of "allostasis") (Wingfield et al. 1998, Wingfield and Sapolsky 2003, Angelier and Chastel 2009). Glucocorticoid hormones have been suggested to play a key role in this context, particularly in birds (Wingfield and Hunt 2002, Angelier and Wingfield 2013). Specifically, corticosterone is considered as one of the principal mediators in the physiological allostasis: circulating corticosterone levels increase in response to a stressful situation, leading to physiological and behavioural changes that promote the individual survival over other activities (i.e. reproduction) (Wingfield et al. 1998, Breuner et al. 2008). However, when the corticosterone stress response has a low adaptive value, it can be down-regulated in order to provide fitness benefits to individuals (Lendvai et al. 2007, Lendvai and Chastel 2008, 2010, Heidinger et al. 2010, Goutte et al. 2011, Addis et al. 2011), which makes corticosterone a key tool for understanding the responses of individuals to environmental perturbations (Wingfield and Sapolsky 2003, Hau et al. 2010, Wingfield et al. 2011), including brood parasitism (Ibáñez-Álamo et al. 2012, Mark and Rubenstein 2013).

There is, however, another hormone that could play a critical role in the brood parasitism context because of its importance regulating parental care in birds: prolactin, a pituitary hormone associated to the expression of avian incubating and brooding behaviours (Buntin 1996). The maintenance of parental behaviour is linked to elevated levels of prolactin whereas low levels are usually related to breeding failure and nest desertion (Sockman et al. 2006, Angelier and Chastel 2009, Ouyang et al. 2011). In birds, prolactin can also be affected by environmental stressors, and circulating levels of this hormone decrease after exposure to an acute stressor, which is related to the disruption of current parental care and nest desertion in order to redirect the energy from reproduction to self-maintenance (Angelier et al. 2007, Angelier and Chastel 2009). Several studies have shown that prolactin stress response can be down-regulated in order to maintain the parental care if current reproduction has an important fitness value (Chastel et al. 2005, Angelier et al. 2007, Angelier and Chastel 2009, Heidinger et al. 2010). In addition, it has been proposed that the magnitude of the decrease in prolactin levels in response to the standardised-stress protocol might reflect the parental investment in the current reproduction (Angelier and Chastel 2009). Although some studies have pointed out an effect of corticosterone on prolactin secretion (Angelier et al. 2009a, Tartu et al. 2015), most studies failed to find a link between these hormones, suggesting that they could mediate different behavioural responses to acute stressors (Angelier et al. 2013, 2016, Krause et al. 2015). Despite its importance in the physiology of avian breeding, prolactin has been largely overlooked in previous studies on the hosts´ physiology, which have focused mainly on corticosterone (see above). The combined study of both corticosterone and prolactin could provide a new perspective in understanding how brood parasitism impacts both the physiology and behaviour of host species.

Here we experimentally parasitized natural nests of common blackbirds (*Turdus merula* Linnaeus; blackbird hereafter) with non-mimetic eggs to study the effect of brood parasitism on the hormonal state of hosts during incubation. First, we assessed how hosts adjust their physiological state when they must cope with a parasitic egg. We predicted that parasitized females will show higher corticosterone levels than non-parasitized individuals given the important role of glucocorticoids in the response of birds to environmental stressors (Sapolsky et al. 2000, Landys et al. 2006); however, prolactin levels will remain unaffected in parasitized blackbirds given that previous studies performed in this species have shown absence of nest desertion (i. e. maintenance of parental effort) (Soler et al. 2015) and lack of propensity to assume potential ejection costs when egg ejection is hindered (Ruiz-Raya et al. 2015). Furthermore, an increase in corticosterone levels might affect body reserves (Sapolsky et al. 2000), which would result in a worse body condition of parasitized females. Second, we studied whether brood parasitism affects the stress response of hosts since it has been experimentally proven that birds modulate their stress response according to the value of their current reproduction (Lendvai et al. 2007). We expected that the magnitude of the corticosterone and prolactin response to the standardised-stress protocol will be higher in parasitized females as their current reproductive value is lower compared to non-parasitized birds. Finally, we investigated whether corticosterone and prolactin were correlated in blackbirds to provide new information on the potential relationship between these two hormones under different ecological contexts. Thus, if corticosterone and prolactin are functionally related in blackbirds, they should be correlated and modulated according to the same factors (Angelier et al. 2013).

Methods

Study system

This study was conducted in the Valley of Lecrín (Southern Spain, 36° 56′ N, 3° 33′ W) from late March to May 2015. The area is dominated by orange groves, in which blackbirds usually nest. See Ibáñez-Álamo and Soler (2010) for a detailed description of the study population. The blackbird is a potential but rarely parasitized (Grim et al. 2011) host of the common cuckoo (*Cuculus canorus*; cuckoo hereafter) and has frequently been used as a model species in egg-rejection experiments (e.g. Grim et al., 2011; Martín-Vivaldi et al., 2012; Polačiková and Grim, 2010; Ruiz-Raya et al., 2016, 2015, Samas et al., 2014, 2011, Soler et al., 2017, 2015), providing us detailed information about their response to experimental foreign eggs.

Experimental procedure and blood sampling

Since the beginning of the breeding season we actively searched for blackbird nests in the study area. Once a nest was located, we checked it to determine its content and visited each nest every two days to obtain data on laying date and clutch size. We created two experimental groups of nests: parasitized and non-parasitized nests. In the first one, blackbird nests were experimentally parasitized by introducing a large non-mimetic model egg during incubation (between the fifth and tenth day from the onset of laying). In non-parasitized nests, we followed the same procedure but nests were not experimentally parasitized. We conducted a sequential assignment of nests to the parasitized or non-parasitized group in order to control for possible differences in the hormonal levels of the females prior to the experiment. As model eggs, we used common quail (*Coturnix coturnix*) eggs (weight: 12.2 ± 0.04 g; size: 32.6 ± 0.1 x 25.3 ± 0.1 mm; n = 49), which are slightly larger than blackbird eggs (weight: 6.6 ± 0.1 g; size: 30.4 ± 0.2 x 21.1 ± 0.1 mm; n = 40). This difference hinders the action component of egg ejection thus extending the period of time the parasitic egg is present in the nest (three days; see below). Model eggs were coloured red with acrylic paint the day before being placed in the blackbird nest in order to ensure egg recognition. Previous studies have demonstrated that eggs painted red are good non-mimetic model eggs (Soler and Møller 1990, Avilés et al. 2004, Martín-Vivaldi et al. 2012) easily detected as a parasitic egg by this species (Ruiz-Raya et al. 2015, Soler et al. 2015). Each model egg was used only in one trial and discarded afterwards. All nests were inspected daily to look for possible cracks or broken eggs (ejection costs).

Blood samples were taken both from non-parasitized and experimentally parasitized females. Three days after the start of the experiment, all females were captured just after sunrise by using a mist net placed near the nest. We took the blood samples after a period of three days mainly because of two reasons: first, corticosterone-induced changes in prolactin levels happen progressively and during several days before returning to the initial levels (Angelier et al. 2009a). Secondly, we use a time frame wide enough to detect possible relationships between corticosterone and body condition. To assess the hormonal levels of blackbirds as well as their hormonal response to stress, females were bled following the standardised technique described by Wingfield (1994). Immediately after capture, an initial blood sample (400 - 500 μl) was collected from the brachial vein with a 25-gauge needle and 80 μl heparinized microhematocrit tubes (baseline levels). We quantified the time elapsed between the capture and the end of the initial blood sampling to control for the possible effect of handling time on hormone levels, especially for corticosterone. Thus, all blood samples used in corticosterone assays were taken within 3 min after capture. After the first bleeding, females were kept in an individual cloth bag and suspended off the ground. For stress-induced hormonal levels, a second blood sample was taken 30 min after capture from the brachial vein of the other wing (stress-induced levels). This period of time has been previously used to obtain maximal stress-induced corticosterone levels for this species (Ibáñez-Álamo et al. 2011) based in the findings of several investigations comparing different time periods in several species, including the blackbird (Partecke et al. 2006, Adams et al. 2011, Mark and Rubenstein 2013). During the handling time, eggs were covered with cotton to reduce the heat loss. Individuals were ringed so each female was used only once. All birds were released near the nest in 5-15 minutes after the last blood sampling and returned to the nest to continue with incubation within the next hour (pers. obs.). There were no cases of nest desertion attributable to our experimental manipulation. Blood samples were kept cold and were centrifuged at 4500 RCF for 3 min as soon as possible (maximum 5 hours after collection). Plasma was separated and stored at 20 °C until the hormonal assay.

Body condition

After bleeding, all females were weighed twice to the nearest 0.1 g and tarsus length was measured twice to the nearest 0.01 mm. In all cases measures were made by the same researcher (FRR). We calculated the average weight and tarsus length for each female in order to estimate the body condition for each individual. Thus, we calculated the "scaled mass index" (SMI) following (Peig and Green 2009, 2010) for each individual *i*:

$$SMI_i = M_i \cdot (\frac{L_0}{L_i})^b$$

Where M_i and L_i are the body mass and the tarsus length of individual *i*, respectively; L_0, the arithmetic mean value of tarsus length in our study population (L_0 = 34.95 mm, n = 37) and *b*, the slope of a standardised major axis (SMA) regression of log body mass on log tarsus length. Unlike residual index, which assumes isometry (i. e. proportion of body components remain constant as the size increases), the scaled mass index considers allometry (i.e. relative length of different body parts changing with size) by estimating a scaling exponent and including it in the calculation, which allows to determine the structural effects of growth on mass-length relationships (Peig and Green 2009, 2010).

Hormone assays

All hormonal analyses were performed at the Centre d'Études Biologiques de Chizé (CEBC-CNRS, Villiers en Bois, France). Plasma concentrations of corticosterone were determined after diethyl-ether extraction by a radioimmunoassay (RIA) as detailed in (Lormée et al. 2003). Plasma concentrations of prolactin were also determined using an heterologous RIA assay following the method described by Cherel et al. (1994) and validated for blackbirds (Préault et al. 2005). All samples were run in one assay for both hormones (intra-assay variations, corticosterone: 10.8%; prolactin: 4.8%). The minimal detectable corticosterone and prolactin levels were 0.5 and 6 ng/ml respectively and no samples fell below these limits.

Statistical analyses

We ran separated general linear models (LM) to analyse differences between parasitized and non-parasitized females regarding both baseline and stress-induced hormone levels. As predictors, our initial models included experimental parasitism (yes/no), clutch size (number of eggs in the nest during the trial), the interaction between parasitism and clutch size, and date (day of the breeding season). Non-significant terms were sequentially dropped until the model with lowest AIC was obtained. To assess the effect of the capture stress protocol on both corticosterone (log transformed) and prolactin, we performed Linear Mixed Models (LMM) by using *nlme* (R package v.3.1-122 (Pinheiro et al. 2014)) as the same individuals were sampled twice during the protocol. In this case, our initial models included the following predictors: experimental parasitism, sampling (baseline or stress-induced), clutch size and date. Model selection was performed following Zuur et al. (2009): firstly, the optimal structure for random effects was estimated by including all fixed effects and their interactions in the model (beyond optimal model), and comparing different models, adjusted by REML, with an increased complexity in random structure: models with no random effect, only random intercept (female identity), and finally considering both a random intercept and random slopes (female identity and sampling, respectively). The final corticosterone model included both the random intercept and random slopes whereas the final prolactin model included only the random intercept. To assess the optimal structure for fixed effects, we compared nested models for the fixed components while maintaining the optimal random structure previously determined. In this case, ML estimation was used instead of REML. After determining the best structures for both fixed and random effects, we adjusted the final models by REML. Finally, model validation was performed by visual inspection of the residuals graphs in order to verify the assumptions of normality of the residuals and homogeneity of the variances. We followed Johnson (2014) to obtain R^2 from random slopes and random intercept LMM's by using *MuMIn* (R package v.1.15.6 (Barton 2016)). Thus, we obtained two values of R^2: the marginal R^2 ($R^2_{LMM(m)}$), which describes the proportion of variance explained by the fixed factor(s) alone; and the conditional R^2 ($R^2_{LMM(c)}$), which describes the proportion of variance explained by both the fixed and random factors. On the other hand, we also ran correlations between corticosterone levels, prolactin levels and body condition in order to study the potential biological relationships between these variables. We report the effect size, R^2 and p-value for all significant effects. All analyses and graphs were performed using R version 3.2.3 (R Core Team 2015).

Results

We performed experiments in 49 blackbird nests. However, in 7 cases the experimental egg disappeared from the nest during the day before the blood sampling, so these females were not sampled as their hormone levels could have returned to values similar to those prior to our manipulation. Furthermore, 5 females were excluded from the hormonal assays given that the bleeding time exceeded 3 minutes. We finally report results from 37 blackbird females: 19 parasitized and 18 non-parasitized.

Baseline hormonal levels and body condition

Experimental parasitism had an important effect on corticosterone levels ($F_{1,33} = 5.78$; $p = 0.02$), which were significantly higher in parasitized than in non-parasitized individuals (estimate = 1.82; se = 0.75; t = 2.41; $p = 0.02$; $R^2 = 0.12$; Fig. 1A). Clutch size, date or the interaction between treatment and clutch size did not show a significant effect on baseline corticosterone levels (all cases $p > 0.45$).

Prolactin levels were not affected by the experimental treatment since we did not find differences between parasitized and non-parasitized females ($F_{1,30} = 0.07$; $p = 0.79$; Fig. 1B). Clutch size, date or the interaction between treatment and clutch size did not show an effect on prolactin levels (all cases $p > 0.34$). We did not find correlation between corticosterone and prolactin levels (parasitized females: Pearson's $r = -0.15$, $p = 0.56$; non-parasitized females: Pearson's $r = -0.02$, $p = 0.94$).

Body condition differed between groups ($F_{1,35} = 8.83$; $p = 0.005$) as non-parasitized individuals were in better body condition than parasitized ones (estimate = 4.30; se = 1.45; t = 2.97; $p = 0.005$; $R^2 = 0.18$). Clutch size, date or the interaction between parasitism and clutch size did not affect body condition (all cases $p > 0.11$). Interestingly, we found a negative correlation between corticosterone levels of parasitized females and body condition: parasitized females with higher corticosterone levels showed poorer body condition (Pearson's $r = -0.53$, $p = 0.02$; Fig. 2). However, we did not find any relationship between body condition and corticosterone levels for non-parasitized females (Pearson's $r = 0.14$, $p = 0.60$, Fig. 2). Finally, body condition did not show any relationship with prolactin levels for non-parasitized (Pearson's $r = -0.43$, $p = 0.08$) or parasitized females (Pearson's $r = 0.11$, $p = 0.64$).

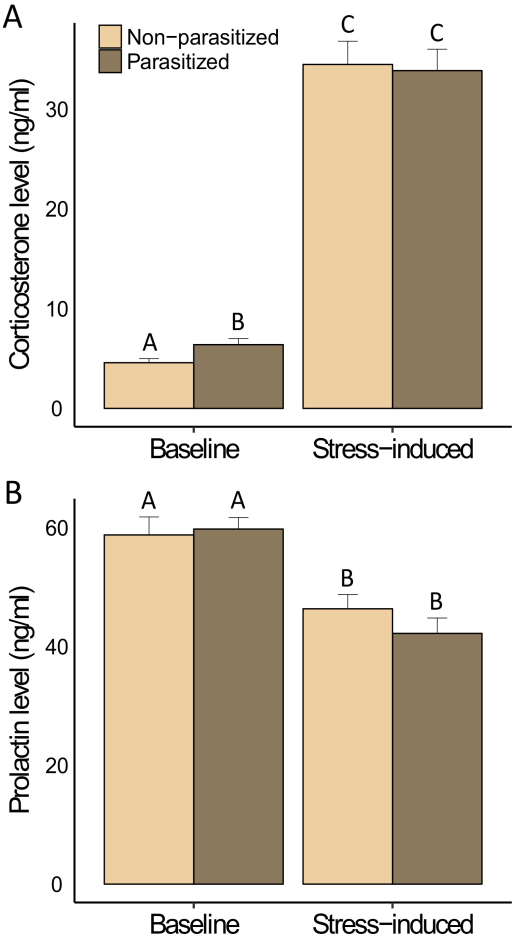

Figure 1: **Effect of the parasitic egg in the hormonal levels of hosts.** Baseline and stress-induced corticosterone (A) and prolactin (B) levels for parasitized and non-parasitized blackbird females. Different letters above bars indicate significant differences at $p < 0.05$. Data are expressed as means + se.

Hormonal response to stress

The stress protocol affected corticosterone levels ($F_{1,33}$ = 582.51; $p < 0.0001$; Fig. 1A) and blackbirds responded to the capture by significantly increasing their corticosterone levels (LMM for log-transformed corticosterone: estimate = 0.88; se = 0.05; df = 33; t = 18.26; $p < 0.0001$; $R^2_{LMM(m)}$ = 0.86; $R^2_{LMM(c)}$ = 0.97; Fig. 1A). Furthermore, the magnitude of this increase showed a marginally significant tendency to be higher in non-parasitized females ($F_{1,33}$ = 4.02; p = 0.053; Fig. 1A). Stress-induced corticosterone levels did not depend on brood parasitism ($F_{1,28}$ =

0.04; p = 0.85; Fig. 1A). Clutch size and date did not affect the stress-induced corticosterone levels (all cases $p > 0.46$).

Regarding prolactin, the capture stress protocol had an important effect on stress-induced prolactin levels of both parasitized and non-parasitized females ($F_{1,35}$ = 173.28; $p < 0.0001$), which experienced a significant decrease relative to baseline levels (LMM: estimate = -12.44; se = 1.64; df = 35; t = -7.58; $p < 0.0001$; $R^2_{LMM(m)}$ = 0.34; $R^2_{LMM(c)}$ = 0.86; Fig. 1B). Interestingly, this decrease in prolactin levels was more pronounced in parasitized females ($F_{1,35}$ = 5.00; p = 0.03; Fig. 1B). Absolute stress-induced prolactin levels did not depend on brood parasitism ($F_{1,35}$ = 1.36; p = 0.25; Fig. 1B). Clutch size and date did not affect the stress-induced prolactin levels (all cases $p > 0.31$).

We did not find a correlation between stress-induced corticosterone and stress-induced prolactin levels (parasitized females: Pearson's r = -0.01, p = 0.97; non-parasitized females: Pearson's r = 0.19, p = 0.46). However, the magnitude of the prolactin response to stress was correlated with the female's body condition since those individuals with a lower body condition suffered a higher decrease in their prolactin levels on both treatments (non-parasitized females: Pearson's r = 0.50; p = 0.036; parasitized females: Pearson's r = 0.59; p = 0.008; Fig. 3).

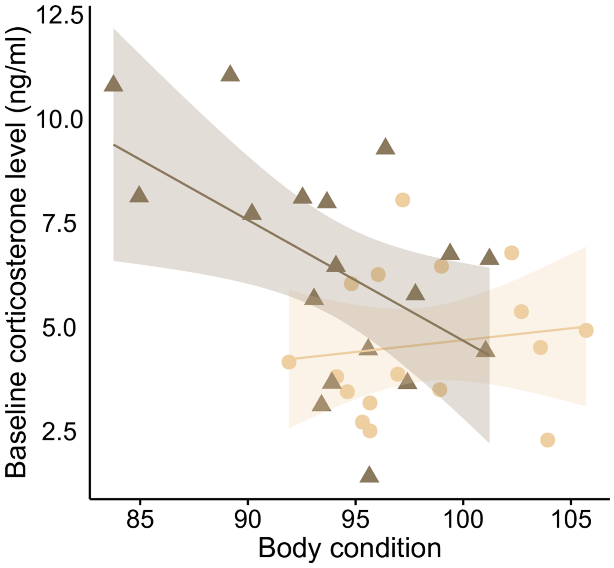

Figure 2: **Baseline corticosterone levels and body condition.** Relationship between baseline corticosterone levels and body condition (SMI) for parasitized (triangles) and non-parasitized blackbird females (circles). Shade areas represent 95% confidence intervals.

Discussion

Our study offers novel results that considerably expand our understanding on the effects of brood parasitism on the physiological state of hosts. First, we found that parasitized females showed higher corticosterone levels than non-parasitized females. Secondly, experimental brood parasitism significantly affected the physical state of parasitized blackbirds, which showed a poorer body condition than non-parasitized ones. Thirdly, we found no differences in prolactin levels due to brood parasitism. Fourthly, the reduction of prolactin levels due to the standardised-stress protocol was more pronounced in parasitized females. Finally, corticosterone and prolactin appear to be functionally unrelated in the common blackbird, suggesting that these two hormones provide different pieces of information about the anti-parasitic responses of this species. To the extent of our knowledge, this is the first experimental study showing the effects of brood parasitism on both the physiological and physical state of hosts during incubation.

Brood parasitism, hormones and body condition

Blackbird females parasitized with a non-mimetic egg had significantly higher corticosterone levels than non-parasitized females confirming that, under certain circumstances, parasitized nests become a physiological stressful environment for hosts (Ibáñez-Álamo et al. 2012), even from incubation. Our results contrast with those found by Mark and Rubenstein (2013) showing the absence of physiological costs during the incubation stage in naturally parasitized females of the rufous-and-white wren (*Thryophilus rufalbus*). These differences could be explained by the low recognition rates of cuckoo eggs exhibited by their model species (Mark 2013), and suggest that the extra parental care needed for the incubation of an additional (parasitic) egg does not seem to be high enough to modify host's hormonal responses. Corticosterone is a well-known mediator in metabolic processes affecting the energetic state of individuals by mobilizing energy from body reserves to deal with environmental stressors (Sapolsky et al. 2000, Landys et al. 2006). In our study, parasitized females suffered a worsening in body condition, probably due to protein or lipid mobilization from storage sites associated to the rising of baseline corticosterone levels. In fact, we found a significant negative correlation between corticosterone levels of parasitized females and their body condition since higher values of corticosterone levels corresponded to parasitized females with a worse body condition (Fig. 2). This relationship was not found in non-parasitized females, which indicates that brood parasitism was responsible for the reduced body condition. One possible explanation is that, as found in previous studies, recognition of parasitic eggs by blackbird females leads to several behavioural changes including an increase in female activity at the nest, such as touches given to eggs and time spent on nest checking (Ruiz-Raya et al. 2016, Soler et al. 2017). These behaviours likely involve an additional energy demand for parasitized females which could be satisfied in the short term by using body reserves. Interestingly, the physical state of parasitized females could affect the host decision: a lower body condition could contribute to the acceptance of the parasitic egg as it can make ejection more difficult and potentially costlier, so a higher motivation would be needed to promote this anti-parasitic defence (Soler et al. 2012). However, nothing is known about the effect of body condition on the decision making during the egg rejection process, and further studies are therefore needed on this subject.

Our results show that prolactin levels of blackbird females remained unaffected by the presence of a non-mimetic parasitic egg in the nest (Fig. 1B). Prolactin has been classically proposed as the hormone responsible for the expression of parental care in birds (Silver 1984, Hall et al. 1986, Sharp et al. 1988, Buntin 1996, Lormée et al. 2000, Sockman et al. 2006) and can be used as a good proxy of parental effort (i. e. amount and quality of parental behaviour)(Angelier and Chastel 2009). In fact, elevated prolactin levels are positively related to breeding success through a greater parental effort (Miller et al. 2009), while decreasing prolactin levels are linked to processes such as incubation interruption and nest desertion (Cherel et al. 1994; Chastel and Lormée 2002; Angelier et al. 2007, 2015; Groscolas, Lacroix and Robin 2008; Spée et al. 2010). Although a decrease of parental care could be expected in parasitized females because of the lower value of their brood, unaffected prolactin levels found in parasitized females could explain previous findings showing absence of nest desertion as an egg-rejection strategy in blackbirds (Soler et al. 2015), and point out the potential mechanism responsible for the absence of nest desertion in other host species. Thus, plastic responses related to nest desertion found in some host species could be mediated by endocrine mechanisms associated to prolactin, but more studies are needed to expand our understanding about the physiological changes behind the conditional component of anti-parasitic responses.

Unlike corticosterone, we found no correlation between prolactin levels and body condition in both parasitized and non-parasitized blackbirds. In some species such as capital breeders, which rely on stored energy for reproduction, it is expected that prolactin is negatively affected by body condition as individuals with insufficient body reserves would benefit by redirecting energy away from reproduction (O'Dwyer et al. 2006). However, blackbirds exercise a behavioural control on their stored energy and they do not rely on large body reserves during reproduction, when foraging opportunities are greater (Macleod et al. 2005), which would explain why baseline prolactin levels are not affected by the low body condition of parasitized females.

Brood parasitism and the hormonal response to stress

Standardised-stress protocol resulted in a quick and robust increase of corticosterone levels both in parasitized and non-parasitized females. This strong hormonal response to a standardised stressor has been previously described in several bird species (e. g. (Angelier et al. 2013, 2015a, Krause et al. 2014), including blackbirds (Partecke et al. 2006, Adams et al. 2011, Ibáñez-Álamo et al. 2011). Some species can down-regulate their response to an acute stressor as an hormonal tactic to optimize their current reproductive effort, even if this involves survival costs (O'Reilly and Wingfield 2001, Jessop 2001, Wingfield and Sapolsky 2003). In our study, non-parasitized birds were expected to show a lower adrenocortical stress response as the value of their offspring would be higher compared to parasitized females. However, both groups reached corticosterone levels nearly six-fold higher than baseline levels, although this response tended to be slightly weaker in parasitized females (Fig. 1A). The maintenance of a robust stress response in both non-parasitized and parasitized females is expectable from an "emergency life-history stage", a strategy that would allow individuals to minimize the risk of mortality and ensure the future reproduction when dealing with unpredictable perturbations, such as an encounter with a predator or our capture stress protocol (Wingfield et al. 1998). On the other hand, the trend to a weaker response found in parasitized females might be explained by their worse body condition since individuals in poor physical condition usually respond more slowly to stressors than those in better conditions (Heath and Dufty, 1998).

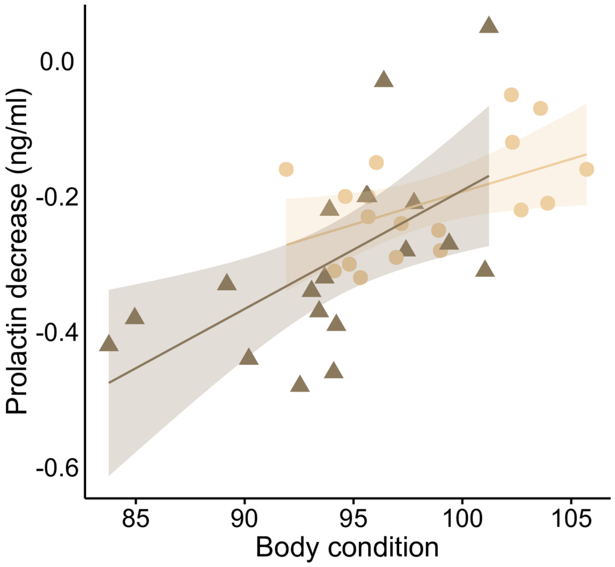

Figure 3: **Prolactin stress response and body condition.** Relationship between the decrease in prolactin levels in response to the stress-induced protocol and body condition (SMI) for parasitized (triangles) and non-parasitized blackbird females (circles). Shade areas represent 95% confidence intervals.

Prolactin levels of both parasitized and non-parasitized blackbirds were affected by the standardised-stress protocol, resulting in a significant decrease of baseline levels in both groups (Fig. 1B). Decreasing prolactin levels have been previously proposed as an adaptive mechanism in birds to cope with stressors allowing them to disrupt the current parental care in order to maximize future reproduction (Chastel et al. 2005). However, in agreement with our prediction, parasitized females showed a more pronounced decline in their prolactin levels than non-parasitized females, indicating that brood parasitism might impact on the parental investment of blackbird females (Angelier and Chastel 2009). Although blackbirds tend to maintain the parental care in parasitized nests (i. e. absence of nest desertion and unaffected baseline prolactin levels), the higher hormonal response to stress exhibited by parasitized females indicates that their parental investment under stressful situations might be lower when compared to non-parasitized females. According to the "brood value hypothesis", individuals are able to modulate their stress response with respect to the value of current reproduction (Lendvai et al. 2007, Lendvai and Chastel 2008). As cuckoo parasitism usually involves important fitness costs on hosts (Davies 2000), parasitized females may be expected to markedly reduce their parental care to redirect some of the energy available from reproduction to survival in response to stressful situations. This response may be especially important in cases where the energy availability is limited, and previous studies have shown that circulating prolactin levels are negatively impacted from a certain threshold of body condition as individuals energetically constrained usually show a sharper and quicker decline of prolactin levels (Cherel et al. 1994, Criscuolo et al. 2002, O'Dwyer et al. 2006, Groscolas et al. 2008, Angelier et al. 2009b, Spée et al. 2010, Riechert et al. 2014). Most of these studies have focused on species with large body reserves such as seabirds, but little is known about smaller species that do not rely on large body reserves for reproduction. In our study, females with a poorer body condition experienced a higher prolactin response to the stress (Fig. 3), which suggests that the amount of energy available is linked to the stress-induced prolactin response in blackbirds. Importantly, most studies focusing on stress-induced prolactin changes in wild birds have been performed in seabirds (reviewed in Angelier et al. (2016)), so this is one of the few investigations done with a passerine showing a decrease in prolactin levels during incubation as a response to the standardised-stress protocol.

Are corticosterone and prolactin functionally linked?

In our study, we did not find any significant relationship between corticosterone and prolactin stress-induced levels for non-parasitized or parasitized blackbird females, suggesting that these two hormones are not strongly linked in this species, regardless of brood parasitism. In addition, as discussed in the previous section, corticosterone and prolactin stress responses seem to be modulated according to different factors in blackbirds. Thus, in this species, the energetic costs associated with experimental parasitism might modulate the response of prolactin, but not corticosterone, to stress. Taken together, our results fit previous studies showing that corticosterone and prolactin are usually unlinked in birds (Angelier et al. 2013, 2016, Krause et al. 2015). Corticosterone stress response is known to be related to physiological changes aimed to redirect energy to cope with stressors (Sapolsky et al. 2000, Landys et al. 2006), whereas the prolactin stress response is linked to parental decision when dealing with environmental perturbations (Angelier and Chastel 2009). Therefore, the combined study of both hormones can provide valuable information to understand some crucial aspects of the host responses to brood parasitism, such as the energetic state of hosts and its potential impact on parental decisions.

In conclusion, our results suggest that the combined study of corticosterone and prolactin can lead to major advances in our understanding of the physiological effects of brood parasitism on hosts. Importantly, the presence of a non-mimetic parasitic egg becomes a stressful situation for female hosts which can modify their hormonal levels, alter their response to the standardised-stress protocol, and can negatively affect their body condition. The study of hormones related to the maintenance of parental care can be crucial to reveal the physiological mechanisms underlying the anti-parasitic responses previously found in several host species, such as acceptance decisions or the absence of nest desertion. These results highlight the need of further studies focused on the endocrine mechanisms behind the life-history decisions of bird species impacted by brood parasitism.

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Competing interests

The authors declare that they have no conflict of interest.

Authors' contributions

FRR, MS and JDIA conceived the study and designed the methodology; FRR, TA and GR performed the field work and collected the data; FRR and OC performed the hormonal assays; FRR analysed the data; FRR wrote the first draft. All authors contributed critically to the manuscript and gave final approval for publication.

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Chapter 5

Phenotypic plasticity in egg rejection: evidence and evolutionary consequences

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Abstract

Rejection of parasitic eggs is the most common and effective defence used by hosts to mitigate the fitness costs imposed by avian brood parasites. Although egg rejection importantly relies on the cognitive abilities of parasitized individuals, both theoretical models and experimental studies have found that some hosts are able to modify their response according to the current conditions of parasitism, which reflects the existence of phenotypic plasticity in host defences. In environments in which the risk of parasitism is variable, plastic responses can be favoured by natural selection as they will allow hosts to avoid potential rejection costs under low risk of parasitism. In this chapter, we review the current evidence of plastic responses in egg rejection and discuss both the evolution and the long-term consequences of phenotypic plasticity for brood parasite-host coevolution. In addition, we suggest addressing the study of egg rejection as a complex process affected by multiple components and governed by decision making and host motivation, which has important implications for host responses. Despite its apparent benefits, phenotypic plasticity is scarce among host species. Therefore, the evolution of phenotypic plasticity in brood parasite-host systems deserves special attention as the maintenance or the loss of plastic responses involve important evolutionary consequences, affecting the long-term outcome of the interaction between brood parasites and their hosts. We conclude with some suggestions to deal with phenotypic plasticity in future egg-rejection studies.

Chapter 5

Introduction

Antagonistic interactions between avian brood parasites and their hosts usually result in a coevolutionary arms race in which adaptations and counteradaptations evolve on both sides (Davies 2000). The high fitness costs imposed by brood parasitism select for host defences at different stages of the breeding cycle: hosts attack brood parasites that approach their nests, reject the parasitic egg, remove the young parasitic chick or refuse to feed parasitic nestlings or fledglings (Soler 2014). However, recognition and rejection of parasitic eggs is the most common and effective strategy used by hosts against brood parasitism (Rothstein 1990, Davies 2000), which has led some brood parasites to evolve sophisticated egg mimicry and host species to fine-tune their abilities to recognize parasitic eggs (Brooke and Davies 1988).

Both theoretical models (Rothstein 1990, Takasu 1998, Robert et al. 1999, Servedio and Lande 2003) and empirical evidence (Martín-Gálvez et al. 2006, 2007) have pointed to the importance of the genetic component of hosts' egg-recognition abilities. In many host species, such abilities are maintained even after generations of allopatry with brood parasites (Lahti 2006, Hale and Briskie 2007, Peer et al. 2011, Soler 2014, Ruiz-Raya et al. 2016) while, in other cases, evolutionary change has led to a decline in the egg-rejection behaviour of some hosts after long period of isolation from parasites (Kuehn et al. 2014). There are occasions, however, when the decline of parasitism rate is accompanied by a decrease in rejection rates too rapid to reflect genetic change in populations (Soler et al. 2012a; Thorogood and Davies 2013). These findings suggest that egg rejection does not rely exclusively on genetically determined cognitive abilities, but hosts are able to modify their rejection decisions under different environmental contexts, such as the risk of parasitism (Stokke et al. 2005; Moskát and Hauber 2007; Soler et al. 2012b). Given the costs linked to the expression of antiparasitic behaviours, natural selection should favour the emergence of flexible host defences in circumstances in which the risk of parasitism is low. For example, highly mimetic parasitic eggs can lead hosts to eject their own eggs by mistake (recognition costs), or to break some eggs of their own during the ejection process (ejection costs)(Davies 2000). Furthermore, cuckoo-hawk mimicry (Davies and Welbergen 2008, Welbergen and Davies 2011) makes it dangerous to approach an intruder that might be a potentially lethal enemy. Thus, when the probability of being parasitized is low, phenotypic plasticity in host defences would allow individuals to avoid such costs. Phenotypic plasticity can be defined as the property of a single genotype to produce different phenotypes in response to distinct environmental conditions (Pigliucci 2001), a relationship usually represented by "reaction norms" (Figure 1) (Schlichting and Pigliucci 1998; Pigliucci 2005). Under this perspective, the slope of reaction norms will reflect the degree of plasticity of genotypes. In those cases in which there is variation within the population for the slope of the reaction norm (non-parallel reaction norms), it can be said that population show a GxE effect (i. e. genotype x environment) (Pigliucci 2005). In highly variable environments, individuals can obtain fitness benefits from their ability to respond plastically when compared with those that do not, so phenotypic plasticity can become adaptive. Behavioural plasticity has been suggested to be particularly important in variable environments since the development and expression of animal behaviour may be immediate, reversible and especially sensitive to environmental changes (West-Eberhard 2003, Fordyce 2006, Snell-Rood 2013).

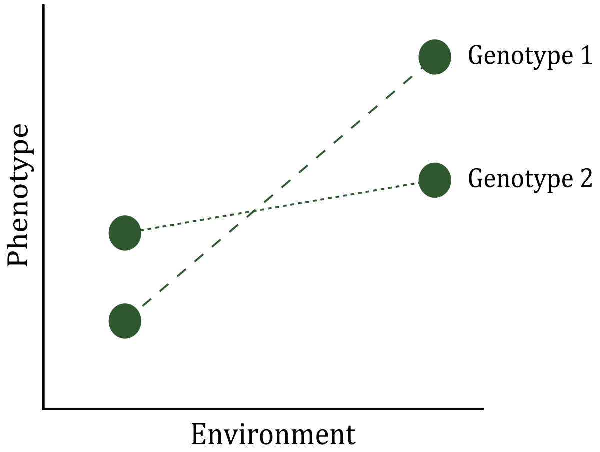

Figure 1: **Genotypic reaction norms used to illustrate phenotypic plasticity.** Dashed lines represent the reaction norms for two single genotypes exposed to two different environments. Different slopes show the different degree of plasticity between genotype 1 (more plastic) and genotype 2 (less plastic). Based on Figure 1 in (Pigliucci 2005).

The strength of interactions between brood parasites and their hosts shows a remarkable spatiotemporal heterogeneity and some hosts have been found able to adjust their defences accordingly (see below). Such flexible defences are important from an evolutionary perspective as they can determine the result of ecological interactions (Fordyce 2006). The existence of flexible defences in hosts has been documented regarding nest vigilance (Davies et al. 2003, Feeney and Langmore 2015), mobbing to adult parasites and nest defence (Lindholm and Thomas 2000, Welbergen and Davies 2009, 2012, Langmore et al. 2012, Thorogood and Davies 2013, Kuehn et al. 2016), egg rejection (see Table 1) and chick rejection (Langmore et al. 2009a). Throughout this chapter, we review the current evidences of flexible defences in hosts of brood parasites by focusing on egg-rejection behaviour and host decision-making. In addition, we discuss both the evolutionary origin and the main consequences of phenotypic plasticity on the coevolutionary history between brood parasites and their hosts.

Chapter 5

Evidence of phenotypic plasticity in egg rejection

The existence of a conditional component in egg rejection behaviour has been predicted by several theoretical models, which widely describe the importance of both the risk of parasitism and potential rejection costs on host responses (Davies et al. 1996, Rodríguez-Gironés and Lotem 1999, Holen and Johnstone 2006, Stokke et al. 2007). In addition to theoretical predictions, many empirical studies (see below) have suggested that phenotypic plasticity is behind the variations in rejection rates found among both host populations and individuals, especially those cases where there is a variable risk of parasitism.

Ecological interactions between brood parasites and their hosts show spatial heterogeneity resulting in the emergence of both coevolutionary hotspots (population with intense interactions between parasites and hosts) and coldspots (populations showing scarce or absent interactions)(Thompson 2005). As a result, host populations vary in the extent of local adaptations against brood parasitism, leading to differences in egg-rejection behaviour among populations (Davies and Brooke 1989, Soler and Møller 1990, Briskie et al. 1992, Soler et al. 1999a). Although such differences may reflect genotypic differences (Martín-Gálvez et al. 2007), studies on metapopulation dynamics have revealed the importance of phenotypic plasticity in explaining variations in rejection rates among host populations. For example, the presence of common cuckoo *Cuculus canorus* (cuckoo hereafter) parasitism has been proven to be the main predictor to explain the spatial variation in host resistance existing among different populations of reed warbler *Acrocephalus scirpaceus* across Europe (Stokke et al. 2008). In British populations of this species, which are not genetically isolated, individuals from unparasitized populations reject eggs at lower rates and show a less aggressive response towards cuckoos than those from parasitized populations (Lindholm and Thomas 2000). Most importantly, reed warblers from both parasitized and unparasitized populations were able to recognize (all populations pecked the model eggs) and eject experimental model eggs but they differ in their tendency to reject them, confirming that phenotypic plasticity was responsible for the differences in host responses between nearby populations (Lindholm 2000). Similarly, some hosts of the shiny cowbird *Molothrus bonariensis*, such as the village weavers on Hispaniola, show higher rejection rates in those areas where the parasite are presents (Cruz et al. 2007), confirming that differences between host population can be explained by the spatial distribution of brood parasites.

In addition to geographic variation, risk of parasitism also varies across a temporal scale as the result of changes in the density of parasite populations over the time. In some cases, when parasitism pressure increases in a certain area, or brood parasites expand their geographical ranges, some hosts respond by increasing their egg-rejection rate. In Guadix, southern Spain, increase in parasitism rate by great-spotted cuckoos *Clamator glandarius* over a decade was followed by a marked increase of egg rejection by common magpies *Pica pica* (Soler et al. 1994, 1998). A similar increase in host defences was also found in one of the major hosts of the common cuckoo in Japan, the azure-winged magpie *Cyanopica cyana*, which was also related to a strong increase in cuckoo parasitism (Nakamura et al. 1998). In other cases, parasite populations experience significant declines over a few years as a result of various ecological factors. In such circumstances, host defences can show a rapid decrease within host populations as parasitism rate decreases. The most extreme response to such decline in parasitism was that found by Soler et al. (2012a) in Los Palacios, southern Spain, where rufous-tailed scrub robins *Cercotrichas galactotes* drastically reduced their egg-rejection rate from about 64.7% to 0% within 10 years following the cuckoos' disappearance from the area. These results are similar to those previously found on Wicken Fen, England, where a decline in cuckoo parasitism from 16% to 2-6% over a 12-year period resulted in a significant decline in egg-rejection behaviour by reed warblers from 75% to 25% during the same period of time (Brooke et al. 1998). Interestingly, this trend has been maintained in this English population during the subsequent 15 years and it may reflect a phenomenon common to other reed-warbler populations across Europe (Thorogood and Davies 2013).

Although changes in host defences as those described above could also be due to rapid evolutionary change, the fact that hosts are able to modify their rejection behaviour in response to changes in parasitism pressure within the same breeding season supports the idea that these variations are due to phenotypic plasticity. For instance, rufous-tailed scrub robins show a drastic decline in rejection rate when cuckoos leave the breeding area to go to their winter quarters (Álvarez 1996; Soler et al. 2012a). Other host species, such as reed warblers, have been also found to weaken their rejection response as the breeding season progresses (Brooke et al. 1998, Lindholm 2000, Thorogood and Davies 2013); although the opposite effect, an increase in rejection rates later in the breeding season, has also been described in some host species of the brown-headed cowbird *Molothrus ater* (Lang et al. 2014). Of course, a seasonal decline in rejection rates could be the consequence of factors linked to life-history traits instead of changes in parasitism pressure, such as inexperienced individuals breeding later (Lotem et al. 1992) or lower chances of re-nesting at the end of the breeding season (Burgham and Picman 1989). However, rufous-tailed scrub robins did not show differences in their rejection rates between young and experienced individuals (Soler et al. 2000), suggesting that phenotypic plasticity is the underlying mechanism behind the within-season changes in the egg-rejection behaviour found in some host species.

Table 1: **Studies showing evidence of host plastic responses towards parasitic eggs.**

Evidence of plastic response	Brood parasite	Host species	Reference
Responses to spatial heterogeneity	*Cuculus canorus*	*Acrocephalus scirpaceus*	Lindholm and Thomas (2000)
	Cuculus canorus	*Acrocephalus scirpaceus*	Lindholm and Thomas (2000)
	Cuculus canorus	*Acrocephalus scirpaceus*	Lindholm (2000)
	Molothrus bonariensis	*Ploceus cucullatus*	Cruz et al. (2008)
Responses to temporal heterogeneity	*Clamator glandarius*	*Pica pica*	Soler et al. (1994)
	Clamator glandarius	*Pica pica*	Soler et al. (1998)
	Cuculus canorus	*Acrocephalus scirpaceus*	Brooke et al. (1998)
	Cuculus canorus	*Cyanopica cyana*	Nakamura et al. (1998)
	Molothrus bonariensis	*Ploceus cucullatus*	Robert and Sorci (1999)
	Cuculus canorus	*Cercotrichas galactotes*	Soler et al. (2012a)
	Cuculus canorus	*Acrocephalus scirpaceus*	Thorogood and Davies (2013)
Seasonal changes in egg rejection	*Molothrus ater*	*Dendroica petechia*	Burgham and Picman (1989)
	Cuculus canorus	*Cercotrichas galactotes*	Álvarez (1996)
	Cuculus canorus	*Acrocephalus scirpaceus*	Brooke et al. (1998)
	Cuculus canorus	*Acrocephalus scirpaceus*	Lindholm (2000)
	Cuculus canorus	*Cercotrichas galactotes*	Soler et al. (2012a)
	Cuculus canorus	*Acrocephalus scirpaceus*	Thorogood and Davies (2013)
	Molothrus ater	*Turdus migratorius*	Lang et al. (2014)
Response to the parasite presence	*Cuculus canorus*	*Acrocephalus scirpaceus*	Davies and Brooke (1988)
	Cuculus canorus	*Anthus pratensis*	Moksnes and Røskaft (1989)
	Cuculus canorus	*Anthus pratensis*	Moksnes et al. (1993)
	Cuculus canorus	*Acrocephalus scirpaceus*	Moksnes et al. (2000)
	Cuculus canorus	*Acrocephalus arundinaceus*	Bartol et al. (2002)
Lack of consistency in egg rejection	*Cuculus canorus*	*Cercotrichas galactotes*	Álvarez (1996)
	Cuculus canorus	*Cercotrichas galactotes*	Soler et al. (2000)
	*Cuculus canorus**	*Sylvia atricapilla*	Honza et al. (2007)
	Molothrus ater	*Quiscalus quiscula*	Peer and Rothstein (2010)
	Cuculus canorus	*Acrocephalus scirpaceus*	Guigueno and Sealy (2012)
Response to parasite retaliation	*Clamator glandarius*	*Pica pica*	Soler et al. (1995)
	Clamator glandarius	*Pica pica*	Soler et al. (1999b)
Recognition without rejection	*Cuculus canorus*	*Acrocephalus scirpaceus*	Lindholm (2000)
	Cuculus canorus	*Acrocephalus arundinaceus*	Moskát and Hauber (2007)
	Cuculus canorus	*Hippolais pallida*	Antonov et al. (2009)
	Cuculus canorus	*Cercotrichas galactotes*	Soler et al. (2012b)
	*Cuculus canorus**	*Turdus merula*	Ruiz-Raya et al. (2015)
	*Cuculus canorus**	*Turdus merula*	Soler et al. (2017)

** Potential host of the common cuckoo*

Flexible responses to brood parasitism are also observable within populations as individuals may respond differently to parasitism and show variations in their individual level of defence, which could be considered valuable evidence of phenotypic plasticity. Several studies have shown that the presentation of a female cuckoo model increases the likelihood of egg rejection in several host species, such as reed warblers (Davies and Brooke 1988), meadow pipits *Anthus pratensis* (Moksnes and Røskaft 1989, Moksnes et al. 1993) and great reed warblers *Acrocephalus arundinaceus* (Bartol et al. 2002) (but see (Lindholm 2000; Soler et al. 2012a)), meaning that individuals exposed to greater risk of parasitism are more likely to reject parasitic eggs, at least in the mentioned species. In fact, naturally parasitized reed warblers reject the parasitic egg more frequently when they discover the cuckoo near the nest (Moksnes et al. 2000). In other cases, individuals show lack of consistency in their rejection behaviour and they vary their response to experimental parasitism as a consequence of the balance between cost and benefits of egg rejection (Soler et al. 2000). In response to repeated parasitism, individual yellow warblers *Setophaga petechia*, may either accept or reject the parasitic egg after recognising it (Guigueno and Sealy 2012). This individual lack of consistency in subsequent parasitism events has been documented even in rarely parasitized species, such as the common grackle *Quiscalus quiscula* (Peer and Rothstein 2010).

One of the most striking examples of conditional host behaviours is that exhibited in response to the mafia tactics used by some parasites, which have been particularly studied in the great spotted cuckoo-magpie system. After parasitism, great-spotted cuckoos systematically punished rejecter magpies by predating those nests where the parasitic egg had disappeared, drastically reducing the reproductive success of hosts and, therefore, the advantage of egg rejection (Soler et al. 1995). The benefit obtained by the great-spotted cuckoo is a greater probability of acceptance of the parasite egg since punished magpies changed their behaviour from rejection to acceptance in subsequent parasitism events (Soler et al. 1999b). Thus, magpie response to the great-spotted cuckoos' mafia tactics reveals the ability of hosts to adjust their response based on previous experience. These changes in host response are more frequent in areas with a high density of brood parasites in which host nests are likely to be parasitized by several cuckoos, which means that magpies can plastically adjust their response according to the risk of suffering a second retaliation (Soler et al. 1999b; Chap. 15).

Host defences are often costly since rejection behaviour may involve the desertion of the complete clutch, recognition errors during egg ejection, the accidental breaking of some own eggs or even retaliation by the parasite. Therefore, flexible defences can be favored by natural selection in order to avoid such costs under certain ecological conditions, such as a low risk of parasitism. The use of video cameras in recognition experiments has made possible the detailed study of host responses to parasitic eggs and it has shown that hosts can recognize more eggs than they finally reject, thus confirming the complex nature of the egg-rejection process. Antonov et al. (2009) found that eastern olivaceous warblers *Hippolais pallida* pecked the experimental egg very often but they did not always eject it because of physical restrictions that made ejection harder, such as the impossibility shown by olivaceous warblers to puncture strong egg shells. But acceptance of previously recognized eggs is not always the outcome of ejection failure, as was revealed by the fact that some host species such as rufous-tailed scrub robins, which can easily eject parasitic eggs, frequently peck the experimental egg but fail to eject it (Soler et al. 2012b). In this case, acceptance may occur if hosts do not have the necessary motivation to assume the potential costs associated with egg ejection, perhaps due to a low risk of parasitism in the area (Soler et al. 2012b). According to this idea, an increase in potential costs of egg ejection would lead to higher acceptance rates, even if hosts have previously recognized the parasitic egg. Indeed, recent studies have experimentally demonstrated that hosts can decide to accept foreign eggs due to physical constraints imposed by certain characteristics of the parasitic egg that do not affect recognition but hinder egg ejection (Ruiz-Raya et al. 2015, Soler et al. 2017).

Why have flexible defences evolved in hosts?

Evidence of flexible behaviours described in the previous section reveal that hosts can adjust their response to the environmental conditions of brood parasitism. But what ecological conditions make plastic responses adaptive for hosts? Why have plastic responses been described in just a few host species? Behaviour, like any phenotypic trait, is either favored or penalized by natural selection as a result of a fitness trade-off between associated costs and benefits under certain ecological contexts. However, ecological conditions are often highly variable and no behavioural trait can be considered consistently optimal, so behavioural plasticity will allow individuals to track rapid environmental changes and respond appropriately. According to (Mery and Burns 2010), the evolution of behavioural plasticity requires four conditions: (i) environmental heterogeneity, (ii) reliable cues, (iii) that benefits of plasticity outweigh the costs, and (iv) the existence of genetic basis to plasticity. Throughout this section, we will discuss the importance of these conditions for the evolution of plastic responses by hosts.

Environmental heterogeneity

Environmental changes force both individuals and populations to rapidly respond and adapt to the current ecological context. This response may occur through genetic changes within populations resulting from microevolutionary processes guided by natural selection. However, environmental heterogeneity sometimes involves variation on such a fine spatiotemporal scale, that individuals respond too rapidly to represent genetic changes within populations. Brood parasite populations are usually variable in space and time, so hosts are likely to experience wide fluctuations in parasitism rates. This variation would make phenotypic plasticity adaptive for hosts and would explain the presence of plastic responses in some host populations. For example, populations of common cuckoo parasitizing reed warblers are usually restricted to small patches in wetland, which makes them prone to local extinction; as a consequence, parasitism rates frequently vary between reed warbler populations and years (Lindholm 1999). In addition, after dispersion, young reed warblers probably occupy territories where the rate of parasitism will be different from that suffered by their parents. But variation in risk of parasitism may also result from annual cuckoo movements among host populations, which might be an adaptive behaviour allowing cuckoos to increase the probability of finding naïve hosts with less effective defences (Lotem et al. 1995, Langmore et al. 2009a, 2012). Recently, the existence of spatiotemporal variation in host use due to habitats constraints has been revealed in the great-spotted cuckoo, indicating that parasitism pressure differs among environments for host species (Baglione et al. 2017). Given this temporal and spatial heterogeneity in parasitism rates, hosts would benefit from the ability to assess changes in the local risk of parasitism and adjust their defences accordingly, leading to the emergence of behavioural plasticity in some host populations.

Reliable cues of parasitism

The ability to rapidly respond to variations in parasitism conditions implies that hosts effectively assess the risk of parasitism in their area. Thus, the evolution of plastic defences requires the use of reliable cues that make possible an accurate monitoring of changes in the risk of parasitism. But how do hosts assess the risk of parasitism to which they are subjected? Hosts might assess the risk of parasitism from direct encounters with parasites. Several studies have found that some hosts increased their rejection rate after observing the parasite near the nest (Davies and Brooke 1988, Moksnes et al. 2000, Bartol et al. 2002); however, this response has been documented in a few species while, in other cases, the presence of the parasite does not seem to be enough to modify the host behaviour (Lindholm 2000; Soler et al. 2012a). Furthermore, some brood parasites such as the cuckoo show secretive habits that make encounters with parasites unlikely (Davies 2000), as well as cuckoo-hawk mimicry makes any inspection approach potentially lethal to hosts (Davies and Welbergen 2008, Welbergen and Davies 2011). Another possibility is that hosts are able to assess the activity of parasites in their own territories and adjust their behaviour based on such information. Brooke et al. (1998) evaluated the effect of proximity to naturally parasitized nests, where parasite activity will be easier to detect, on the rejection rate of reed warblers. Although they did not find greater rejection rates in nests located near parasitized nests, reed warblers from a small unparasitized population just 11 km away did not show rejection behaviour, which suggests that hosts were able to track the cuckoo activity. Therefore, given the scarce and potentially risky nature of direct encounters with parasites, it has been suggested that some hosts may use both direct and indirect cues to assess specific risk of parasitism. Welbergen and Davies (2012) found that nest defence by reed warblers declined with distance to potential cuckoo perches (indirect cue), places from where cuckoo usually locate the host nests and that significantly affect the risk of parasitism (Øien et al. 1996). Interestingly, this effect was found both in parasitized and unparasitized sites, suggesting that direct cues of parasitism, such as the cuckoo presence, can be modulated by the use of indirect cues (Welbergen and Davies 2012).

The existence of reliable cues of parasitism is a necessary condition for the evolution of plastic defences, which could explain the lower degree of plastic responses shown by hosts of other brood parasites. As was suggested by Thorogood and Davies (2013), the degree of specialization between the common cuckoo and its hosts is so high that parasite activity may be a more reliable cue for cuckoo's hosts than for hosts of more generalist parasites. For instance, parasite activity alone might not be a reliable indicator of the risk of parasitism for any particular host species of the brown-headed cowbird given the more generalist nature of this brood parasite, which could difficult the evolution of plastic responses in these hosts species. It is clear (Table 1) that documented cases of plasticity in brown-headed cowbird hosts are much scarcer (only 3 species) than in common cuckoo hosts (8 species). This should not be surprising considering that most cowbird hosts are acceptors and, phenotypic plasticity would be adaptive later, once egg rejection ability has evolved, and, mainly, when the risk of parasitism is highly variable (Soler 2014).

Benefits and costs of plastic responses

Evolution of behavioural plasticity in host responses is necessarily subject to a trade-off between its costs and its benefits, which explain the variation in behavioural plasticity existing among species, populations or individuals. As suggested above, benefits of phenotypic plasticity become noticeable in changing environments, so environmental variation is therefore considered the main selective force for phenotypic plasticity (Moran 1992, Ghalambor et al. 2007, Hendry 2016). Regarding behaviour, spatial and temporal heterogeneity may favour the evolution of different types of behavioural plasticity, namely innate behaviour plasticity and learning (Mery and Burns 2010). Thus, the characteristics of environmental variation will determine the relative benefits of each of these two types of behavioural plasticity. When individuals have to cope with variations within their lifetime, reversible behavioural plasticity will be favoured. In contrast, in those situations where environmental variations occur between generations or exceed the dispersal capacity of individuals, but remain relatively constant within generations, learning costs would be minimized and irreversible plasticity would be favoured (Mery and Burns 2010, Snell-Rood 2013). Some host species may experience significant variations in the rate of parasitism throughout their lifetime, so evolution could favour the appearance of flexible responses (reversible) in such species. On the other hand, when the parasitism pressure remains stable over time, the level of defence could be maintained in the population according to such invariable risk of parasitism (Zölei et al. 2015).

As we mentioned, behavioural plasticity has been proved to be advantageous for hosts when facing environments with variable risk of parasitism. So why do most host species lack this capacity? Given that no trait is infinitely or ideally plastic, the most likely response is that there are restrictions both for the evolution of plasticity and for its maintenance. Studies on the evolution of phenotypic plasticity have suggested the existence of two main types of constraints on the evolution and maintenance of plasticity: *costs*, which lead to lower fitness when a feature is produced through plasticity rather than constitutively; and *limits*, referring to the impossibility of reaching the optimal trait value (DeWitt et al. 1998, Murren et al. 2015). Specifically, costs linked to innate behavioural plasticity may be related to the maintenance of the sensory and regulatory mechanisms to detect environmental changes and a further development of possible motor responses (Snell-Rood 2013). Unlike innate behavioural plasticity, which is usually an immediate response to environmental variations, selection of learning is usually linked to a trial-and-error process. This involves a period of suboptimal behaviour that is usually costly since it requires a considerable investment in time and energy, as well as making errors, what is known as *costs of being naïve* (Mery and Burns 2010). In addition, learning has been also shown to involve important constitutive costs since it requires a remarkable neurobiological and morphological reorganization (Mery and Burns 2010). Therefore, if environmental conditions fail to favour phenotypic plasticity, the costs of plasticity *per se* could lead to the loss of host plastic responses.

Individual experience can enhance the host ability to respond against brood parasitism and results in more effective defences. In some cases, experience is the result of the interaction with other individuals, which can also modify the behaviour and lead to plastic responses. It has been suggested that social transmission of defences (social learning) represents an additional factor in the rapid acquisition of defences by some hosts. For instance, social learning is especially important in the transmission of mobbing behaviour towards cuckoos in reed warblers, which acquire information by observing conspecific from adjacent territories (Davies and Welbergen 2009, Campobello and Sealy 2011). These cues will allow hosts to track fine-scale variations in parasitism risk and respond accordingly in future encounters with parasites. In the case of egg rejection, a similar mechanism of social transmission is unlikely to evolve due to the difficulty for individuals to witness an egg-rejection event on the part of their conspecifics. However, other mechanisms of social transmission have been suggested to explain the extraordinarily rapid increase in rejection rates observed in some host species. Such mechanisms might be based on communicative systems and they would be especially important in species with remarkable cognitive abilities such as the common magpie or the azure-winged magpie (Soler 2011); however, further studies are needed to prove the existence of such mechanisms. Unlike individual learning, social transmission of defences would allow hosts to track environmental changes in risk of parasitism while avoiding the potential costs associated with trial-and-error processes (Mery and Burns 2010), which would benefit the evolution of host plastic defences.

Genetic basis of plasticity

Since phenotypic plasticity is a property of genotypes and it is subject to evolution, an underlying genetic basis would be expected in those host species showing plastic defences. From a general point of view, three models have been proposed to address the genetic basis of plasticity (Scheiner 1993): (i) *overdominance*, which states that plasticity is an inverse function of heterozygosity, (ii) *pleiotropy*, which states that plasticity comes from the differential expression of genes in different environments, and (iii) *epistasis*, which predicts that plasticity results from the interaction between genes that determine the magnitude of the response to environmental factors with genes that determine the mean expression of a trait. Empirical studies reveal that, while there is little evidence for the overdominance model, both pleiotropic and epistatic effects have been proved important in any plastic response, just as is probably the case with most of the phenotypic complex traits (Scheiner 1993, Pigliucci 2005). Therefore, current evidence suggest that specific properties of genotypes could favour the evolution of plastic responses in certain host species.

The egg-rejection process: decision making and the role of motivation

Lack of egg rejection exhibited by some host populations has been considered one of the most puzzling issues in the study of brood parasitism (Stokke et al. 2005), especially considering the high fitness costs linked to rearing the chick of many brood parasites. Over the last few decades, several hypotheses have been proposed to explain the acceptance of parasitic eggs within the framework of the coevolutionary arms race between brood parasites and hosts. Lack of rejection might reflect an initial stage in the arms race between brood parasites and hosts resulting from a recent parasitism, so hosts would not have had enough time to evolve egg-rejection defences (evolutionary-lag hypothesis (Rothstein 1990, Davies 2000)). Another possibility is that acceptance is adaptive under certain circumstances, which would occur whenever costs of maintaining egg rejection are higher that costs of parasitism (evolutionary-equilibrium hypothesis (Lotem et al. 1992, 1995, Lotem and Nakamura 1998)). On the other hand, according to metapopulation dynamics theory, the presence of acceptors within specific populations might be due to gene flow of *acceptor alleles* from non-parasitized populations (Martínez et al. 1999, Soler et al. 1999a), as a possible consequence of the spatial structure of habitats (Røskaft et al. 2002, 2006). In other cases, the evolution of cryptic eggs by the parasite has prevented hosts to evolve recognition abilities, as occurs in some Australian cuckoos (Brooker et al. 1990, Langmore et al. 2009b). Furthermore, as some traits of parasite eggs such as the eggshell thickness can make ejection difficult, some species could accept due to the impossibility to puncture the parasitic egg, which would imply *rejection failures* instead of *recognition failures* (Antonov et al. 2009). However, there is one last possibility to be considered: in some cases absence of egg rejection might reflect *acceptance decisions*, which means that some hosts choose to accept the parasitic egg even after recognition (Soler et al. 2012b; Ruiz-Raya et al. 2015; Soler et al. 2017). Under this framework, host decision-making would play a central role in the egg-rejection process, being affected by the interaction of multiple elements such as clutch characteristics, recognition abilities of host, presence of conditional stimuli and the host genotype (Stokke et al. 2005). The study of cognitive phenotypes in a *judgment and decision-making* framework has been suggested as an important point to gain a better understanding of the processes guiding animal decisions in behavioural ecology studies (Mendensol et al. 2016), which also includes egg-rejection studies in brood parasite-host systems (Ruiz-Raya and Soler, *In prep*). Egg rejection can therefore be understood as a complex and plastic process in which different stages are differentiated, namely: judgment, decision and action itself.

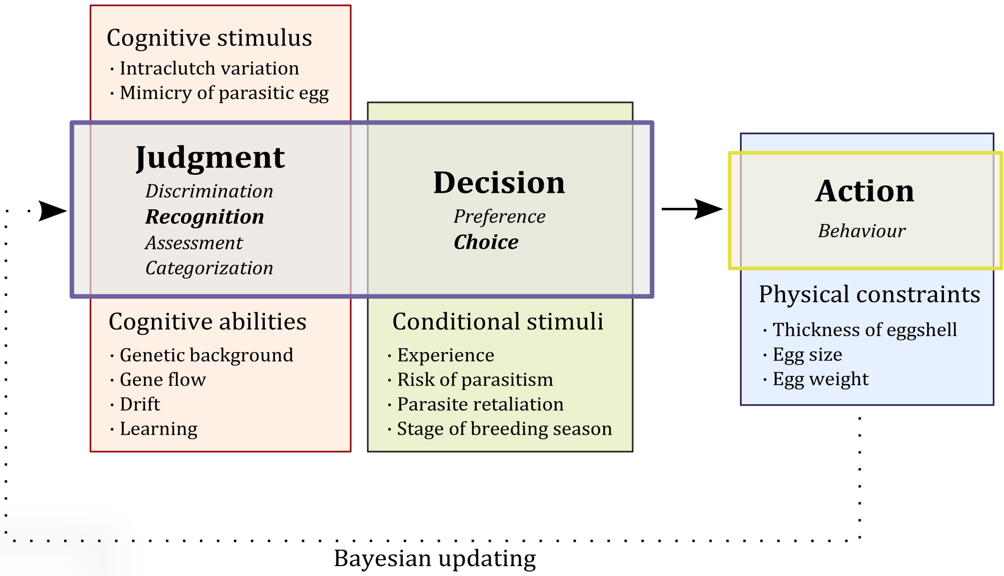

Figure 2: **The egg-rejection process and its three main stages: judgment, decision and action.** Both judgment and decision are involved in the host decision-making (purple box), which will results in the host behaviour (action, yellow box). Each stage of the egg rejection process can be affected independently, which will determine the outcome of the process. Importantly, host are able to add new information regarding parasitism conditions during any phase of the egg-rejection process, even the action stage (i. e. after decision), which may result in an updated decision-making process leading to a new decision (Bayesian updating). In italics, important terms related to each stage of the egg-rejection process are highlighted.

Judgment

Judgments allow hosts to arrive at an understanding of the environment, which is necessary to carry out a specific response towards the parasitic egg. Thus, egg rejection requires that hosts successfully assess information and realize that their nests have been parasitized; that is, recognition of the parasitic egg must occur. As suggested by Ruiz-Raya and Soler (*In prep*), egg-rejection studies require the use of a unified and consistent terminology in which terms such as egg recognition deserve to be properly differentiated from other cognitive abilities affecting judgment, such as discrimination, categorization, or evaluation. According to the authors, *egg discrimination* refers to the cognitive process by which hosts can distinguish two or more different stimuli from the parasitized clutch, and it therefore relies on the intensity of the stimulus and lead to the signal detection needed to initiate the decision-making process (Rodríguez-Gironés and Lotem 1999). On the other hand, *egg recognition* can be considered as the process leading to the host response, which implies that host has been able to identify the odd egg as a parasitic egg. Both intraclutch variation and the mimicry of the parasitic egg will be determining factors affecting both egg discrimination and recognition. Thus, highly-mimetic parasitic eggs will hinder egg discrimination whereas high intraclutch variation will increase the likelihood of committing recognition errors (Stokke et al. 2005). On the other hand, cognitive abilities of hosts can also significantly affect egg recognition. Such cognitive abilities will be determined by the host genotype and, at the population level, egg rejection will be partly affected by the frequencies of different genotypes present in that population. In accordance with the existing parasitism pressure and rejection costs, natural selection will favour or penalize certain genotypes and, along with metapopulation processes such as gene flow or drift, will determine the frequencies of *rejecters* and *acceptors* within populations. When the cognitive stimulus (i. e. the signal) is weak and/or the host's cognitive abilities are not fine enough, the egg-rejection process can be wrecked in the recognition stage and the outcome of the egg-rejection process will be egg acceptance.

Decision

Once the parasitic egg is recognized, hosts must choose among two different options before carrying out the action itself: they have to decide between acceptance or rejection of the parasitic egg. But how do individuals determine which strategy is optimal? During the decision stage, some hosts are able to integrate information from the judgment with that derived from conditional stimuli in a process that will determine their "tendency to rejection" or motivation. *Motivation* is revealed as a crucial concept in the psychology of decision-making and it can be understood as the host's tendency to assume the potential costs of egg rejection under certain parasitism conditions (i. e. trade-offs between costs of egg rejection and risk of parasitism) (Soler et al. 2012b). As previously seen, some hosts vary their response to parasitism according to the current risk of parasitism, their experience or the stage of the breeding season, which leads some of them to accept the parasitic egg. Of course, judgment and decision are deeply linked in the decision-making process since they both depend on the accuracy with which hosts evaluate the available information about parasitism. The combination of prior and new information will allow hosts to improve the evaluation of their current situation, a process known as "Bayesian updating" (Piersma and Gils 2011). As will be seen in the following section, the assessment of these potential costs is continuously updated, even in the last stage of the egg-rejection process (action itself), which can also significantly influence decision-making as it could result in new judgments and therefore new decisions.

Action

Action is the last stage of the egg-rejection process and it can be defined as the specific behaviour resulting from the host decision making. The egg-rejection process can also be interrupted in this last stage since some physical characteristics of parasitic eggs make ejection particularly hard. For example, unusual strong shells difficult ejection to small hosts (puncture ejectors) and can force the acceptance of previously recognized eggs (Antonov et al. 2009). During the action stage, hosts can assess the potential costs of ejection by gathering information on the physical characteristics of the parasite egg that may hinder ejection. For instance, Soler et al. (2012b) suggested that hosts may use weak touches to the parasitic egg as a mechanism to assess the shell strength and therefore the potential costs of egg puncture-ejection. These behaviours contribute to the Bayesian-updating process and allow hosts to add new information to the prior information on parasitism condition, which might lead to new judgments and therefore modify the outcome of the making-decision process (Figure 2). In this context, a low risk of parasitism would involve insufficient motivation to assume potential ejection costs, resulting in acceptance decisions. However, when the conditions of parasitism make the host's motivation higher, hosts could increase the strength and frequency of their pecking in order to puncture the parasitic egg, assuming the possibility of breaking one of their own eggs in the attempt. At this point, if puncture ejection is not possible, higher motivation would be required to desert the nest as it is a much more costly strategy (see Figure 6 in (Soler et al. 2012b)). In grasp ejectors, who eject the parasitic egg by grasping it with the beak and taking it out of the nest, the action stage can be affected by other egg traits such as egg size or weight. In fact, it has been recently shown how such traits can lead to acceptance of previously recognized eggs, or a delay in rejection decisions, when host motivation is not enough (Ruiz-Raya et al. 2015; Soler et al. 2017).

Chapter 5

Phenotypic plasticity and brood parasite-host coevolution

The existence of plastic responses makes it possible for individuals to rapidly respond to changes in ecological conditions affecting their environment. Importantly, these ecological conditions include phenotypes of other individuals with which they interact, and phenotypic plasticity has important consequences on both the strength of ecological interactions and the evolutionary trajectories of the species involved (Agrawal 2001, Fordyce 2006). In brood parasite-host interactions, the existence of fine-tuned plastic responses (i. e. close to the optimal phenotype) allows some hosts to successfully respond to changes in parasitism pressure within populations. When a host is able to rapidly reach optimal behaviour after increases in parasitism rates, parasite fitness will be affected and the potential outcome might be host switching or local extinction of the brood parasite. Phenotypic plasticity would allow certain hosts species, such as reed warbler, to retain their rejection abilities after periods of low parasitism pressure, even when the expression of their defences is reduced (Lindholm and Thomas 2000, Stokke et al. 2008, Thorogood and Davies 2013). This will make it difficult for a future reutilization of the host population by brood parasites since hosts will be able to rapidly respond to changes in the risk of parasitism.

However, although plastic responses have been documented in several host species, phenotypic plasticity in egg rejection seems to be the exception rather than the rule. In most host species, rejection behaviour is a fixed trait retained in the absence of brood parasitism (even after speciation events; see references above), and continuous coevolutionary cycles have been shown to be absent in some brood parasite – host systems (Soler et al. 1998, Rothstein 2001, Peer et al. 2007), having been emphasized that absence of coevolutionary cycles is the most frequent situation in hosts–brood parasite systems (Soler 2014). So what role does phenotypic plasticity play in most host species? Phenotypic plasticity is crucial to tolerate and then adapt to new environmental conditions, allowing populations to move more easily to another adaptive peak (Price et al. 2003, Ghalambor et al. 2007). Therefore, plastic responses would be especially important in the early stages of brood parasitism (Soler 2014), allowing hosts to benefit from the ability to adjust their behaviour to the new selection pressure. As long as the risk of parasitism is variable and unpredictable, hosts that show plastic responses will have higher fitness than those that do not, and phenotypic plasticity will be adaptive. By contrast, if the pressure or virulence of parasitism increases and is maintained over time, plastic responses may cease to be adaptive because of the costs of phenotypic plasticity itself, which could favour the evolution of the canalized phenotype (Pigliucci et al. 2006). This loss of plasticity can be seen as an alteration in the reaction norm (i. e. a flat reaction norm) derived from selection operating only in the new environment (Pigliucci et al. 2006, Ghalambor et al.

2007). In the absence of parasitism, the evolution of relaxed fixed host-defences as response to reduced risk of parasitism will lead to coevolutionary cycles (Nuismer and Thompson 2006); however, egg-rejection behaviour will be maintained unless recognition errors and/or rejection costs are high. The maintenance of egg rejection shown by many species over long periods of time indicates that such costs are insignificant, which has important implications in the evolutionary trajectory of brood parasites and their hosts: coevolutionary cycles are replaced by successful resistance as the main outcome of brood parasite-host coevolution (Rothstein 2001, Soler 2014), forcing parasites to specialize in previously less suitable host species (Soler 2014). Interestingly, Soler et al. (2104) found that: first, fixed responses and absence of coevolutionary cycles are frequent in host-brood parasite systems; and, second, the few species showing phenotypic plasticity in their responses are usually involved in coevolutionary cycles, probably reflecting initial stages of parasitism.

In some cases, phenotypic plasticity results in the emergence of reciprocal phenotypic changes among players in ecological interactions, which has important evolutionary implications (Agrawal 2001). In brood parasite-host systems, mathematical models suggest that emergence of some parasites' strategies such as mafia behaviour might be promoted by the host's plastic responses (Chakra et al. 2014). When retaliation occurrence is moderate, plastic responses will be beneficial for hosts and, at the same time, brood parasites will benefit from the existence of such plastic responses to force the acceptance of the egg parasites by punishing rejecter individuals. As the mafia strategy expands in the population, hosts would benefit from unconditional acceptance of parasitic eggs, leading the parasite population back to a non-mafia strategy. Thus, the occurrence of the mafia strategy within parasite population will oscillate in time and plastic host responses would be crucial for its evolution. The magpies' ability to express plastic defences is a determinant of the evolution of plastic virulence in parasites (e. g. retaliatory behaviours); while the existence of such plastic virulence will favour the maintenance of plastic defences in hosts (see Chap. 17).

Concluding remarks and future directions

In this chapter, we have seen how some host species are able to modify their egg-rejection behaviour according to the perceived risk of parasitism, which reveals the existence of plastic defences against brood parasitism. Egg-rejection plastic responses are favoured by natural selection under highly variable risk of parasitism since it allows hosts to avoid the expression of costly defences when the risk of parasitism is low. Future work should address the mechanisms used by current hosts to accurately assess the risk of parasitism in their sites, which is a necessary condition for the evolution of plastic responses. In addition, further studies should focus on the costs and limitations linked to the host plastic responses, which will determine the maintenance or loss of plasticity under low parasitism pressure. From a general perspective, studies on brood parasite-host coevolution should consider the potential effects of phenotypic plasticity on both the maintenance of host defences over the time and the long-term outcome of ecological interactions. Recent evidence that some potential host species may recognize more eggs than they eventually reject indicates that egg rejection can be viewed as a complex process in which multiple components interact, and where decision-making and host motivation play a central role. On the other hand, the existence of phenotypic plasticity remains untested in many potential host species. Thus, egg recognition experiments in different populations and/or under different risk of parasitism need to be made in most potential host species. These results, when available, will open a new avenue of research in which comparative studies would allow to answer crucial questions about the evolution of egg rejection. More work is also required to clarify the possible role of social learning on the defence acquisition by hosts and its importance on egg rejection. Finally, in view of the significant decline experienced by some parasites during the last years, phenotypic plasticity will be crucial to understand how host populations adapt to such changing environment.

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Chapter 6

Egg rejection in blackbirds *Turdus merula*: a by-product of conspecific parasitism or successful resistance against interspecific brood parasites?

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Abstract

Traditional theory assumes that egg recognition and rejection abilities arise as a response against interspecific brood parasitism (IBP). However, rejection also appears in some species that are currently not exploited by interspecific parasites, such as *Turdu*s thrushes. Recent evidences suggest that rejection abilities evolved in these species as a response to conspecific brood parasitism (CBP). To test these two alternative hypotheses, we performed an experimental study by parasitizing nests of the common blackbird (*Turdus merula*) with conspecifics or heterospecific eggs under different risk of parasitism (presence of interspecific or conspecific parasites near the nest). Common blackbird is a potential host of the common cuckoo (*Cuculus canorus*) but suffers low levels of CBP too. We found that blackbirds were able to recognize and eject heterospecific eggs at high rates whereas most of conspecifics eggs were not recognized and, therefore, accepted. Ejection rates of conspecific eggs did not exceed 13%, even in situations of high risk of CBP (blackbird female placed near the nest), which contradict the main prediction derived from the CBP hypothesis. Conversely, ejection rates of experimental eggs simulating IBP were much higher (80-100%). Furthermore, female blackbirds were more aggressive towards cuckoos than towards blackbird dummies. Our results considered together support the IBP hypothesis, indicating that recognition and rejection of parasitic eggs in blackbirds have probably evolved due to previous cuckoo parasitism. The current absence of IBP in blackbirds may be due to the highly efficient rejection abilities in this species. Thus, these abilities have been retained in absence of brood parasitism due to the low costs involved for blackbirds, resulting in a successful resistance against interspecific brood parasitism.

Introduction

Interspecific brood parasitism (IBP hereafter) generally imposes high fitness costs on hosts since the parasitic chick is usually better at competing for food or evicts all host offspring (Davies 2000, 2011). Under this strong selective pressure, many hosts have evolved defences against brood parasitism operating at every phase of the breeding cycle. Meanwhile, brood parasites have also evolved counter-defences in response to successive stages of host defence, resulting in a coevolutionary arms race between brood parasites and their hosts (Davies 2000, 2011, Soler 2014a).

Rejection of the parasitic egg is the most widespread and effective defence used by hosts against IBP (Davies 2000). In response to this, brood parasites have evolved mimetic eggs whose degree of mimicry is related with the strength of host rejection (Brooke and Davies 1988, Stoddard and Stevens 2011). Therefore, it has usually been assumed that recognition and rejection abilities in hosts arise as a response against IBP (Rothstein 1990, Davies 2000, Soler 2014a).

But rejection behaviour also appears in species that are currently not exploited by interspecific brood parasites, which has traditionally been considered as evidence of ancient history of IBP (Rothstein 1990, Davies 2000, Soler 2014a). However, it has also been suggested that conspecific brood parasitism (CBP hereafter; i.e. parasitic females laying eggs in nests of their own species (Yom-Tov 1980)) could also account for egg rejection (Grendstadl et al. 1999, Stokke et al. 2002, Peer et al. 2011). This argument has been used regarding thrushes in previous studies (Grendstadl et al. 1999, Polačiková and Grim 2010, Grim et al. 2011, Polačiková et al. 2013, Samas et al. 2014a). Samas et al. (2014a), in an experimental study with two species of *Turdus* thrushes: the common blackbird (*Turdus merula*; blackbird hereafter) and the song thrush (*Turdus philomelos*), concluded that egg discrimination in thrushes has evolved as a response to CBP instead of IBP based on the ejection rate of conspecific eggs found in their study (20-40%) and the existence of CBP in their blackbird populations (CBP rates of 3.1% and 0% in the areas of sympatry and allopatry with the common cuckoo - *Cuculus canorus*; cuckoo hereafter -, respectively). They found that conspecific eggs were ejected more often in the population of higher breeding densities, which is interpreted as a response of blackbirds to the perceived risk of conspecific parasitism. In their work, Samas et al. (2014a) assume (1) that thrushes are unsuitable hosts that have not been involved in a long-term coevolutionary history with the cuckoo, and (2) that blackbird defences have to decline in the absence of the selection pressures that favoured them (i. e. IBP). Based on these two points, they proposed that IBP is unlikely to be the factor responsible of the evolution of egg rejection in thrushes and suggested that CBP constitutes an evolutionary scenario comparable to IBP that could produce the same antiparasitic adaptations in hosts. This is an important conclusion with great impact in the field of brood parasitism that deserves to be studied in detail. On the other hand, these arguments have been recently discussed by Soler (2014b), who suggested that conclusions from Samas et al. (2014a) were based on unclear predictions and, therefore, should be treated with caution. As Soler (2014b) argued, the evolution of abilities to discriminate and eject conspecific eggs is rare in species that suffer CBP because of two reasons. First, due to the high similarity between host eggs and those laid by the conspecific female, which entails that hosts of conspecific parasites require a more subtle level of recognition than those who are exploited by interspecific parasites, making recognition much more difficult to evolve than in hosts of interspecific brood parasites (Petrie and Møller 1991, Rothstein and Robinson 1998, Peer and Sealy 2004, Soler et al. 2011). Second, while IBP imposes dramatic fitness costs to hosts (see above), costs resulting from CBP are much lower, which reduces the strength of selection for defences to evolve (Petrie and Møller 1991, Davies 2000, Lyon and Eadie 2008, Soler et al. 2011, Poláček et al. 2013). In fact, current available information shows that CBP almost never selects for egg rejection: CBP has been documented in 234 avian species (Yom-Tov 2001), but egg rejection has only evolved in a few species. In altricial birds, the evolution and maintenance of rejection defences as consequence of CBP has only been reported in the house sparrow (*Passer domesticus*) (Kendra et al. 1988, Moreno-Rueda and Soler 2001, Soler et al. 2011) and Eurasian tree sparrows (*Passer montanus*)(Poláček et al. 2013), species for which an evolutionary history of relationships with interspecific brood parasites is also likely (Soler et al. 2011). Furthermore, there are no reasons to think that the existence of rejection abilities in blackbirds could not have evolved in response to IBP because the maintenance of rejection abilities (successful resistance) in the absence of brood parasitism is a frequent long-term outcome of the relationships between interspecific brood parasites and their hosts (Rothstein 2001, Honza et al. 2004, Peer and Sealy 2004, Soler 2014a). According to calculations in Soler (2014a), 29.7% of potential host species that are not currently parasitized reject nearly 100% of nonmimetic eggs.

In European thrushes, parasitism by the cuckoo was documented in all six species that occur in Europe, but parasitism rates were lower than those in current cuckoo hosts (Moksnes and Røskaft 1995), so European thrushes are currently considered not impacted by IBP. Despite this, thrushes species are able to reject foreign eggs from the nest at high rates (Davies and Brooke 1989a, Grim et al. 2011, Martín-Vivaldi et al. 2012, Soler et al. 2015) and some species are reluctant to feed cuckoo nestlings experimentally introduced in their nests (Grim 2006, Grim et al. 2011). Moreover, aggression towards cuckoo dummies has been experimentally demonstrated in thrushes (Røskaft et al. 2002, Grim et al. 2011), suggesting that IBP was the selective force that selected for egg rejection in this group. Previous studies have classified blackbirds as either suitable (Davies and Brooke 1989a) or unsuitable host (Grim et al. 2011) for the cuckoo. Grim et al. (2011) concluded that blackbirds were not involved in long-term coevolution with the cuckoo because no cuckoo gens have been found for any *Turdus* species. However, this conclusion is based on an analysis of cuckoo and host eggs from collections of European museums (Moksnes and Røskaft 1995) and takes into account a relatively short period of time (only a few centuries) of the interactions between cuckoos and their hosts (tens of thousands of years; (Gibbs et al. 2000)). Furthermore, under this scenario, the experimentally demonstrated existence of aggression towards cuckoo dummies and reluctance to feed cuckoo nestlings in thrushes (Grim et al. 2011) remain unexplained. These defences are especially developed in the blackbird, which attack more frequently a cuckoo dummy than a predator one (49.2% vs. 33.3%) and were reluctant to feed even lone cuckoo nestlings (Grim et al. 2011).

Thus, the origin and maintenance of rejection abilities in thrushes is an interesting evolutionary question that deserves more attention, especially given that previous studies that have addressed this issue did not show conclusive results (Samas et al. 2014a, b). Therefore, the main aim of this study is to clarify whether the cause of rejection behaviour in the blackbird is a by-product of conspecific parasitism (CBP hypothesis) or evolved in the past as a defence against interspecific brood parasites (IBP hypothesis). To do so, we carried out an artificial parasitism experiment with blackbirds by manipulating the risk of IBP or CBP simultaneously. Our experimental design expands previous research in two important aspects. First, the risk of IBP or CBP is directly manipulated by presenting a dummy of a cuckoo or a blackbird, respectively. Second, we distinguish between recognition abilities and rejection of the parasitic model eggs. Studies of artificial parasitism focused on discrimination abilities should do such differentiation (Ruiz-Raya et al. 2015) due to the existence of plastic responses of hosts against the parasitic egg (Davies et al. 1996, Antonov et al. 2008a, b, 2009, Stokke et al. 2008, Svennungsen and Holen 2010, Soler et al. 2012, Ruiz-Raya et al. 2015). We tested the following predictions on different aspects of anti-parasitic defences (see Table 1):

Recognition of parasitic eggs

If IBP selected for egg discrimination, then heterospecific eggs (Figure 1b) should be much better recognized than conspecifics eggs (Figure 1a) given that a much finer level of discrimination is required to recognize conspecific compared to heterospecific eggs (Prediction 1a)(Reeve 1989). In contrast, (Prediction 1b) if CBP selected for egg discrimination, cognitive abilities needed to recognize parasitic eggs evolved in blackbirds as a response to conspecific eggs (highly mimetic eggs both in size and colour). Under this scenario, we predicted that heterospecific (less mimetic) and conspecific eggs should be recognized at a similar level, as occurs in house sparrows (*Passer domesticus*) (Moreno-Rueda and Soler 2001), the only species in which rejection abilities have probably evolved as a consequence of CBP (Soler et al. 2011). However, it is well-known that egg recognition is usually conditioned by the degree of egg mimicry (Brooke and Davies 1988, Davies and Brooke 1988). Thus, even if the egg discrimination ability would have evolved under selection from CBP, the non-mimetic heterospecific eggs will probably be more rejected than the highly mimetic conspecific eggs. Therefore, in order to be conservative, our prediction here is that conspecific eggs should be recognized at a similar or slightly smaller rate than heterospecific eggs.

Table 1: **Summary of predictions derived from IBP and CBP hypotheses.**
$\leq$ means a similar or lightly smaller rate.

Prediction	IBP Hypothesis (a)	CBP Hypothesis (b)
1. Recognition	CBP eggs $<$ IBP eggs	CBP eggs $\leq IBPeggs$
2. General ejection rate	CBP eggs $<$ IBP eggs	CBP eggs $\leq IBPeggs$
3. Ejection and risk of parasitism	Higher under IBP risk	Higher under CBP risk
4. Aggression	Blackbird $<$ Cuckoo	Blackbird $>$ Cuckoo

Ejection of parasitic eggs

(Prediction 2a) If IBP selected for egg discrimination, then heterospecific eggs should be ejected at higher rates than conspecifics eggs (see Prediction 1a). Conversely, (Prediction 2b) if CBP selected for egg discrimination (i.e. egg recognition have evolved in blackbirds to be able to recognize mimetic eggs) then both conspecific and heterospecific eggs will probably be ejected at similar rates. Moreno-Rueda and Soler (2001) found that house sparrow (*Passer domesticus*), a species with CBP, rejects mimetic (i.e. conspecific) and non-mimetic (i.e. heterospecific) eggs at similar rates. However, for similar reasons to those explained in Prediction 1b, our prediction is that conspecific eggs should be ejected at a similar or slightly smaller rate than heterospecific eggs.

Ejection and risk of parasitism

(Prediction 3a) If IBP selected for egg discrimination, ejection rates should be higher in situations of higher risk of IBP. The plastic response of hosts in egg rejection behaviour according to the perceived risk of parasitism (i. e. after they have encountered a cuckoo near their nests) has been documented in many cases (Moksnes and Røskaft 1989, Moksnes et al. 1993, Lindholm and Thomas 2000, Bartol et al. 2002). However, in this context, it is also predictable that conspecific model eggs are accepted, even in a situation of high IBP risk, if abilities to discriminate conspecific eggs are not fine enough (see Prediction 1a). On the contrary, (Prediction 3b) if CBP selected for egg discrimination, ejection rates should be higher in situations of a clear risk of CBP.

Aggression

(Prediction 4a) If IBP selected for egg discrimination, then cuckoo dummies should be more attacked than blackbird or control dummies. Many host species are able to recognize brood parasites near their nests and respond to them aggressively (Gill et al. 1997, Davies and Welbergen 2008, Welbergen and Davies 2009, Campobello and Sealy 2010). Alternatively, (Prediction 4b) if CBP selected for egg discrimination, then blackbird dummies should be more attacked than cuckoo or control dummies.

Ethical Note

The filming of adults or placement of dummies did not cause any negative effect on blackbird egg hatchability relative to natural nests. Research has been conducted according to relevant national (Real Decreto 1201/2005, de 10 de Octubre) and regional (permissions provided by Consejería de Medio Ambiente de la Junta de Andalucía) guidelines.

Methods

Study site and species

We carried out experiments in the Valley of Lecrín (Southern Spain, 36° 56′ N, 3° 33′ W; 580 m a.s.l.) from March to May 2014. The study area is dominated by orange groves, in which blackbirds usually nest. For a detailed description of the population, see (Ibáñez-Álamo and Soler 2010). The common cuckoo is not currently present in the study site but there are evidences of their presence in the area until thirty years ago (personal information).

The blackbird, one of the most common thrushes in Europe, is a potential host species for cuckoos, but it is currently rarely parasitized (Grim et al. 2011). This species has frequently been used as a model species in egg-recognition experiments (e.g. (Polačiková and Grim 2010, Grim et al. 2011, Samas et al. 2011, 2014a, Martín-Vivaldi et al. 2012, Ruiz-Raya et al. 2015, Soler et al. 2015)), which have provided us detailed information about their response to experimental foreign eggs.

Experimental procedure and data collection

We actively searched for blackbird nests in the study area throughout the breeding season of 2014. Once a nest was located, we checked it using a mirror to determine its content. The nest was visited every two days to obtain data on laying date and clutch size. To determine blackbird responses to parasitism, nests were experimentally parasitized and parents were exposed to different parasitism risk situations. We created six different treatments by combining two factors: type of parasitic egg (conspecific or heterospecific) and risk of parasitism (risk of CBP, risk of IBP and control). Each nest was randomly assigned to one of these six treatments. CBP was simulated by introducing a real conspecific egg from previously deserted nests of the same population (Figure 1a). To simulate IBP, we used real house sparrow (*Passer domesticus*) eggs from deserted nests of a captive population maintained at the University of Granada (Figure 1b). The use of real eggs excludes the potential problems of other types of model eggs used in artificial parasitism experiments (i.e. clay, plasticine...) like an increased costs of rejection and/or nest desertion (Martín-Vivaldi et al. 2002). Furthermore, house sparrow eggs are similar in size to cuckoo eggs from the south of Spain (Martín-Vivaldi et al. 2002). We used non-manipulated real eggs of house sparrows to ensure that all parasitized nests (CBP and IBP) were in the same conditions. Eggs were introduced into the nests during the laying (minimum of two eggs laid) or incubation (at most the ninth day from the onset of lay) stages and each nest was tested only once. Previous studies have shown that blackbirds reject experimental eggs at similar rates in both laying and incubation stages (e.g. (Davies and Brooke 1989a, Honza et al. 2005, Polačiková and Grim 2010, Grim et al. 2011)). Before experimentally parasitizing a blackbird nest, we numbered all eggs near the blunt pole using a non-toxic marker; previous studies have shown that marks on the blunt pole does not affect host responses to eggs (Polačiková et al. 2013, Hauber et al. 2014, Samas et al. 2014a).

Figure 1: **Blackbird nest parasitized with a (a) conspecific or (b) heterospecific egg.** Parasitic eggs are indicated with a white arrow.

We placed a video camera (Panasonic HDC-SD40) near the nest (2 – 2.5 m) and filmed normal blackbird behaviour for 1.5 hours before egg introduction ("previous session"). After the experimental parasitism, we continued filming for the following two hours ("egg session") in order to record the blackbirds' responses to the parasitic egg and their nest attendance. We followed a standardized procedure previously used in other studies with this species (Ibáñez-Álamo and Soler 2012, Ruiz-Raya et al. 2015, Soler et al. 2015). The video camera was placed as high as possible in order to film all the eggs; unfortunately, this was not possible in all cases so we used differences in touches between "previous" and "egg" session to determine the recognition of the parasitic model eggs. The placement of a camera near the nest did not affect blackbird behaviour in relation to egg-recognition experiments (Ruiz-Raya et al. 2015, Soler et al. 2015).

Immediately after introducing the parasitic egg, a painted wooden dummy was placed near the blackbird nest (2-3 m) in order to simulate a risk of parasitism. In all cases we ensured that dummies were easily seen from the nest and the surrounding area. We used female cuckoo (simulating risk of IBP), blackbird (simulating risk of CBP) or turtle dove dummies (*Streptopelia turtur*, control). The turtle dove is a frequent species in our study area that shows a neutral interaction with the blackbird (pers. obs.). We used three specimens of each dummy type. Blackbird responses did not differ between specimens, so we pooled the data (results not shown). After placing the dummy close to the blackbird nest, one of us hid in the area with a camouflage tarpaulin and observed the response of the focal female blackbird towards dummies for 5 min after she appeared in the vicinity of the nest and became aware of the dummy. We noted the latency to the first arrival and the minimum distance from the dummy. The reaction of blackbirds was noted following the scale (from 1 to 4) proposed by Moksnes et al. (1990): (1) "No reaction", when females remain near the nest ignoring the dummy and even returned to the nest in some cases and began to incubate the eggs, (2) "distress calls", when blackbird stay in the area and uttered distress or alarm calls, (3) "mobbing", when females performed flights around the dummy or dives close to it but without touching it, and (4) "attack", when blackbird attacked the dummy with a strong contact. Following the methodology used by Røskaft et al. (2002), we pooled "no reaction" and "distress calls" behaviours as "no aggression", and "mobbing" and "attack" behaviour as "aggression". We scored one additional response as "fear" when the blackbird suddenly left the area of the nest obviously frightened after seeing the dummy, in some cases with a strong alarm call. We considered "fear" as "no aggression" for the aggression analyses. We presented only one type of dummy (cuckoo, blackbird or turtle dove) near each nest. To standardize this data, all observations were made by the same author (FRR).

After the two recording hours, we checked for the introduced egg. If the model egg remained in the nest, we checked it again after 24 hours and continued visiting the nest for the following five days to determine the ejection time. This five-day period has been used in other egg-rejection experiments conducted in thrushes (e.g. (Honza et al. 2005, 2007, Polačiková and Grim 2010, Samas et al. 2011, Ruiz-Raya et al. 2015, Soler et al. 2015)). When the model egg disappeared, we assigned the ejection time considering that the ejection occurred between the last two visits adding 12 hours to the time (in hours) of the last visit in which the introduced model egg was still present. We considered the egg was "accepted" if it remained intact at the nest for five days after its introduction. All eggs (blackbird and introduced) were inspected during each visit to look for possible cracks or broken eggs (ejection costs) or mistakenly ejected eggs (recognition costs). We estimated the CBP rate in our population from those nests found during the nest building or laying stage (one egg) by checking these nests every day during the laying stage in order to find cases in which two eggs were laid per day (Samas et al. 2014a).

Variables and statistical procedures

We used the recordings to extract information related to nest attendance and egg recognition. We analysed three different variables to assess the nest attendance of females after encountering the dummies: (1) the time taken by the females to return to the nest (latency), (2) time that females spent at the nest per hour (time at the nest) and (3) time spent by females inspecting the nest, corrected by the time spent at the nest (nest checking). Regarding egg recognition, we used three variables following the procedure of Ruiz-Raya et al. (2015): (1) "first-contact touches first visit" (number of times the female touched the eggs with its bill from her arrival to the nest until she sat on the nest for the first visit), (2) "first-contact touches per visit" (similar to the previous variable but for the complete filmed period corrected by the number of visits) and (3) "incubation touches" (number of times the female touched the eggs with its bill during interruptions of incubation corrected by the incubation time). For analysis, we utilized mainly Generalized Linear Mixed Models (GLMM) by using *lme4* (R package v.1.1-10 (Bates D et al. 2015)). We included female identity as random factor and the following predictors: dummy (species of dummy placed near the nest), session (before and after the experimental parasitism), D*S (interaction between dummy and session) and clutch size (number of eggs in the nest during the trial). Conspecific and heterospecific model eggs were analysed separately and Laplace approximation of likelihood was used for the parameter estimation. This approach does not allow F-test for fixed effects, so we report the 2 statistics from the likelihood ratio test (LRT) between models. We performed an additional analysis to assess differences in recognition between conspecific and heterospecific model eggs considering only the "egg session". To do this, we performed a negative binomial generalized linear model (GLM) by using *MASS* (R package v.7.3-45 (Venables and Ripley 2015)) in order to deal with overdispersion. Time at the nest was analysed by using Linear Mixed Model (LMM). In this case, we adjusted our model by REML using the *lme4* R package and checked the model assumptions.

To assess the response of females to experimental parasitism (ejection) we used Generalized Linear Models (GLM's: binomial error and logit link function). We included the following predictors in the model: egg, dummy, E*D (interaction between egg and dummy) and clutch size. We also used GLM's (binomial error and logit link function) to analyse the response of female towards dummies regarding aggression (aggression or no aggression, see above) and fear (yes or no). Differences between levels were compared by using *multcomp* (R package v.1.4-1 (Bretz et al. 2015). All analyses were performed using R version 3.1.1 (R Core Team 2014).

Results

We conducted our experiment in 104 blackbird nests. 14 of them were not used in the ejection analyses, because they were predated (11 nests) or deserted (3 nests) before the end of the trial. We assumed that nest desertion is not a response to experimental parasitism in blackbirds (Soler et al. 2015). We found two nests where two new eggs were laid per day, so estimated CBP rate in our blackbird population was 2.9% (n = 68).

Table 2: **Models used from analyses of egg recognition.** Results from LRT for the recognition models of the three variables used: first-contact touches first visit, first-contact touches per visit and incubation touches. In all cases, significant predictors are in bold.

	df	χ^2	p	df	χ^2	p
First-contact touches first visit						
Dummy	2	0.16	0.92	2	4.10	0.13
Session	1	1.00	0.32	**1**	**113.6**	**<0.001**
D*S	2	4.11	0.13	2	5.00	0.08
Clutch size	2	0.46	0.50	2	2.38	0.12
First-contact touches per visit						
Dummy	2	1.44	0.49	2	0.38	0.82
Session	1	0.58	0.45	**1**	**63.59**	**<0.001**
D*S	2	0.99	0.63	2	1.54	0.46
Clutch size	2	0.06	0.80	2	1.29	0.26
Incubation touches						
Session	1	1.97	0.16	**1**	**245.1**	**<0.001**
Dummy	2	0.21	0.90	2	1.40	0.50
D*S	2	4.16	0.12	2	2.86	0.24
Clutch size	2	1.65	0.20	2	0.17	0.68

Recognition of parasitic eggs

We found differences in recognition between conspecific and heterospecific parasitic eggs in all variables used in our recognition analyses (Figure 2). Heterospecific model eggs introduced in the nest ("egg session") were more touched by females than their own eggs ("previous session") for first-contact touches in the first visit (LRT: χ^2 = 113.6, df = 1, p < 0.001; N = 104; Table 2a; Figure 2), taking all the visits together (first-contact touches per visit, LRT: χ^2 = 63.59, df = 1, p < 0.001) and considering touches during incubation (LRT: χ^2 = 245.1, df = 1, $p < 0.001$). However, females touched conspecific eggs as often as they touched their own eggs for all recognition variables (all cases $p > 0.5$; Table 2a; Figure2). These results indicate that blackbirds are able to recognize heterospecific but not conspecifics eggs, which supports the IBP hypothesis (Prediction 1a). Other predictors, such as risk of parasitism, did not explain variation in recognition touches by blackbirds for both heterospecific and conspecific parasitic eggs (Table 2a).

Considering only the "egg session", heterospecific eggs were significantly more touched than conspecific parasitic eggs for first-contact touches first visit ($t = 3.87$, df = 1, $p < 0.001$, N = 104), the number of first-contact touches per visit ($t = 4.30$, df = 1, $p < 0.001$, N = 104) and incubation touches ($t = 2.09$, df = 1, $p = 0.04$, N = 104).

Egg ejection

Model egg (conspecific or heterospecific) was the only predictor that explained the variation in the response of blackbirds to experimental parasitism (GLM: $\chi^2 = 71.15$, df = 1, $p < 0.001$; Table 2b; Figure 3). Thus, heterospecific eggs were significantly more ejected than conspecific parasitic eggs (Tukey HSD: $p < 0.001$), which fits with the IBP hypothesis according to Prediction 2a. Ejection rate of conspecific parasitic eggs was lower than that of heterospecific eggs irrespectively of the risk of parasitism (GLM: $\chi^2 = 2.63$, df = 2, $p = 0.27$, Table 2b), which also supports the IBP hypothesis (see Prediction 3a). Clutch size had no effect on blackbird rejection responses to experimental parasitism (see Table 2b). No recognition or ejection costs were found in our study.

Table 3: **Generalized linear model used to test blackbird rejection behaviour to our experimental manipulation.** In all cases, significant predictors are in bold

	df	χ^2	p
Egg	**1**	**71.15**	**< 0.001**
Dummy	2	1.11	0.57
E*D	2	2.63	0.27
Clutch size	2	2.10	0.15

Response towards dummies

Blackbird responses (in terms of aggression) towards dummies were significantly different depending on the species of dummy placed near the nest (GLM: $\chi^2 = 91.03$, df = 2, $p = 0.001$, N = 104). Females were more aggressive towards cuckoo (45.7% of cases) than blackbird (17.1% of cases) (Tukey HSD: $p = 0.049$; Figure 4) or turtle dove dummies (5.9% of cases) (Tukey HSD: $p = 0.01$). No differences were found between blackbird and turtle dove dummies regarding aggression by blackbirds (Tukey HSD: $p = 0.49$). These results support the IBP hypothesis according to prediction 4a.

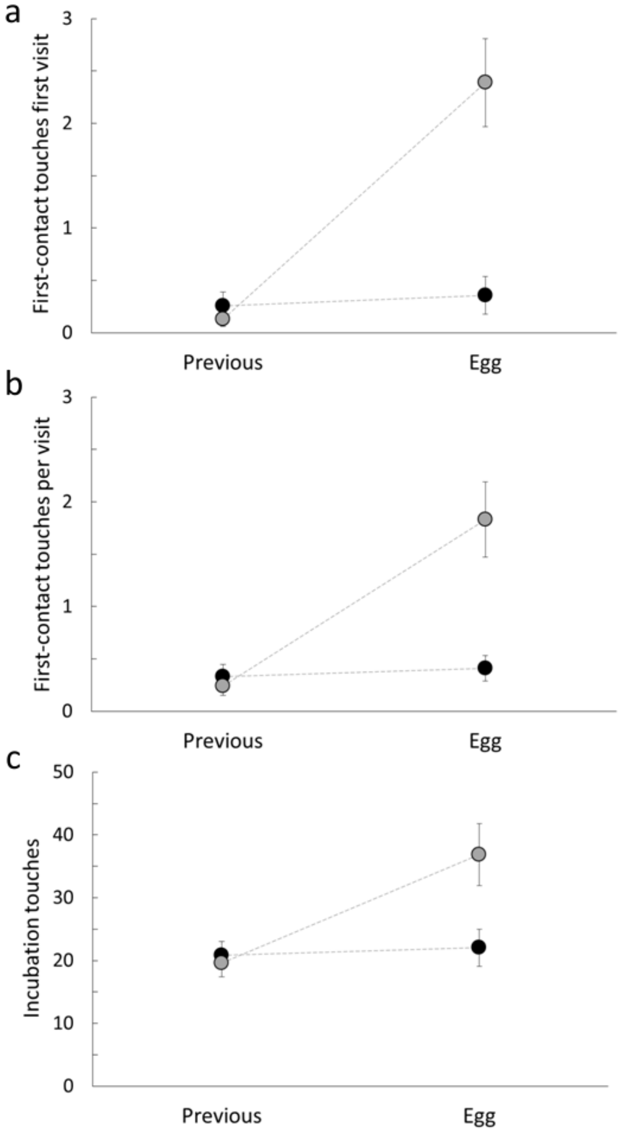

Figure 2: **Recognition of conspecific (black circles) and heterospecific model eggs (grey circles).** Differences between "previous session" (before parasitism) and "egg session" (after parasitism) for nests that received either conspecific or heterospecific eggs, regarding: a first-contact touches first visit (number of touches in the first visit), b first-contact touches per visit (number of touches for all visits corrected by the number of visits) and c incubation touches (number of touches during incubation corrected by the incubation time); see Methods section for a more detailed explanation of each variable. We show nests separately (i. e. receptors of conspecific or heterospecific eggs) in the "previous session" in order to clearly illustrate differences between both sessions for the two types of nests. Values are presented as means ± SE.

The type of dummy placed near the nest also had a significant effect in the "fear" response (see Methods for a detailed explanation) of blackbirds (GLM: $\chi^2 = 61.68$, df = 2, $p < 0.001$, N = 104). Thus, females were more often scared in the presence of a cuckoo dummy (31.4% of cases) than in the presence of a blackbird (2.9% of cases) (Tukey HSD: p = 0.03) or a turtle dove dummy (2.9% of cases) (Tukey HSD: p = 0.03). However, there were no differences for this behaviour between blackbird and turtle dove dummies (Tukey HSD: p = 0.1).

Latency of females to arrival was not affected by the type of dummy placed near the nest, either for conspecific (LRT: $\chi^2 = 1.93$, df = 2, p = 0.38, N = 101) or for heterospecific eggs (LRT: $\chi^2 = 0.43$, df = 2, p = 0.81). Furthermore, females did not modify their time at the nest after encountering any dummy near the nest for both conspecific (F = 0.97, ddf = 53.99, p = 0.39, N = 88) and heterospecific eggs (F = 0.78, ddf = 28.00, p = 0.47). In contrast, nest checking behaviour was affected by the type of egg introduced, and partially by the type of dummy presented. We found a significant increase in nest checking in nests parasitized with heterospecific eggs (LRT: $\chi^2 = 32.32$, df = 1, $p < 0.001$, N = 88) regardless of the type of dummy placed near the nest (LRT: $\chi^2 = 3.18$, df = 2, p = 0.2). Regarding those nests parasitized with conspecific eggs, we found a significant increase in nest checking only in those females who had encountered a cuckoo dummy (LRT: $\chi^2 = 5.87$, df = 2, p = 0.05).

Discussion

The origin and evolution of rejection abilities in species that are not frequently exploited by interspecific brood parasites has been previously addressed in several studies (e. g. (Grendstadl et al. 1999, Polačiková and Grim 2010, Grim et al. 2011, Polačiková et al. 2013, Samas et al. 2014a)). In this work, we experimentally tested for the conspecific or interspecific origin of the rejection behaviour in the blackbird by artificially parasitizing natural nests with conspecific or heterospecific eggs under different risk of parasitism. However, since recognition is not always followed by rejection of the parasitic egg (Moskát and Hauber 2007, Antonov et al. 2008b, 2009, Soler et al. 2012, Ruiz-Raya et al. 2015), it is necessary to conduct experimental studies that provide information on both rejection and discrimination abilities. Regarding recognition, heterospecific eggs were easily recognized by females from the first contact with the parasitic egg. Female blackbirds touched the heterospecific model eggs repeatedly both on arrival at the nest and during incubation (Figure 2) indicating egg recognition. However, when nests were parasitized with conspecific eggs, females did not recognize them during the two first hours after the experimental parasitism, which supports the IBP hypothesis according the Prediction 1a. Clearly, the high similarity between host eggs and conspecific model eggs makes difficult the recognition of the latter. This supports the previously suggested idea that discrimination abilities are much more difficult to evolve in hosts suffering only CBP than in those parasitized by heterospecifics (see above).

The initial absence of recognition of CBP eggs was confirmed in the ejection rate, since acceptance was the most common response against conspecific parasitic eggs (Figure 3). Consequently, conspecific eggs were ejected at much lower rates (less than 13%) compared with ejection rates of heterospecific eggs (nearly 100%), which also supports the IBP hypothesis (Prediction 2a). Although the CBP hypothesis may also predict a higher ejection rate of heterospecific than conspecific eggs (see Prediction 2b above), the very low ejection rates of conspecific eggs found in our study do not support the CBP hypothesis. This is not striking because experimental studies have shown that many rejecters of IBP eggs often show low or no ability to reject CBP eggs (Davies and Brooke 1989a, Welbergen et al. 2001, Croston and Hauber 2014), but see (Procházka and Honza 2003, Honza et al. 2004). In our study, the few cases of ejection of a conspecific egg could be explained by the fine ability of blackbirds to recognize IBP eggs, which would enable them to recognize some CBP eggs, probably those less similar to their own eggs, in terms of colour or shape, as occurs in some species (e. g. (Marchetti 2000)). Regarding ejection rate and the perceived risk of CBP or IBP, we found that conspecific eggs were systematically ejected at low rates regardless of the presence of a blackbird dummy (Figure 3), contradicting Prediction 3b derived from the CBP hypothesis. Conspecific model eggs were also ejected at low rates in the presence of a cuckoo dummy, which could be expected according the IBP hypothesis (Prediction 3a) since, in this context, recognition of conspecific eggs is more difficult to evolve (see Prediction 1a). In fact, blackbirds were usually not able to discriminate against conspecific eggs (see above). Taken together, these results fit again the IBP hypothesis. The low ejection rates of conspecific eggs found in our study contrast with those found by Samas et al. (2014a), further considering that our blackbird population presents a three times higher density (2.9 pairs/ha, (Ibáñez-Álamo 2010)) than those used by them in their predictions of high density populations in New Zealand (Samas et al. 2014a) (1 pair/ha max, (Samas et al. 2013)), which should increase the rejection of conspecific eggs by hosts in our study area (Hoi et al. 2010, Samas et al. 2014a). This result calls into question the use of indirect information on breeding density for manipulating the risk of IBP or CBP, which should be done by presenting directly dummies of cuckoo or blackbirds, respectively. The higher ejection rates of conspecific eggs found by Samas et al. (2014a) could be explained by a population bottleneck during the blackbird's introduction in New Zealand or due to a higher difference in inter-individual egg variability in their population, which would facilitate recognition of conspecific eggs. Clearly more studies are needed to clarify these differences between populations. Finally, the absence of differences in ejection of heterospecific model eggs despite the presence of a cuckoo near the nest could be due to the high ejection rates in all cases (close to 100%). In fact, strong ejection of heterospecific eggs has been previously found in other experimental studies in blackbirds (Grim et al. 2011, Samas et al. 2011, 2014a, Martín-Vivaldi et al. 2012, Ruiz-Raya et al. 2015, Soler et al. 2015).

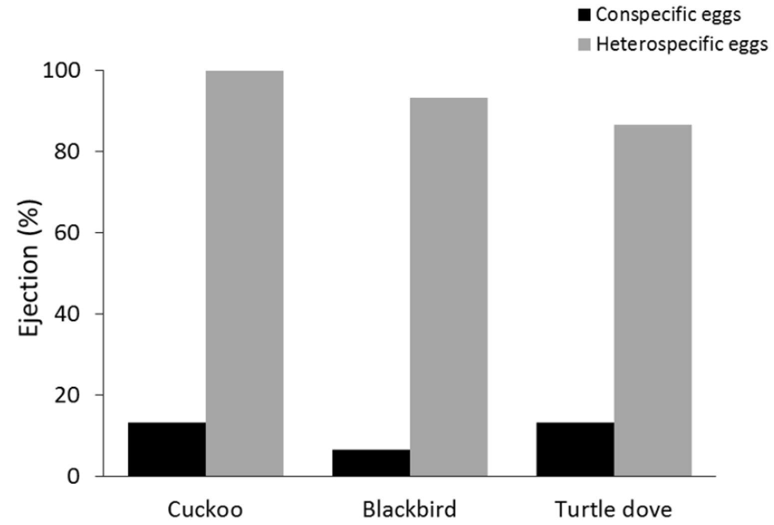

Figure 3: **Ejection.** Percentage of conspecific (black) and heterospecific eggs (grey) ejected under different risk of parasitism (presence of a cuckoo, blackbird or a turtle dove dummy).

We found a particularly aggressive response of blackbirds towards cuckoo dummies (Figure 4), indicating that they were perceived by females as an important risk of parasitism (Gill et al. 1997, Davies and Welbergen 2008, Požgayová et al. 2009, Welbergen and Davies 2009, Campobello and Sealy 2010) and supporting the IBP hypothesis according with the Prediction 4a. Thus, in our study, aggressions were specifically directed towards cuckoo dummies, which suggest that in this case the blackbird behaviour is a response to the threat presented by the parasite and not a result of generalized nest defence, as has been suggested in previous studies (Grim and Honza 2001). Although we can predict the existence of plasticity in host behaviour towards cuckoo dummies regarding allopatry or sympatry with the parasite (Røskaft et al. 2002), the aggression rates found in our study are similar to those found for other potential cuckoo hosts, including some of the most common hosts (Moksnes et al. 1990). Furthermore, the aggressive response towards cuckoo dummies found in our study area is not surprising because it has also been reported in other blackbird populations (Grim and Honza 2001, Grim et al. 2011). This is so, even after considering the scary effect of the cuckoo-hawk mimicry, which usually reduces the aggression to cuckoos by hosts (Davies and Welbergen 2008, Welbergen and Davies 2011). Despite this cuckoo-hawk mimicry, cuckoo dummies were usually perceived by females as a brood parasite instead of predator (i. e. sparrowhawk) as the time spent by females at the nest did not decrease and the latency to arrival did not increase specifically in the presence of a cuckoo dummy, as occurs in other unusual hosts after encountering a predator near the nest (Požgayová et al. 2009). Furthermore, we found a specific increase in the time the females spent checking their nests after encountering a cuckoo dummy in those nests parasitized with conspecific eggs. In the case of parasitism with heterospecific eggs, females increased their time checking the nest regardless of the type of dummy. These results seem to indicate that recognition of a parasitic egg alerts the females and they spend more time inspecting the clutch; but it also mean that, even if the parasitic egg is not recognized, the presence of a cuckoo near the nest is perceived by blackbirds as a specific threat of parasitism. Interestingly, fear behaviour was also a frequent response of females towards cuckoo dummies, but not towards blackbird or turtle dove dummies, which may be explained in these cases by the cuckoo-hawk mimicry. However, in these cases, the shock was limited to the first visual contact with the cuckoo dummy, as blackbirds often remained in the nest area and did not delay the arrival to the nest compared to the turtle dove or blackbird dummies. Previous studies have shown that some species delay their return to the nests after perceiving a risk of predation (Schaef and Mumme 2012), which does not occur in the blackbird after encountering a cuckoo dummy. We also reported some cases of aggressive behaviour towards blackbird dummies, although significantly less than towards cuckoo dummies (Figure 4). In these cases, intraspecific territoriality may explain the response towards blackbird dummies if females perceived them as potential competitors for food or nest sites (Creighton 2001), especially considering the high breeding density in our population (see above).

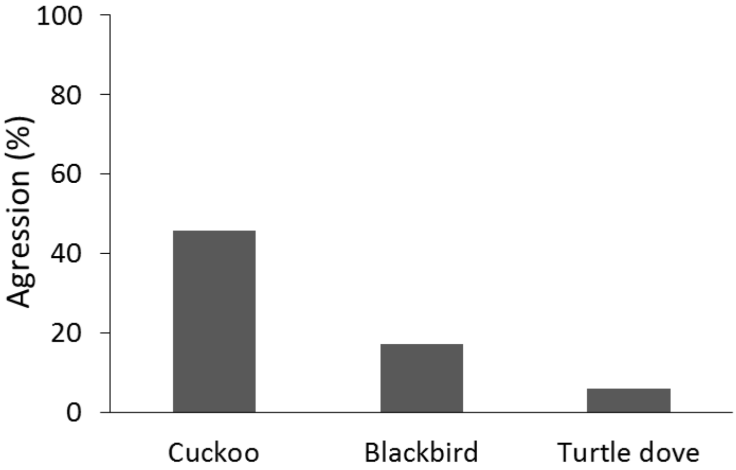

Figure 4: **Aggression of blackbirds towards the three different dummies (percentage of cases).**

Although some previous studies considered the blackbird as an unsuitable cuckoo host (Grim and Honza 2001, Grim et al. 2011, Samas et al. 2014a), our results suggest that the existence of rejection abilities in blackbirds probably evolved in response to IBP as a consequence of historical interactions with the cuckoo. Cuckoos are not currently present in our study area, but the unmistakable song of cuckoo males was frequently heard only 30 - 40 years ago according to locals living in the area. Given that real unsuitable host species such as those breeding in inaccessible nest sites or those feeding nestlings with seeds are pure acceptor species (Davies and Brooke 1989a, Moksnes et al. 1991), rejection of foreign eggs by thrushes is an indication of past parasitism (Rothstein 1990, Davies 2000, Soler 2014a). The patterns found by Grim et al. (2011) (see above) can be understood under the IBP hypothesis taking into account that reciprocal adaptations between brood parasites and their hosts occurring at all stages of the breeding cycle and that different lines of defence can evolve in all those stages (Davies 2011, Soler 2014a). Thus, current absence of parasitism in blackbirds may be due to the highly efficient rejection ability in this species, which would provoke the cuckoo to switch to other host species with less developed defences (Soler 2014a). As a matter of fact, most currently non-parasitized potential hosts of the cuckoo show a rejection rate of nearly 100% (Davies and Brooke 1989a, Moksnes and Røskaft 1995, Martín-Vivaldi et al. 2012), which has been retained over very long time periods including speciation events (Bolen et al. 2000, Rothstein 2001, Peer and Sealy 2004), even in the absence of CBP (Briskie et al. 1992, Peer and Sealy 2000, Bolen et al. 2000, Lorenzana and Sealy 2001, Underwood et al. 2004). Soler (2014a), showed that about 30% of potential host species of brood parasites present an ejection rate of nearly 100%. In the case of blackbirds, non-mimetic eggs are ejected at high rates and, in many cases, nearly 100% (Davies and Brooke 1989a, Grim et al. 2011, Martín-Vivaldi et al. 2012, Soler et al. 2015, this study). Moreover, many of the rarely used potential hosts of the cuckoo show high rejection rates of non-mimetic eggs, in many cases higher than current frequently used hosts (Davies and Brooke 1989a, 1998, Moksnes et al. 1991, Moskát and Fuisz 1999, Medina and Langmore 2015).

According to traditional theory, costs associated with maintaining the non-functional traits will determine the persistence of such traits for long periods of time (Lahti et al. 2009). Thus, the maintenance of egg rejection in species that are currently not affected by IBP has been considered in many cases as an evolutionary enigma because a rapid decline of rejection abilities would be predicted (Brooke et al. 1998). This decline of the rejection behaviour would give rise to coevolutionary cycles that would allow parasites to return later to the previous population (Soler et al. 1998) or host species (Davies and Brooke 1989a, b, Marchetti 1992). Although several experimental studies have shown evidences of rejection and recognition costs in some species (Davies and Brooke 1988, Marchetti 1992, Lotem et al. 1995, Lahti 2006, Samas et al. 2014a), the absence of such costs is the rule instead of the exception (Soler 2014). This is also true for the blackbird: one study has reported low costs (Samas et al. 2014a), but all others have found absence of costs (Martí-Vivaldi et al. 2002, Hale and Briskie 2007,Ruiz-Raya et al. 2015, Soler et al. 2015, this study). Furthermore, only recognition errors in non-parasitized nests would select for the loss of egg recognition abilities (Davies et al. 1996) and this type of error has only been reported once in blackbirds (Samas et al. 2014a), but not in others studies in this species (Ruiz-Raya et al. 2015, Soler et al. 2015, this study) or in any other host species (Rothstein and Robinson 1998, Stokke et al. 2005, Peer et al. 2007, Cruz et al. 2007). Therefore, if there are no costs for maintenance of the rejection abilities, brood parasite-host coevolution might result in successful resistance, preventing future exploitation of host species by parasites (Davies 2000, Soler 2014a). Indeed, successful resistance is a very frequent outcome of brood parasite-host interaction and high rejection rates are maintained in some potential host species that are currently not exploited by interspecific parasites [reported in 54 host species (29.7%) (Soler 2014a)].

Recognition and rejection of parasitic egg is a widespread defence used by host against IBP (Davies 2000) but is absent in most species suffering CBP. The existence of CBP in the blackbird populations used by Samas et al. (2014a) is considered as one of the lines of evidence supporting their conclusion that egg rejection evolved in response to CBP instead of IBP. However, their reported rates of CBP in blackbirds (3.1% and 0% in sympatric or allopatric areas with the cuckoo, respectively) are extremely low according to theoretical predictions, as occurs in our study area (2.9%) and other blackbird populations (3.9% and 5.0%, (Ibáñez-Álamo 2010)). Could percentages of CBP of this magnitude support the hypothesis that egg recognition evolved to counter CBP? To answer this question we used the Davies et al.'s (1996) signal-detection model. This methodology allowed Underwood et al. (2004) to estimate the level of CBP necessary to select for conspecific egg rejection in the black-billed magpie (*Pica hudsonia*); specifically, the values of CBP predicted by the model were 22% - 49% (based on two different assumptions). In the case of blackbirds, even assuming fairly high costs for CBP (i. e. the loss of a chick in a parasitized nest) and considering the rejection costs found by Samas et al. (2014a), the signal detection model predicts that values of CBP occurrence needed for the evolution of responses against conspecific eggs in the blackbird would range from 55% to 65%. Thus, it can be concluded that extremely low CBP rates reported in blackbirds (see above) do not support that CBP is an important pressure favouring the evolution of egg discrimination. Furthermore, it deserves to be emphasized that rejection based on discrimination is absent in most species suffering CBP, including those with a high frequency of CBP. For instance, in cliff swallows (*Hirundo pyrrhonota*) CBP is detected in about 24% of nests but egg rejection occurs only when the experimental egg is added before the host female has laid its first egg, but never thereafter (Brown and Brown 1989); which also occurs in other species that readily reject conspecific model eggs (Stouffer et al. 1987). This indicates that absence of rejection is based on a lack of egg recognition instead of a physical impairment to reject conspecific eggs (Lyon and Eadie 2008).

Conclusion

Our results fitted all predictions based on the IBP hypothesis but none of those based on the CBP hypothesis. Female blackbirds recognized easily heterospecific but not conspecific eggs. As occurs in most of non-parasitized potential hosts of the cuckoo, blackbirds showed a high ejection rate (independent of perceived risk of parasitism) and, furthermore, high aggression towards cuckoo dummies, suggesting a historical interaction between these two species. In addition, CBP occurrence in natural blackbird populations is well below the expected theoretical levels that will allow for the evolution of such defenses due to CBP alone. Finally, current absence of cuckoo parasitism in the common blackbird may be the consequence of the very low recognition and ejection costs found in this species, which will result in the maintenance of antiparasitic defences, leading to successful resistance. All of these pieces of evidence together strongly suggest that the evolutionary origin of egg recognition and rejection abilities in this species has probably been cuckoo parasitism.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

FRR, MS, GR and JDIA designed the study. FRR and GR performed the fieldwork. FRR and TA processed the recordings and analysed the data. FRR wrote the first draft. FRR, MS, GR, TA and JDIA edited drafts and contributed to writing. All authors read and approved the final manuscript.

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Chapter 7

Non-incubating blackbird males can recognize and eject experimental parasitic eggs

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Abstract

It has been traditionally assumed that only the incubating sex can recognize and eject parasitic eggs. However, this assumption has rarely been explored despite its important implications for the evolutionary relationship between parasites and hosts. Here we used information on previous egg-rejection experiments made by us to explore the recognition abilities of both males and females of the common blackbird Turdus merula towards experimental eggs with a variable degree of mimicry. We found that both sexes recognized non-mimetic eggs similarly, supporting the idea that visits to the nest can favour the evolution of rejection abilities. In contrast, only females recognized mimetic eggs, which indicate that, although recognition abilities can evolve in both sexes, they are subsequently refined in females driven by their more frequent interactions with parasitic eggs. In addition, we filmed an ejection event carried out by a male, which suggests that this sex might play an unexpected role in the evolution of egg ejection behaviour in this species. Our results provide new insights into the main anti-parasitic defence in birds, egg rejection, and highlight the need of considering the non-incubating sex in egg-recognition studies.

Chapter 7

Introduction

Bird species exploited by obligate brood parasites usually suffer significant fitness costs because parasitic chicks frequently kill or evict all host´s offspring, or they are better competing for food (Rothstein 1990, Davies 2000). In response, many hosts have evolved defences at all stages of the breeding cycle, including active nest defence, rejection of parasitic eggs and chicks, or parent reluctance to feed parasitic fledgling (Davies 2011, Soler 2014). Among these lines of defence, egg rejection is the most effective and widespread strategy to minimize the fitness costs associated to rearing a parasitic chick (Davies 2000). Thus, given the high selective pressure imposed by brood parasitism, both parents would be expected to invest in egg rejection. However, despite the significant advances in the study of the co-evolutionary relationship between brood parasites and their hosts over the last three decades, the role played by each sex in egg rejection remains poorly studied.

Traditionally, females are assumed to be the main responsible for anti-parasitic defences towards parasitic eggs since they typically incubate in most bird species (e. g. (Davies and Brooke 1988, Lotem et al. 1992)). This assumption has been confirmed by experimental studies showing that, in those species in which only females incubate, females were the responsible for egg rejection (Rohwer et al. 1989, Soler et al. 2002, Požgayová et al. 2009). Instead, in host species where both parents incubate, both males and females have been found to be able to reject parasitic eggs (Soler et al. 2002, Honza et al. 2007). There is evidence, however, of egg recognition and rejection by males even though they are not involved in the incubation, as is the case of northern orioles Icterus gulbula, a host species of the brown-headed cowbird Molothrus ater (Sealy and Neudorf 1995). Thus, according to the authors, rejection behaviour could also evolve in males even if they play no role in incubation, but frequently visit the nest; this prediction however has not been confirmed in any other host species (Požgayová et al. 2009).

The question of which sex is responsible for the host response towards parasitic eggs has important implications for the evolution of anti-parasitic defences in host populations. If both mates rather than only females can respond to parasites, defensive traits are expected to spread faster within the population (Rothstein 1975b, Sealy and Neudorf 1995). Thus, the study of sex-roles in the rejection behaviour is crucial to understand the long-term outcome of the co-evolution between brood parasites and their hosts. In this study, we explore the role of non-incubating males in the host response against parasitic eggs. To do so, we use the common blackbird Turdus merula (blackbird hereafter) as our model species, a potential host species whose egg-rejection behaviour is well known. Blackbird females are able to recognize and eject foreign eggs at high rates (Grim et al. 2011, Samas et al. 2011, Ruiz-Raya et al. 2015, 2016, Soler et al. 2017), and the sex role in the response towards parasitic eggs has been previously explored (Soler et al. 2002). In their study, Soler et al. (2002) found that all ejection events (n =

5) were performed by females whereas no male approached the nests during the filming period (n = 9). However, egg rejection is a complex process involving three different stages: judgment, decision and action (Ruiz-Raya and Soler, in press). First, hosts gather information from the environment (i.e. eggs), which might result in egg recognition. Second, information from the judgment stage, as well as those from conditional stimuli, is integrated by hosts to choose between accept or reject the parasitic egg. Finally, hosts will carry out the action of rejection itself. This process suggest that recognition abilities should not be inferred from ejection rates alone given that there could be acceptance decisions. In fact, recent studies have experimentally demonstrated the existence of acceptance decisions in blackbird females (Ruiz-Raya et al. 2015, Soler et al. 2017), highlighting the importance of differentiating egg recognition and ejection also in males. Here, using a dataset from egg-recognition experiments in which the behaviour of both parents was video-monitored, we assessed the recognition abilities of both blackbird males and females towards several types of model eggs showing a variable degree of mimicry.

Methods

General field procedure

We combined both unpublished and published data from our previous experiments on the egg-rejection abilities of the common blackbird carried out in a blackbird population located in the Valley of Lecrín, Southern Spain (Ruiz-Raya et al. 2015, 2016, Soler et al. 2017). As a general procedure, natural blackbird nests were artificially parasitized with experimental eggs. Our dataset includes information on the parents´ response to four different types of experimental eggs showing an increasing degree of mimicry: (1) *natural blackbird eggs*, collected from deserted clutches; (2) *mimetic experimental eggs*: eggs painted mimetic; (3) *cuckoo-sized non-mimetic eggs*: real house sparrow *Passer domesticus* eggs collected from deserted nests (see (Ruiz-Raya et al. 2016)), whose size is similar to the cuckoo eggs of southern Spain (Martín-Vivaldi et al. 2002); (4) *non-mimetic eggs*: experimental eggs painted in red. Furthermore, an additional group of non-parasitized blackbird nests was used as control. Some types of experimental eggs, specifically mimetic and red-type eggs, included eggs that differed in size (small, medium and large eggs). We did not consider the effect of egg size in egg recognition since it has previously been proven that egg size does not affect egg recognition in blackbirds, but exerts its effect on egg rejection by hindering the ejection itself once the parasitic egg is recognized (Soler et al. 2017). All nests were checked every 24 h to determine whether the model egg was ejected. We consider the experimental egg was accepted if it remained in an active nest for five days.

Just after the experimental parasitism, a video camera (Panasonic HDC-SD40) was placed near the nest (1.5–2.5 m) to film the blackbird response towards the experimental egg for the following 1.5 – 2 hours. We used a standardized procedure previously used to study the incubation behaviour of this species (Ibáñez-Álamo and Soler 2012). From recordings, we extracted information on (i) nest attendance and (ii) egg recognition from those cases where information of both male and female was available (64 nests). Data on the females´ response to experimental eggs were previously published (see references above). However, information regarding nest attendance of adults and the egg-recognition abilities of blackbird males remain unpublished.

Statistical analysis

We used *glmmADMB* (R package v. 0.8.3.3 (Fournier et al. 2012, Skaug et al. 2016)) in order to fit generalized linear mixed models (GLMM) to analyse two different variables related to nest attendance: (i) number of visits to the nest per hour; and (ii) time spent by blackbirds at the nest per hour. Egg-recognition data were analysed by generalized linear mixed model using *lme4* (R package v. 1-13 (Bates D et al. 2015)). In this case, following the methodology used in our previous studies (Ruiz-Raya et al. 2015, 2016, Soler et al. 2017), egg-recognition abilities of blackbirds were assessed from the number of times that they touched the eggs with its bill during the first visit (first-contact touches). Egg-touching behaviour can be considered a reliable indicator of egg recognition in blackbirds since: first, it occurs more frequently in parasitized than in non-parasitized nests (non-parasitized females occasionally touch their eggs during incubation); and, second, clutches with non-mimetic eggs are significantly more touched than those containing mimetic eggs (Soler et al. 2017). All statistical models included nest identity as a random factor and the following fixed predictors: type of parasitic egg, blackbird sex, their interaction, clutch size and its interaction with sex. Model selection was performed by dropping non-significant terms and then using likelihood ratio tests (LRT) to compare full and reduced models. We used *lsmeans* (R package v.2.27-2 (Lenth 2016)) for post hoc comparisons. Overdispersion was tested by using the *overdisp function* and the final model validation was carried out by a visual inspection of residuals. All analyses and graphics were performed using R version 3.4.0 (R Core Team 2017).

Results

Male and female behaviour during incubation

Blackbird males are not directly involved in egg incubation but they visited the nests during the filming time (24.3% of nests, N = 263), checking inside and occasionally feeding the female during incubation. As expected, blackbird females visited the nest more often than males ($\chi^2 = 92.0$; df = 1; $p < 0.0001$) regardless the type of parasitic egg in the nest (sex x egg: $\chi^2 = 6.44$; df = 4; p = 0.17). Furthermore, females spent significantly more time in the nest than males ($\chi^2 = 167.90$; df = 1; $p < 0.0001$) in all experimental groups (sex x egg: $\chi^2 = 5.71$; df = 1; p = 0.22). Clutch size partially affected nest attendance as blackbird females, but not males, visited more frequently (clutch size x sex: $\chi^2 = 5.26$; df = 1; p = 0.02; Fig. 1a) and spent more time (clutch size x sex: $\chi^2 = 8.7$; df = 1; p = 0.003; Fig. 1b) at those nests containing a higher number of eggs.

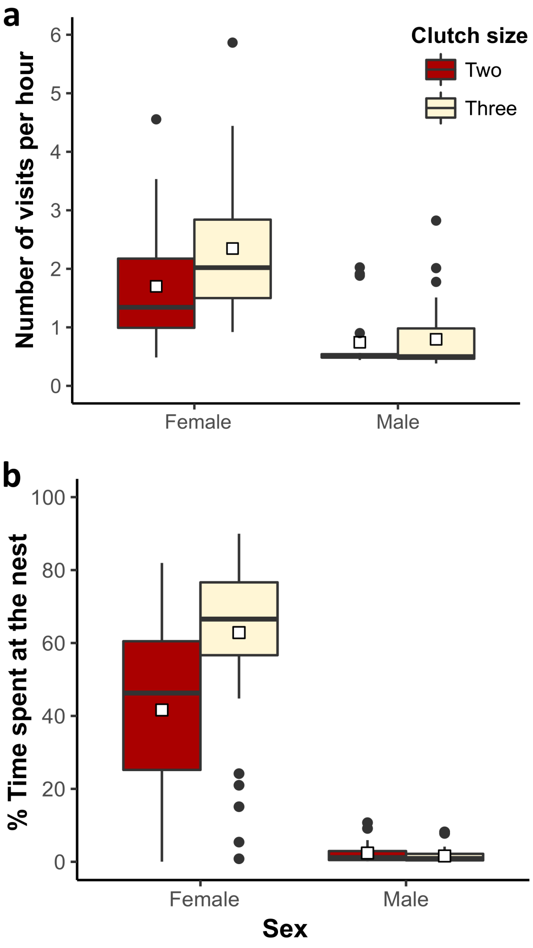

Figure 1: **(a) Number of visits to the nest per hour and (b) percentage of time spent by the blackbirds at the nest regarding the clutch size.** Box plots show the median (black line), the mean (white box), 25th and 75th percentiles (coloured boxes), with whiskers denoting the 5th and 95th percentiles. Black points indicate outliers.

Recognition abilities in each sex

Although both sexes increased their touches to the eggs as the degree of mimicry decreased (Fig. 2), the effect of egg mimicry differed between males and females (sex x egg: χ^2 = 14.96; df = 4; p = 0.005). Compared to control nests, both males and females touched more often in those nests parasitized with clearly dissimilar experimental eggs, as cuckoo (females: z = - 3.40; p = 0.02; males: z = -3.38; p = 0.03) or red egg models (females: z = - 3.37; p = 0.03; males: z = -3.84; p = 0.01). Furthermore, we found no significant differences in number of touches between control nests and those parasitized with real blackbird eggs for both females (z = - 1.70; p = 0.80) and males (z = - 0.09; p = 0.99). However, while blackbird females tended to touch more often mimetic eggs compared to the control (z = -3.08; p = 0.06), we found no differences in the number of touches provided by males between control nests and those parasitized with mimetic eggs (z = - 0.82; p = 0.99). Finally, we found no effect of clutch size on the number of touches regardless the sex (χ^2 = 1.38; df = 1; p = 0.24).

We filmed one case in which a blackbird male ejected a non-mimetic egg (red type) by grasping it with its bill (see Video1 in Supp Inf). We filmed a total of 43 ejection events, showing that 2.3% of them were carried out by males and 97.7% by females.

Discussion

Here we investigated the existence of sex differences in the egg-rejection behaviour in blackbirds. During incubation, parental care of blackbirds relies mainly on females (Ibáñez-Álamo and Soler 2012), who visited the nest more frequently and stayed at it for longer than males (Fig. 1). Furthermore, we found that females increased their nest visits and attendance in nests with larger clutches (Fig. 1), which could be related to higher incubation requirements of larger clutches leading to longer incubation periods (Moreno and Carlson 1989, Dobbs et al. 2006). Previous studies have shown that, in other single-sex incubator species such as swallows *Hirundo rustica*, females increased their incubation time in response to artificially enlarged clutches (Jones 1987). Despite the preponderant role of the female during incubation, our recordings showed that blackbird males occasionally visited and inspected the clutch too. Males visits to the nest during the incubation stage have been previously described in blackbirds (Ibáñez-Álamo and Soler 2012) and they might favour the evolution of recognition abilities (Sealy and Neudorf 1995). In fact, we found that blackbird males are able to recognize and eject parasitic eggs even though they are not actively involved in egg incubation. This finding contradicts the general hypothesis stating that incubation is a prerequisite for egg rejection (Davies and Brooke 1988, Lotem et al. 1992, Soler et al. 2002). Instead, our results show that non-incubating individuals (males) can also evolve recognition abilities, which supports the hypothesis that visits to the nests could favour the evolution of anti-parasitic host responses during the egg stage (Sealy and Neudorf 1995). We found that both blackbird females and males recognized non-mimetic parasitic eggs. However, unlike blackbird females (Soler et al. 2017, Roncalli et al., in prep., this study), males did not recognize mimetic experimental eggs, which indicates that recognition abilities are less fine-tuned in blackbird males compared to females (Fig. 2). From an overall perspective, the most likely explanation for the more precise egg-recognition abilities shown by females is that the evolution of fine-tuned recognition abilities could be limited in males because they have fewer opportunities than females to observe the clutch and learn the aspect of their own eggs. This argument assumes that both sexes rely on learning mechanisms for egg recognition, which seems to be the most widespread egg-recognition mechanism among the cuckoo's hosts (Rothstein 1974, 1975a, Lotem et al. 1995, Lahti and Lahti 2002, Lyon 2007, Lang et al. 2014). Alternatively, males of some host species could rely on discordance mechanisms for egg recognition, thus avoiding the potential costs linked to learning mechanisms when polymorphism in egg phenotype exists (Liang et al. 2012).

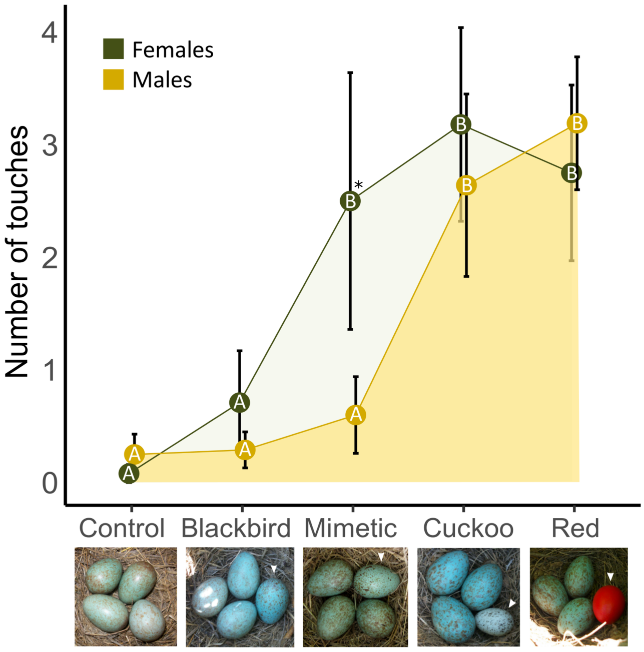

Figure 2: **Number of touches to the eggs by both males and females in relation to the degree of mimicry of the parasitic egg.** Different letters indicate significant differences at $p < 0.05$. Values are presented as means ± SE. **Differences between blackbird and mimetic eggs in the edge of significance (p = 0.06).*

Although most of the filmed ejections were carried out by females, we filmed one case in which a blackbird male ejected a non-mimetic egg by grasping it with its bill (see Video1 in Supp Mat.), which means that males, although less frequently than females, are also able to eject eggs in this species. But, why is egg-ejection by males so rare? One possibility would be that, as females visit the nest more frequently than males do, they have more opportunities to reject the parasitic egg, which would decrease the probability of ejection by males. On the other hand, the less precise recognition abilities shown by males could lead to a delay in their ejection decisions compared to females, which would increase the chances of the ejection being carried out by the female. Furthermore, this delay in the ejection decision would make difficult for the ejection event to take place within the filming time and therefore to be documented.

Egg rejection abilities exhibited by some potential host species which are currently not exploited by interspecific brood parasites, as is the case of the common blackbird, have been proposed to be a consequence of conspecific brood parasitism (Samas et al. 2014) or, alternatively, the result of past parasitism by a interspecific brood parasite (Soler 2014, Ruiz-Raya et al. 2016). According to our results, egg recognition abilities exhibited by blackbird males towards non-mimetic eggs, but not against mimetic ones, support the interspecific-origin hypothesis. In addition, neither male nor female are able to recognize real blackbird eggs introduced into their nests, which would be expected if the recognition abilities were the result of parasitism by conspecifics (Ruiz-Raya et al. 2016). The evolution of rejection abilities in non-incubating males is intriguing and probably requires very strong selective pressures, with a long duration of sympatry with the brood parasite. But, were blackbirds so heavily exploited by interspecific brood parasites in the past? Certain ecological factors linked to the biology of blackbirds, such as their main habitat or the characteristics of their nests, could have facilitated cuckoos to parasite this species in the past. If we assume that the original habitat preferred by cuckoos are open forests where caterpillars are abundant, and that species nesting in this habitat and with conspicuous nests would be preferentially exploited (Soler 2014), it could be concluded that blackbirds (or their ancestors) were likely heavily parasitized in the past. Non-incubating males of current hosts species, such as males of the great reed warbler *Acrocephalus arundinaceus*, have been found to play a crucial role in cuckoo mobbing and nest defence (Požgayová et al. 2009). Thus, an intense brood parasitism in the past would have selected for blackbird males actively participating in nest defence against parasites and, this relationship of males with brood parasites, together with the visits of males to the nest could have facilitated the initiation of the evolution of egg recognition in males.

Two main conclusions can be derived from this study. First that it is crucial to examine egg recognition abilities of hosts in addition to their egg rejection capacity since, otherwise, the former could be underestimated (Soler et al. 2012); and second, that we have found support for the hypothesis that non-incubating males that occasionally visit the nest can evolve egg rejection behaviour, so they could be involved in the host response against parasitic eggs. This fact entails important consequences for the evolution of anti-parasitic responses within host populations, so additional studies addressing the sex role on anti-parasitic host defences are required.

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Chapter 8

Rejection of parasitic eggs: an updated terminology for a complex process

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> *"Definitions are temporary verbalizations of concepts, and concepts - particularly difficult concepts- are usually revised repeatedly as our knowledge and understanding grows"*
>
> Ernst Mayr

Introduction

The tricky reproductive habits exhibited by avian brood parasites, who exploit the parental care that other species provide to their offspring, and obligate them to work hard to rear the parasitic chick, has fascinated naturalists and researchers for centuries. Over 2300 years ago, the parasitic life-style of the common cuckoo *Cuculus canorus* was already known by Aristotle: *"The cuckoo [. . .] makes no nest, but lays its eggs in the nests of other birds"* (Aristotle 1862, B-IX, p. 249). However, it was not until the eighteenth century that knowledge on the cuckoo's reproductive biology began to develop, and several authors provided descriptions on the way in which cuckoos lay their eggs, some characteristics of the parasitic egg, or the eviction of host young by the newly-hatching cuckoo chick (reviewed in (Schulze-Hagen et al. 2009)). Charles Darwin (1859), in his *The Origin of Species*, for the first time addressed the study of brood parasitism from an evolutionary perspective, suggesting that natural selection was the responsible for the evolution of cuckoo behaviour. In the first half of the 20th century, some intriguing aspects of the cuckoo's breeding behaviour were revealed as a result of pioneering field observation (e. g. (Chance 1922, 1940)). Nowadays, experimental studies have led to major advances in the understanding of brood parasite-host coevolution, and it is known that costs of parasitism are an important selective pressure as host fitness is dramatically reduced, thus leading to the evolution of host defences and subsequent parasite counter-adaptations in all stages of the breeding cycle (Davies 2000, Soler 2014).

Egg rejection is likely the best-studied hosts' defence against brood parasitism and, as a consequence of the astonishing advance in our understanding of this adaptation, a wide array of concepts referring to the hosts' cognitive abilities driving this process have emerged. However, some important concepts related to animal cognitive abilities and decision making, which have recently been reviewed, are absent in most works on egg rejection. At the same time, some terms throughout the brood-parasitism literature can become confusing due to the lack of unified terminology that standardizes their use, which can be disadvantageous for the scientific advance in this field. For instance, egg discrimination is often understood as a synonym of the complete process of egg rejection, which has been widely used to infer recognition abilities of hosts. However, recent experimental studies have allowed to know the complex nature of the egg-rejection process, in which several stages can be differentiated and decision-making play a central role, revealing that hosts are able to recognize more parasitic eggs that they finally reject (see below). Furthermore, some terms frequently used in behavioural ecology, including discrimination, have recently been defined within a decision-making framework (Mendelson et al. 2016), which could also be extended to the study of hosts' response to parasitic eggs. Here, we call for the use of an updated terminology in egg-rejection studies by incorporating some new concepts, and offering a more precise delimitation of some already existing terms, based in the above mentioned advances.

Historical use of some common terms in egg-rejection studies

Taking a look at the terminology used in the study of host's responses to parasitic eggs, it seems fair to conclude that brood parasitism literature is dominated by two ubiquitous terms: egg recognition and egg discrimination. The former has been widely used in brood parasitism studies to refer to the cognitive process by which hosts are able to identify the parasitic egg from their clutch, which is the first necessary step towards egg rejection. From the classical studies carried out by Stephen Rothstein on the mechanism behind egg recognition (Rothstein 1974, 1975, 1978), this term usually includes mechanisms and cognitive rules based on both recognition "by discordancy" (rejection of the egg in the minority, or odd egg) and "template-based recognition" (rejection of the egg by comparing visual features of parasitic eggs to a template of the host's own eggs, which can be innate or learned), which is also called “true recognition”. On the other hand, the term egg discrimination takes on a different nuance in some studies since it frequently includes an action component; that is, it is usually linked to the specific host response (i. e. egg rejection)(e. g. (Brooke and Davies 1988, Davies and Brooke 1989a, b, Lotem et al. 1995, Welbergen et al. 2001, Lahti and Lahti 2002, Grim et al. 2003, Spottiswoode and Stevens 2011)). Although we can discern some differences in the historical use of these two terms, the delimitation between both is diffuse and they are often used interchangeably. Importantly, discrimination usually refers to the three-stage process of egg rejection, and it is often used to assess and infer recognition abilities in hosts, which entails important implications that may hamper our understanding of the host response. In the next section, we suggest that recognition and rejection need to be studied individually given the complex nature of the host responses. Furthermore, according to recent work on animals’ cognitive phenotypes (Mendelson et al. 2016), the terms recognition and discrimination deserve to be redefined and consistently used in the literature on brood parasitism.

There are also some terms not directly related to the egg rejection process that deserve, given their wide use, be nuanced in order to standardize their meaning. This may be the case, for example, of those terms used to describe the host response against parasitic eggs. We propose that the term ejection be specifically used to refer to those cases when the parasitic egg is removed from the nest. On the other hand, the term rejection includes a wider range of host response such as nest desertion, the burial of the parasitic egg and, in addition, egg ejection. Another terms which could become confusing are those referring to costs linked to host defences, the so-called rejection costs (Davies 2000). This term has historically included costs derived from errors in the recognition leading to the ejection of an own egg (which has been denominate as recognition errors); as well as costs resulting from the breaking of an own egg during ejection, or the loss of total fitness in that reproductive event in those host species whose rejection mechanism is based on nest desertion. Although variations that may exist in the use of these terms do not have important implications in our understanding of the host’s response, their frequent, and often inconsistent, use in egg-rejection studies makes them worthy of being included in our proposed terminology. In order to simplify, we propose the terms recognition costs and rejection costs to refer to costs arising from the judgment stage and action stage, respectively (Table 1).

Proposed updated terminology

The host response against the parasitic egg is a sequential process in which different stages can be differentiated: first, the host has to realize that its nest has been parasitized, and identify which eggs are theirs and which is a foreign egg; if this occurs, the individual must decide how to respond; and, finally, the specific response behaviour must be carried out (Stokke et al. 2005, Soler et al. 2012). Although egg rejection is frequently used to infer recognition abilities in hosts (e. g. (Davies and Brooke 1989a, Marchetti 2000, Soler et al. 2000, Lahti and Lahti 2002, Avilés et al. 2004, Spottiswoode and Stevens 2011)), recent experimental evidences have confirmed that egg rejection does not necessarily follow egg recognition, but host response is a complex and frequently a plastic process in which decision-making occupies a prominent place, and where several stages can be differentiate: recognition, decision and action itself (Antonov et al. 2009, Soler et al. 2012, 2017, Ruiz-Raya et al. 2015). Therefore, recognition abilities should not be inferred from the final host response but they need to be studied separately, which requires a delimitation of terms as accurate as possible. Of course, this natural process happens regardless of the terms that we use for study; however, the terminology used to study the host response should reflect the complex nature of the egg-rejection process, and a well-established terminology would avoid misunderstandings and will favour scientific advance.

Progress in the study of cognitive phenotypes has made possible a more precise delimitation of terms related to animal decisions, highlighting the convenience of adopting a unified conceptual framework for the study of animal decisions in some specific domains of behavioural ecology studies (Mendelson et al. 2016). According to these authors, the decision-making process arises from the interaction of two main components: judgments, "the acquisition and processing of information to arrive at an understanding of the world"; and decisions, "the process by which individuals use the judgments to arrive at a course of action". In this framework, discrimination and recognition are two important aspect linked to the host judgment, whose outcome will condition the host decision-making. As a result, the process will lead to a specific behaviour on the part of the host, the action itself or host response. In our opinion, brood parasitism terminology should adapt these significant advances in the science of animal decision-making for a better approach to the cognitive mechanisms that allow for recognition, the processes that guide decision making, and the characteristics of a specific behaviour. Therefore, we rely on these three main components of animal behaviour to propose an updated terminology for use in brood parasitism studies addressing the egg-rejection process (see Table 1).

Table 1: **Stages of the egg-rejection process (judgment, decision and action) and terms proposed for a unified terminology in the study of the host response to parasitic eggs.** Some terms are based on the decision-making ontology developed by Mendelson et al. (2016).

Judgment	*Discrimination*	Basic sensory process by which hosts can distinguish two or more different stimuli from their clutch. Necessary for egg recognition.
	Categoritation	Assignment of two or more stimuli (e. g. own eggs) to a specific set; and host ability to differentiate from other stimulus (e. g. parasitic egg)
	Assessment	Acquisition of measurable information related to a single stimulus (e. g. parasitic egg); referring to fitness consequences of that stimulus.
	Recognition	Cognitive process by which a previously processed stimulus is associated by host with a foreign egg, leading to the host response.
	Recognition costs	Loss of individual fitness linked to the host's response to the parasitic egg. It involves the ejection of an own egg by mistake.
Decision	*Preference*	Cognitive encoding of a ranking of options, independently of the behavioural outcome. If recognition occurs, host preference will be egg rejection.
	Motivation	Host propensity to assume the potential costs linked to egg rejection.
	Choice	Selection of a certain option by the host over other alternatives (rejection or acceptance). Motivation will determine the final choice over the previous preference.
	Egg-touching	Behaviour by which the host softly touches the eggs with its bill as a mechanism for obtaining additional information on the parasitic egg (post-recognition assessment).
	Egg-pecking	Behaviour by which the host repeatedly knock the parasitic egg with its beak. It is usually used by puncture-ejector species to break the eggshell or gain additional information of the eggshell strength (post-recognition assessment).
Action	*Grasp ejection*	Egg rejection behaviour by which hosts eject the parasitic egg by grasping it with its bill. It usually involves low rejection costs.
	Puncture ejection	Egg rejection behaviour by which hosts eject the parasitic egg by making a hole in the eggshell and gripping it to fly away. It usually involves higher rejection costs.
	Nest desertion	Egg rejection behaviour consisting in the abandonment of the entire parasitized clutch.
	Rejection costs	Loss of individual fitness linked to the host's response to the parasitic egg. They involve the accidental breaking of an own egg during the ejection of a parasitic egg, or the abandonment of the complete clutch.

Chapter 8

Discussion

Is an updated terminology needed in egg-rejection studies?

Several studies have called for the use of standardised terminologies in ecology, arguing their benefits in improving efficiency and communication in ecological research (Wells and Richmond 1995, Hall et al. 1997, Davis and Thompson 2000, Colautti and Richardson 2009). However, attempts to standardise ecological terminology has also received criticisms, which claim that this purpose is frequently unnecessary and unworkable, and highlighting potential benefits of flexible terminologies (Hodges 2008). In this article, we do not pursue an exhaustive lexical review of terms used in egg-rejection studies and the hazards of its potential misuse. Instead, we intend to incorporate recent advances in both the knowledge of animal decision-making and the processes guiding the host response to the study of the complex process of egg rejection, which will undoubtedly be reflected in the terminology employed by the inclusion of new terms and the more precise delimitation of some concepts previously used in egg-rejection studies. We are convinced that the emergence of new concepts, and the redefinition of some pre-existing terms, is a logical and necessary consequence of the advance in our knowledge of the host defensive response against parasitic eggs. The use of an updated and unified terminology will benefit the development of future work on egg-rejection behaviour, and thus, on brood parasitism, by stimulating the emergence of new ideas as a result of a more detailed understanding of the mechanisms that underlie the host's response against the parasitic egg.

Host judgment and the recognition-rejection dichotomy

Through brood parasitism literature, the specific response to the parasite egg has often been used to infer host recognition abilities. In this context, the term discrimination has often been used to describe the entire process by which the host acquires information, evaluates such information and, more frequently, performs a response to the parasite egg. In many cases, the judgment stage of the egg-rejection process will lead, if host have the necessary discrimination and recognition abilities, to the recognition of the parasitic egg. However, the host response might not reflect the degree of recognition shown by certain host species as the egg-rejection process can be interrupted in subsequent stages (decision or action). The historical use of the term discrimination not only includes the cognitive abilities of hosts, but involves the rejection of the parasitic egg, which may be imprecise and confusing if acceptance decisions occur. Therefore, in the light of recent progress in the study of the egg rejection process, we emphasize the idea that studies on egg rejection will benefit from the separation between egg recognition and egg rejection. Furthermore, according to recent advances in the study of cognitive phenotypes and animal decisions (Mendelson et al. 2016), we suggest the use of discrimination to refer to, not to the complete egg rejection process, but to a purely cognitive process related to the host's ability to differentiate stimuli (Table 1), being the first step in the judgment stage of the egg-rejection process. On the other hand, the term recognition would imply the association of a previously processed stimulus with a foreign (or own) egg (Table 1), which will trigger the host response as it will be crucial for the decision stage. Under this new perspective, these concepts are both linked to the judgment stage since they allow host to gain a better understanding of its current environment. But does recognition necessarily follow discrimination? Obviously, discrimination and recognition are closely linked concepts since the former is a necessary condition for recognition to occur. However, some nuances may arise when we consider the specific cognitive rule used by hosts during egg rejection. Under "template-based recognition" or "true recognition", the perceived stimuli are compared to a template of the host's own eggs, which will determine the recognition of one of these stimuli as a foreign egg. In this case, discrimination ability of the host to differentiate a stimulus different from that of its own eggs will determine egg recognition. However, under "discordancy" mechanisms, discrimination and recognition abilities appear not to be so linked as this cognitive rule will mainly rely on the discrimination abilities of hosts, while recognition is not required. Several studies manipulating proportions of host and foreign eggs have shown that, as the proportion of foreign egg increases, hosts decrease their propensity to ejection (Moskát et al. 2010, Stevens et al. 2013). Interestingly, brood parasites can exploit this potential conflict between cognitive rules in order to make rejection decisions harder, providing valuable information on the strategies used by brood parasites to cheat hosts (Stevens et al. 2013). Finally, once the differences between discrimination and recognition are established, other concepts related to the cognitive capacities of the host can be considered in the host response. For instance, under circumstances of a high degree of intra-clutch variation or events of multiple parasitism, several stimuli could be perceived from the clutch, so hosts could be able to assign two or more stimuli to the same set (its own clutch) allowing differentiate them from another stimulus (parasitic egg): categorization (Table 1).

Host decision and the concept of motivation

During last years, the study of the decision making has acquired relevance within the field of behavioural ecology (Piersma and Gils 2011), including brood parasitism (Stokke et al. 2005, Soler et al. 2012). Thus, given the flexible nature of host response, terms like preference and choice (Table 1) deserve to be considered in the context of egg rejection, and therefore need to be incorporated to the scientific terminology in order to gain a better understanding of the specific host responses. The term motivation, the host's tendency to assume the potential costs of rejection under certain parasitism conditions (Table 1), has become a crucial concept in the psychology of decision-making, and recent studies suggest that host motivation (or rather, the lack of motivation) is, at least sometimes, behind acceptance shown by some host species (Soler et al. 2012, 2017, Ruiz-Raya et al. 2015). Definitely, the inclusion of the concept of motivation in studies addressing egg rejection is a turning point in our understanding of the host's response to the parasite egg since, as acceptance decisions may occur, the study of the egg-rejection process requires a clear differentiation between recognition and action in the host response, with several ecological factors (e. g. risk of parasitism, experience, egg traits) affecting host decisions, even when the parasitic egg is recognized. Importantly, information on the parasitism condition is updated throughout the complete process of egg rejection and hosts can be able to assess certain characteristics of the parasite egg once it has been recognized. This aspect has been included in recent works focusing on the role of behaviours such as egg-touching or egg-pecking (Soler et al. 2012, 2017, Ruiz-Raya et al. 2015), which could be used by hosts to gain information on some special characteristics of the parasitic egg. Therefore, terms related to these assessment mechanisms should undoubtedly be included in any attempt to establish an updated terminology on host response.

Finally, the last stage in the egg-rejection process is action, which refers to the specific host behaviour resulting from the decision-making process (Soler et al. 2012). Several studies have found that, even if the parasitic egg is recognized, the egg-rejection process may be interrupted at this point. Some egg traits, such as eggshell strength, mass or size, can significantly affect the host response by directly impeding ejection (Antonov et al. 2009) or by reducing the host motivation due to an increase in potential costs of ejection (Soler et al. 2012, 2017, Ruiz-Raya et al. 2015). Thus, the specific characteristics of the parasitic egg could modify the host decision and lead to egg acceptance in order to minimize potential rejection costs.

In conclusion, we propose an updated terminology for its consistent and unified use in egg rejection studies, including concepts that reflect the major advances in the field of animal decision-making, as well as those classically used in this field. The three-stage process underlying the host response to parasitic eggs (judgment, decision and action) can govern other host responses such as chick rejection, so the main aspect of this terminology would be applicable to the study of the host defences during the nestling stage. We believe this will lead to a more rapid scientific advance in the understanding of the egg-rejection process and stimulate future works on brood parasitism.

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General discussion

The complex process of egg rejection

Antagonistic interactions between brood parasites and their hosts provide some of the clearest examples of co-evolution that can be found in nature (Rothstein 1990). Host species have evolved defences to counter the fitness costs that brood parasitism entails, which favours the evolution of sophisticated strategies allowing parasites to evade such defenses. The first part of the thesis addresses the study of the egg rejection process, investigating how the different stages that compose this anti-parasitic response (judgment, decision, and the action itself) can be separately affected by diverse factors. Under this perspective, the host propensity to assume the potential costs of egg rejection (motivation), will determine the outcome of the whole process (Soler et al. 2012a). Costs associated to egg rejection vary depending on the characteristic of the parasitic egg, but also on the egg-rejection mechanism used by the host (Davies 2000). Therefore, the way in which the host carries out the action of rejecting the parasitic egg during the last stage of the egg-rejection process is important. Given that nest desertion is the most costly strategy to deal with parasitic eggs, it is usually restricted to small host species whose small beak prevents egg ejection (Rohwer and Spaw 1988). In Chapter 1, it is found that nest desertion cannot be considered an unequivocal response against brood parasitism in a medium-sized host species such as the blackbird. This finding suggests caution in egg-rejection studies focused on medium or large hosts. Occurrence of nest desertion in blackbirds can be explained by the fact that this behaviour is not a specific response evolved to combat brood parasitism, but may be a response to other ecological perturbations, such as the risk of predation or partial egg predation (Székely et al. 1996, Ackerman and Eadie 2003). As discussed in Chapter 4, the study of the endocrine pathways involved in the expression of parental care could elucidate the physiological mechanisms underlying the absence of nest desertion in medium or large-sized host species since levels of prolactin, a crucial hormone in the regulation of parental care (Buntin, 1996), remained unaffected after experimental parasitism.

The physical restrictions imposed by some characteristics of the parasitic egg can make it difficult to eject, even leading to egg acceptance. However, lack of egg rejection exhibited by some host species of interspecific brood parasites has traditionally been assumed to reflect recognition failures (Rothstein 1990, Lotem and Nakamura 1998, Davies 2000). Interestingly, it has been shown that, at least sometimes, absence of egg rejection may reflect acceptance decisions, and that hosts can recognize more eggs than are finally rejected (Antonov et al. 2009, Soler et al. 2012a, 2017, Ruiz-Raya et al. 2015). The host decision-making preceding the action stage of the egg-rejection process involves the joint action of several factors that condition the outcome of the egg-rejection process, such as the clutch characteristics, the recognition abilities of hosts, the risk of parasitism, parasite retaliation or the intrinsic costs of anti-parasitic host responses (Stokke et al. 2005). The lower rejection rate associated to large experimental eggs found in Chapter 1 suggests that the egg-rejection process might be hampered in the action stage itself since large eggs are likely more difficult to eject. On the other hand, in Chapter 2, we experimentally tested if some characteristics of the parasite egg that do not affect egg recognition could influence the outcome of the egg rejection process by impacting other stages of this process. We found that egg mass importantly influenced the rejection of previously recognized non-mimetic eggs, demonstrating for the first time that an egg trait that do not affect recognition (egg mass) may lead to acceptance decisions even if the parasitic egg is recognized (i. e. more frequently touched). These acceptance decisions are probably due to a low propensity of hosts to assume potential costs linked to the ejection of a slightly heavier egg (Soler et al. 2012a). This thesis therefore provides important support to the idea that hosts can recognize more eggs than are finally rejected, and more importantly, that, at least sometimes, absence of egg rejection reflects acceptance decisions.

The experimental manipulation of certain characteristics of the parasitic egg allows us to verify that some of these traits affect particular stages of the egg rejection process. In this line, Chapter 3 delves deeply into the relationship between recognition and ejection of parasitic eggs by showing that both the degree of mimicry (understood as the background colour and spotting pattern of parasitic eggs) and the size of the parasitic egg can modify the outcome of the egg-rejection process by impacting two different components of this process. While non-mimetic eggs were more easily recognized and therefore more frequently ejected, egg size did not affect egg recognition. However, as it was expected, egg size exerts an effect on egg rejection by impacting the action component of the egg-rejection process. Egg size seems to hinder the physical action of egg rejection and therefore modify the final host decision leading to egg acceptance. Taken together, results from Chapters 3 complements the results discussed in Chapter 2 on the effect of egg mass, demonstrating that characteristics of the parasitic egg can impact separately the three stages of the egg-rejection process (judgment, decision and action stage).

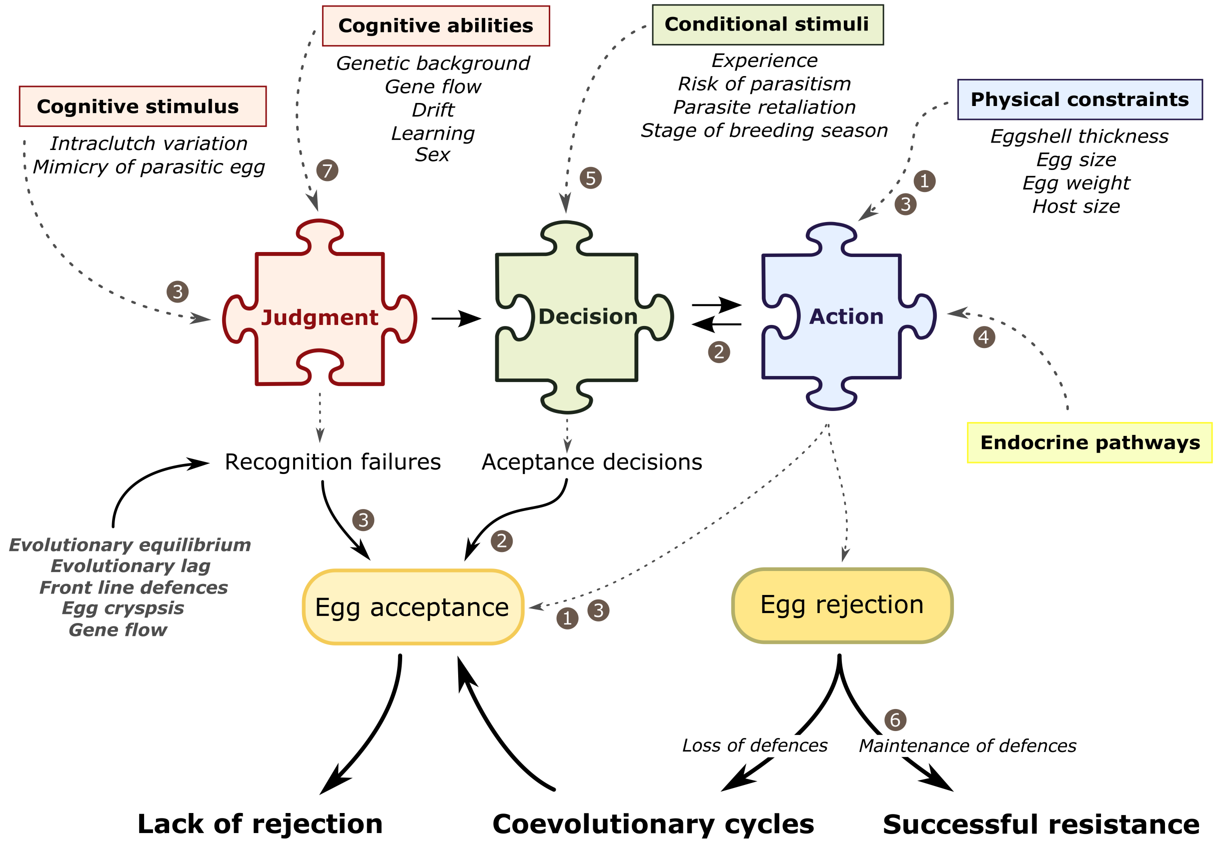

Figure 1: **General concept map.** Egg rejection is a three-stage process (puzzle pieces) that can be affected separately by different factors (colored squares). The proximate mechanisms affecting the egg-rejection process (dashed lines) are addressed in the different chapters of this thesis (circles with numbers indicate the specific chapter).

As discussed in Chapters 1, 2 and 3, costs associated with egg rejection can importantly determine both the egg-rejection mechanism and the outcome of the rejection process. Given such costs, natural selection should favour the evolution of plastic responses under certain ecological conditions (Pigliucci et al. 2006, Ghalambor et al. 2007). Evidence for these plastic responses has been accumulating in the brood parasitism literature, and Chapter 5 reviews the main examples of phenotypic plasticity in egg rejection, showing that some hosts are able to modify their responses to parasitic eggs according to different conditions of parasitism. These conditions include both temporal and geographic variations in the risk of parasitism, effects of the parasite presence, or parasite retaliation after a first event of egg rejection. Thus, in addition to a genetic basis, evolution of behavioural plasticity requires both the existence of environmental heterogeneity and that benefits of plasticity overcome its costs (Mery and Burns 2010). However, the evolution of plastic host responses also need that hosts evaluate, in the most accurate manner, the risk of parasitism to which they are subjected, which can be achieved in some cuckoo hosts by the use of both direct and indirect cues of parasitism (Thorogood and Davies 2012, Welbergen and Davies 2012). Therefore, as seen in previous chapters (Chapter 2 and 3), decision-making will be crucial for the host response to parasitic eggs, which will be conditioned by the cognitive abilities of the host, the clutch characteristics, the costs linked to the host's response, and external factors such as the risk of parasitism. The egg-rejection process can then be analysed from a "judgment and decision-making" framework, as has been suggested for other components of animal behaviour (Mendelson et al. 2016). Briefly explained, "judgments" allow hosts to evaluate information to obtain a vision of the current situation in order to, once integrated with that information derived from conditional stimuli, to carry out the "decision making". Therefore, once the situation is evaluated, the "choice" of individuals will depend on their propensity (motivation) to assume the potential costs of this behavior (in the case of hosts, the costs associated with egg-rejection). Importantly, these processes are susceptible to be affected by both internal (i. e. recognition abilities) and external (e. g. risk of parasitism, clutch characteristics) factors that, together, determine the response of the host.

Given the potential advantages of phenotypic plasticity, which will allow hosts to quickly adjust their response to parasitism conditions, it is intriguing that, in most host species, egg rejection is a fixed response that is retained even in the absence of brood parasitism (Soler 2014). Probably, plastic responses are advantageous in initial stages of parasitism, where the risk of being parasitized is variable; however, when the parasitism pressure increases, natural selection would favour the evolution of fixed egg-rejection behaviour due to the costs of plasticity per se. Afterwards, these fixed responses will be maintained unless their costs are high, which would lead to the appearance of co-evolutionary cycles. However, costs for the maintenance of egg rejection are rare, leading to successful resistance as a common long-term outcome of the co-evolutionary relationship between brood parasites and their hosts (Soler 2014). This fact, as discussed in Chapter 6, may explain the occurrence of egg rejection abilities in species that are currently not exploited by interspecific brood parasites. But even if host responses exhibit a low degree of plasticity in relation to conditional stimuli, decision-making will remain as the keystone of the egg-rejection process.

Endocrine consequences of brood parasitism

As stated above, both the behavioural and morphological consequences of brood parasitism are well known (Davies and Brooke 1988, Davies 2011). However, the impact of parasitism on the hormonal status of hosts has been neglected, despite the relevance of endocrine pathways for both the modulation of individuals' responses to stressful situations and the regulation of parental care in birds. Advances in the study of the hormonal processes behind egg rejection may therefore contribute to better understand the proximate mechanisms underlying host responses. Chapter 4 evaluates the impact of non-mimetic parasitic eggs on the physical and hormonal status of the host, revealing that brood parasitism can become a stressful factor for hosts during incubation, as occurs during the chick (Ibáñez-Álamo et al. 2012) or fledgling stages (Mark and Rubenstein 2013). Corticosterone levels, the hormone that regulates the response to stress (Wingfield et al. 1998, Breuner et al. 2008), were higher in parasitized individuals compared to non-parasitized individuals, which could be related to the mobilization of energy from body reserves to cope with the current environmental stressors (Sapolsky et al. 2000, Landys et al. 2006). In fact, we found a negative relationship between host corticosterone levels and body condition. Furthermore, parasitized individuals showed a significantly lower body condition than non-parasitized females, which, to the best of our knowledge, reveals for the first time the negative effect of brood parasitism on the physical state of hosts. Prolactin, on the other hand, does not appear to play such a clear role in the host's hormonal response to the parasitic egg given that no differences were found in levels of this hormone between parasitized and non-parasitized females. However, since decreases in prolactin levels are associated with disruption of parental care and nest desertion (Sockman et al. 2006, Angelier and Chastel 2009, Ouyang et al. 2011), unaffected prolactin levels found in parasitized females suggest that blackbirds tend to maintain the clutch care even if they recognized the parasitic egg, which may partially explain the endocrine mechanism behind the absence of nest desertion as egg-rejection mechanism described for medium-sized hosts in Chapter 1.

The hormonal response to the standardized stress protocol provides important information on the endocrine strategies of individuals to cope with environmental stressors since it can be modulated according to different ecological factors, such as the value of their current reproduction (Lendvai et al. 2007, Lendvai and Chastel 2008). Circulating levels of corticosterone increased markedly in both parasitized and non-parasitized individuals, but finally no differences were found in stress-induced corticosterone levels between the two groups. This corticosterone increase can be considered as a hormonal strategy of individuals to minimize risks, favouring self-maintenance when dealing unpredictable perturbations (Wingfield et al. 1998). Prolactin levels decreased in response to the stress protocol in both experimental groups but this decrease tended to be higher in parasitized females. This could be related to the lower body condition exhibited by parasitized females since, in other species, a sharper and quicker decline of prolactin levels has been found in energetically constrained individuals (Cherel et al. 1994, Criscuolo et al. 2002, O'Dwyer et al. 2006, Groscolas et al. 2008, Angelier et al. 2009, Spée et al. 2010, Riechert et al. 2014). In this way, we found a negative relationship between body condition and the prolactin decrease in response to the stress protocol. Finally, Chapter 4 shows that corticosterone and prolactin are unrelated in blackbirds, suggesting that each of them is involved in different processes. The combined study of these hormones in future works on brood parasitism may reveal the endocrine mechanisms underlying anti-parasitic hosts behaviours, not only during incubation, but also in other stages of the breeding cycle helping us to better comprehend the host-parasite co-evolutionary process.

Evolutionary origin of egg rejection

The rejection of parasitic eggs is the most widespread defence among hosts of obligate brood parasites (Rothstein 1990, Davies 2000), and is traditionally assumed to have evolved as a defence against interspecific brood parasitism (Davies and Brooke 1989b). But what is the origin of the recognition abilities in those species that are not currently impacted by interspecific brood parasitism, as is the case of the common blackbird? Could the egg-rejection behaviour exhibited by these species be the result of parasitism by conspecifics (Samas et al. 2014), or does it reflect a past exploitation by interspecific brood parasites? Egg-rejection experiments detailed in Chapter 6 provide crucial information supporting the interspecific origin of the egg-rejection abilities showed by the common blackbird. These evidences are based mainly on the low recognition and rejection rates of conspecific compared to cuckoo model eggs exhibited by blackbirds, as well as the particularly aggressive response of blackbirds towards cuckoo dummies compared to blackbird female dummies. Given that rates of conspecific brood parasitism present in the studied blackbird populations are extremely low, and that costs linked to rear a conspecific chick are usually lower than those imposed by young of interspecific brood parasites, it seems fair to conclude that conspecific brood parasitism is not an important pressure leading the evolution of egg rejection abilities in blackbirds. In fact, unlike hosts species exploited by interspecific brood parasites, egg rejection is absent in most species suffering conspecific brood parasitism (Soler et al. 2011). Therefore, the most likely explanation for the egg rejection abilities exhibited by blackbirds is that they were historically exploited by a common cuckoo race, but they became winners of the co-evolutionary arms race thus forcing the cuckoo to switch to another host species (Soler 2014). This fact can explain why some brood parasites, such as the common cuckoo, avoid potential host species with accessible nests and suitable diets for raising the parasitic young, as is the case of the common blackbird. Cross-fostering experiments have revealed that the newly-hatched cuckoo chick is able to eject blackbird eggs from the nest, and that blackbird diet, mainly based on earthworms, is suitable for rearing the parasitic chick (Grim et al. 2011). Blackbirds show fine egg-rejection abilities (Moskát et al. 2003, Hale and Briskie 2007, Samas et al. 2011, Martín-Vivaldi et al. 2012, Ruiz-Raya et al. 2015, Soler et al. 2015, 2017), aggressive behaviour against cuckoo dummies (Grim et al. 2011, Ruiz-Raya et al. 2016), and refuse to feed cuckoo chicks (Grim 2006, Grim et al. 2011), so such highly effective anti-parasitic defences would explain the absence of current parasitism in this species (Ruiz-Raya et al. 2016). Absence of costs for the maintenance of the egg-rejection abilities would result in successful resistance, a long-term outcome of the antagonistic relationship between brood parasites and their hosts that is more frequent than expected and prevent the future exploitation by brood parasites (Soler 2014).

Sex roles in egg rejection

The cognitive abilities of hosts, and therefore recognition abilities, will play a decisive role in the judgment stage of the egg-rejection process. These abilities are determined by the genetic background of hosts, but they are also a consequence of the co-evolutionary history between brood parasites and hosts. Host species recently exploited by brood parasites will exhibit less evolved recognition abilities compared to those host species that have shared a co-evolutionary history with brood parasites for a long time (Rothstein and Robinson 1998). But the evolution of recognition abilities, particularly those related to template-based mechanisms, also depend on the opportunities of hosts to learn the aspect of their own eggs, which can importantly differ between males and females, if they have different roles during incubation. These potential differences in cognitive abilities between males and females are assessed in Chapter 7. Although traditional theory assumes an exclusive role of incubating individuals (usually females) in egg rejection(Soler et al. 2002), we found that non-incubating males can evolve recognition abilities if they occasionally visit the nest, which fit previous results in other host species (Sealy and Neudorf 1995). However, although blackbird males can recognize, and even eject non-mimetic eggs, female's egg-rejection abilities are much more accurate than males´ abilities as they are able to recognize and eject mimetic eggs as well (Chapter 1 and 3), likely as a consequence of their greater association to the eggs during the incubation stage. Interestingly, the fact that blackbird males recognize non-mimetic rather than mimetic eggs supports the idea that egg-recognition abilities evolved in response to interspecific brood parasitism in this species, as concluded in Chapter 6. This result would support then the hypothesis that blackbirds suffered strong parasitism pressures in the past, which match with the predictions based on the ecological characteristics of this species (Martín-Vivaldi et al. 2012, Soler 2014).

Conclusions

1. Desertion of nest experimentally parasitized by cuckoo-sized eggs can be due to environmental factors other than brood parasitism, so nest desertion should not be considered a response unequivocally directed to brood parasitism in medium or large-sized species.

2. Egg-recognition experiments, especially those carried out in medium or large-sized hosts, should include control groups to elucidate if nest desertion is a response particularly directed to experimental parasitism.

3. Egg acceptance may reflect, at least in some cases, acceptance decisions instead of recognition failures. The host's lack of propensity to assume the costs linked to the ejection of a slightly heavier egg (low motivation) led to the acceptance of previously recognized eggs, confirming that some egg traits can modify the host's decision without affecting egg recognition.

4. The egg-rejection process can independently be affected at each of its stages, which would lead to acceptance of the parasite egg. This can be provoked by recognition constraints imposed by highly mimetic eggs, or by physical constraints linked to larger eggs. Delays in egg ejection can therefore be a consequence of the delay in the decision to reject rather than reflecting a delay in recognition.

5. Host´s recognition abilities should not be inferred from the rejection rate as hosts can recognize more eggs than they finally reject. The egg-touching behaviour exhibited by blackbirds, as occurs with the egg-pecking behaviour in other host species, can be used as a reliable indicator of egg-recognition abilities of hosts.

6. Plastic responses are favoured by natural selection under highly variable risk of parasitism allowing hosts to avoid the expression of costly defences in situations of low risk of parasitism, particularly important at initial stage of parasitism. Costs of plasticity, as well as the absence of costs for the maintenance of the canalized phenotype, could explain the low occurrence of plastic responses among host species.

7. The higher baseline corticosterone levels showed by parasitized females indicate that brood parasitism can be a hormonal stressor during incubation. On the other hand, the presence of a parasitic egg did not modify the baseline prolactin levels of blackbird females, which could shed light on the endocrine mechanism behind the absence of nest desertion found in egg-rejection experiments in blackbirds.
8. Brood parasitism negatively affected the body condition of parasitized individuals likely due to energy mobilization from body reserves mediated by the increase in corticosterone levels. In fact, a negative relationship between corticosterone levels and body condition was found in parasitized females.
9. Brood parasitism did not affect the corticosterone stress response; however, the stress-induced prolactin decrease tended to be more pronounced in parasitized females, which could be related to their lower body condition.
10. Corticosterone and prolactin are unrelated in blackbirds as they seem to be modulated according to different ecological factors. Therefore, the combined study of these hormones in future works on brood parasitism will provide complementary information on the host´s anti-parasitic behaviour.
11. Blackbirds recognized and ejected interspecific, but not conspecific eggs, and were particularly aggressive towards cuckoo dummies, suggesting that the evolutionary origin of the rejection abilities in this species is probably linked to a past co-evolutionary relationship with interspecific brood parasites.
12. The low costs associated with the maintenance of egg rejection abilities may explain the high rejection rates found in potential host species that are not currently exploited by interspecific brood parasites, such as the common blackbird.
13. Occasional visits to the nest could favour the evolution of anti-parasitic defences in males even though they are not involved in egg incubation. In blackbirds, a potential host species where only the female incubates, males have been found to be able to recognize and eject parasitic eggs.
14. The terminology used in egg-rejection studies should include updated concepts reflecting recent advances in the field of animal decision-making, as well as the updated version of concepts classically used in brood parasitism studies.

Conclusiones

1. El abandono de nidos experimentalmente parasitados con huevos de tamaño similar a los huevos de cuco puede deberse a factores ambientales no relacionados con el parasitismo de cría, por lo que el abandono del nido no debería ser considerado una respuesta inequívocamente consecuencia del parasitismo de cría en especies hospedadoras medianas o grandes.
2. Los experimentos de reconocimiento de huevos, especialmente aquellos realizados en hospedadores de tamaño medio o grande, deberían incluir grupos control para determinar si el abandono del nido es una respuesta provocada por el parasitismo experimental.
3. La aceptación del huevo parásito puede reflejar, al menos en algunos casos, decisiones de aceptación en lugar de errores en el reconocimiento. La baja inclinación por parte del hospedador a asumir los costes asociados a la expulsión de un huevo ligeramente más pesado (baja motivación) condujo a la aceptación de huevos previamente reconocidos, confirmando que algunos rasgos de los huevos parásitos pueden modificar la decisión del hospedador, aunque no afecten al reconocimiento del huevo.
4. El proceso de rechazo del huevo puede verse afectado de manera independiente en cada una de sus etapas y dar lugar a la aceptación del huevo parásito. Esto puede ser provocado por restricciones de reconocimiento impuestas por huevos altamente miméticos, o bien por restricciones físicas relacionadas con huevos de mayor tamaño. Los retrasos en la expulsión del huevo pueden, por lo tanto, ser consecuencia del retraso en la decisión de rechazar en lugar de reflejar un retraso en el reconocimiento.
5. Las habilidades de reconocimiento del hospedador no deben inferirse a partir de la tasa de rechazo, ya que el hospedador puede reconocer más huevos de los que finalmente son rechazados. El comportamiento de dar toques a los huevos por parte de los mirlos, al igual que ocurre con el comportamiento de picoteo de huevo en otras especies hospedadoras, puede ser usado como un indicador fiable de las capacidades de reconocimiento de los hospedadores.

6. Las respuestas plásticas son favorecidas por la selección natural en contextos donde el riesgo de parasitismo es altamente variable, permitiendo a los hospedadores evitar la expresión de defensas costosas en situaciones de bajo riesgo de parasitismo, lo que podría ser particularmente importante en la etapa inicial del parasitismo. Los costos de la plasticidad, así como la ausencia de costos para el mantenimiento del fenotipo canalizado, podría explicar la baja presencia de respuestas plásticas entre las especies hospedadoras.

7. Los mayores niveles de corticosterona basal mostrados por las hembras parasitadas indican que el parasitismo de cría puede ser un factor de estrés hormonal durante la incubación. Por otro lado, la presencia de un huevo parásito no modificó los niveles basales de prolactina de las hembras de mirlo, lo cual podría arrojar luz sobre el mecanismo endocrino detrás de la baja tasa de abandono encontrada en experimentos de rechazo de huevos en el mirlo.

8. El parasitismo de cría afectó negativamente a la condición corporal de los individuos parasitados, probablemente como una consecuencia de la movilización de energía desde las reservas corporales mediada por el aumento de los niveles de corticosterona. De hecho, se encontró una relación negativa entre los niveles de corticosterona y la condición corporal de las hembras parasitadas.

9. El parasitismo de cría no afectó a la respuesta de la corticosterona al estrés; sin embargo, la disminución de prolactina en respuesta al estrés tendió a ser más pronunciada en las hembras parasitadas, lo que podría estar relacionado con su más baja condición corporal.

10. La corticosterona y la prolactina no están relacionadas en los mirlos y parecen estar moduladas por diferentes factores ecológicos. Por lo tanto, el estudio combinado de estas hormonas en trabajos futuros de parasitismo de cría proporcionará información complementaria sobre el comportamiento antiparasitario de los hospedadores.

11. Los mirlos reconocieron y expulsaron huevos interespecíficos, pero no conespecíficos, y fueron particularmente agresivos con los modelos de cuco, lo que sugiere que el origen evolutivo de las habilidades de rechazo en esta especie está probablemente vinculado a una pasada relación coevolutiva con parásitos de cría interespecíficos.

12. Los bajos costes asociados al mantenimiento de las capacidades de rechazo del huevo pueden explicar las altas tasas de rechazo encontradas en potenciales especies hospedadoras que no son actualmente explotadas por parásitos interespecíficos de cría, como es el caso del mirlo común.

13. Las visitas ocasionales de los machos al nido podrían favorecer la evolución de defensas antiparasitarias en este sexo, a pesar de no estar implicado en

la incubación. En los mirlos, una potencial especie hospedadora donde sólo las hembras incuban, los machos son capaces de reconocer y expulsar huevos parásitos.

14. La terminología utilizada en los estudios de rechazo de huevos debe incluir conceptos actualizados que reflejen los recientes avances en el campo de la toma de decisiones en animales, así como la versión actualizada de conceptos utilizados clásicamente en estudios de parasitismo de cría.

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