BEHAVIOURLA AND PHYSIOLOGICAL RESPONSES TO BROOD PARASITISM AND NEST PREDATION IN TWO PASSERINE SPECIES

Tesis doctoral de Gianluca Roncalli

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RESPUESTAS COMPORTAMENTALES Y FISIOLÓGICAS FRENTE AL PARASITISMO DE CRÍA Y A LA DEPREDACIÓN DE NIDOS EN DOS PASERIFORMES

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TESIS DOCTORAL

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A mio padre

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SUMMARY/RESUMEN

SUMMARY

Brood parasitism and nest predation are two of the most important selective pressures in birds, particularly in altricial species, which are the species whose nestlings, being born unfeathered, blind and helpless, must be cared by parents. Avian brood parasitism in one of the best examples of a coevolutionary process, where hosts evolve a series of adaptations to counteract and limit the fitness costs imposed by brood parasites. Among these defensive adaptations, the recognition and rejection of the parasitic egg are decisive. Nest predation, on the other hand, is a classic example of agonistic interaction, being the most important force shaping nestlings' life-history traits since it represents the first cause of mortality for avian offspring. The main aim of this thesis is to expand our knowledge on how these two extremely important selective pressures shape some behavioural and physiological responses in birds.

In the **first part** of the thesis we provide new information on the breeding biology of a poorly known bird species, the Western Bonelli's warbler *Phylloscopus bonelli*, which is also a potential host for the common cuckoo *Cuculus canorus* in Southern Europe (**Chapter 1**). This novel and detailed information is essential not only for expanding our understanding of this species but also to draw attention to the potential risks that it might face in the near future, considering the reduction that this species has suffered in the study area during recent years.

The **second part** of the thesis focus on brood parasitism. Using the Bonelli's warbler, we first investigated a methodological question (**Chapter 2**), whether different characteristics of the parasitic egg (size and material) used in egg-rejection experiments could affect the egg-rejection behaviour of the host. This kind of methodological studies, even though seldomly done, are of key importance in order to determine the validity of experiments in the field. We found that plasticine may misrepresent the responses to experimental parasitism, at least in small host species, because this material facilitates egg ejection, provoking a decrease in nest desertion rate. We also found that small parasitic eggs could be ejected by this nest-abandon species and that warbler nests parasitized with large eggs were more often deserted, thus indicating that nest desertion occurs because of the constrains imposed by the size of parasitic eggs.

Within the framework of this second part, and linking with the next one, we also studied the interaction between these two important selective pressures: brood parasitism and nest predation (**Chapter 3**). In this case we used another (presumably) former host species of cuckoos, the common blackbird (*Turdus merula*) which suffer high nest predation levels. Several studies on parasite-host systems have investigated adaptations and counter-adaptations associated to this biological interaction, but only recently researchers have understood that the recognition and rejection of parasitic eggs are part of a complex process, in which the decision of rejecting mainly depends on the costs associated to that

action, the external stimuli perceived by the host or its internal status of motivation. Therefore, phenotypic plasticity of egg-rejection behaviour might play an important function in the promotion of evolutionary changes. Predation could be one of the environmental pressures that may affect the egg-rejection process, particularly affecting the cost-benefit trade-off. Nevertheless, studies that explore the interaction between predation and brood parasitism are limited. We found that blackbirds exposed to risk of an adult predator showed a partial increase in egg recognition and a significant lower ejection rate, whereas the risk of an offspring (egg) predator did not modify their anti-parasitic behaviours. Interestingly, this effect increased towards the end of the breeding season. This findings open a new research line in the study of brood parasitism and provide new knowledge in our understanding of the regulation of the egg-rejection process.

The **third part** of this thesis focus on nest predation. Studies on the topic have explored the adaptations of prey to predator pressure (i.e. anti-predator strategies), focusing principally on morphological and behavioural defences, while, only recently, researchers have realized that also physiology has a critical role in anti-predator strategies. Predation, and in particular the risk associated to predation, is known to modify some physiological functions of preys like their hormonal responses. However, very little is still known about its effect in other critical physiological systems. The immune system is definitely an important physiological component, which promotes the survival of an organism by defending it from external pathogens, diseases and infection. Despite recent evidence showing that predation risk can alter the immune response of prey, this link has been greatly overlooked. In the first chapter of this part we explored whether a short-term nest predation risk, typical of a predator encounter, can provoke changes in the immune system of nestlings (**Chapter 4**). Using blackbirds again, we experimentally tested several levels of nest predation risk and measured a complete set of immunological variables in order to capture the most detailed picture of this potential relationship. We found that nest predation risk induced an increase in ovotransferrin, immunoglobulin levels and the number of lymphocytes and eosinophils, suggesting a general activation of the immune response which will prepare nestlings to cope with the possible inflammation or infection provoked by a predator attack. Interestingly, only high and extreme levels of risk caused the immunological changes, indicating that nestlings would be able to modulate their immune responses according to the perceived level of threat.

Finally, we tested whether a long-term increase in nest predation risk, more typical of the ecology of fear concept, could also entail immunological changes in nestlings (**Chapter 5**). Changes in the immune system can impose important costs in organisms, thus, it is possible that the responses to short- or long-term predation risk are different. In addition, the nestling period is particularly critical for birds as, during this phase, they invest most of their resources in the development and this could interfere with the immunological response to predation risk. This is actually what we found. Our manipulation of predation risk during the whole nestling period induced a reduction of

immunoglobulins but an increase in lymphocytes, suggesting different effects depending on the duration of the threat and the costs associated with the immunological changes. Interestingly, in the last two studies (Chapters 4 and 5), we found that the condition of the organism (i.e. health status or body condition) affected the immunological responses to nest predation. For instance, only those nestlings without endoparasites or in good body condition were able to increase their immune response for the short-term manipulation, while body condition and growth rate mediated the immunological responses for the long-term manipulation.

This thesis confirms that both brood parasitism and nest predation, through the behavioural and physiological mechanisms that induce, are two decisive selective pressures that strongly shape the evolution of the adaptations in altricial birds. Predation risk is able to trigger an immune response in the organism and this might have important consequences in developing organisms, such as nestlings, as it can alter the normal trade-offs between immunity and the physiological processes of the development. Further we demonstrated the interplay between predation and brood parasitism, offering a new perspective of the forces that may shape the evolution of the anti-parasite defences in host.

RESUMEN

El parasitismo de cría y la depredación de nidos representan dos de las más importantes presiones selectivas en las aves, sobre todo en las especies altriciales, en las que los pollos, naciendo sin plumas, ciegos e indefensos, dependen totalmente de los cuidados parentales. El parasitismo de cría en aves es uno de los mejores ejemplos de coevolución, en el que los hospedadores han desarrollado una serie de adaptaciones para contrarrestar y limitar los costes en la eficacia biológica causados por los parásitos. Entre estas adaptaciones, las más importantes son el reconocimiento y el rechazo del huevo parasito. Por otro lado, la depredación de nidos es un clásico ejemplo de interacción antagónica que está considerada la fuerza más importante en modular los caracteres de las estrategias vitales, ya que representa la primera causa de mortalidad para los pollos de las aves. El objetivo principal de esta tesis es ampliar el conocimiento de cómo estas dos importantes presiones selectivas modulan las respuestas comportamentales y fisiológicas en las aves.

En la **primera parte** de la tesis proporcionamos nueva información sobre la biología reproductiva de una especie de aves poco conocida, el mosquitero papialbo *Phylloscopus bonelli*, que también es un hospedador potencial del cuco común *Cuculus canorus* en el sur de Europa (**Capitulo 1**). Esta novedosa y detallada información es importante, no solo para ampliar nuestro conocimiento sobre su biología reproductiva, sino también para prevenir los riesgos potenciales que esta especie podrá sufrir en un futuro próximo, considerando la reducción que el mosquitero papialbo ha sufrido en el área de estudio en los últimos años.

La **segunda parte** de esta tesis se centró en el parasitismo de la cría. Utilizando el mosquitero papialbo, investigamos primero una cuestión metodológica (**Capítulo 2**), es decir si diferentes características de los modelos de huevos parásitos (tamaño y material) utilizados en los experimentos de expulsión de huevos pueden afectar el comportamiento de rechazo del hospedador. Este tipo de estudios metodológicos, aunque hayan sido realizados raramente, son muy importantes para determinar la validez de estos experimentos. Hemos encontrado que la plastilina puede afectar de manera importante las respuestas al parasitismo, al menos en los pequeños hospedadores, ya que este material facilita la expulsión del huevo, provocando una disminución en la tasa de abandono del nido. También encontramos que los huevos parásitos pequeños pueden ser expulsados por parte de esta especie en la que el abandono del nido es la respuesta más frecuente, y que los nidos de mosquitero parasitados con huevos grandes fueron abandonados más frecuentemente, lo que indica que el abandono del nido ocurre debido a las limitaciones asociadas al tamaño de los huevos parásitos.

En esta segunda parte y en relación con la siguiente, hemos estudiado también la interacción entre estas dos importantes presiones selectivas: el parasitismo de la cría y la

depredación del nido (**Capítulo 3**). En este caso hemos utilizado otra especie (presumiblemente) hospedadora del cuco común en el pasado: el mirlo común (*Turdus merula*). Esta especie sufre altos niveles de depredación de nidos. Numerosos estudios sobre los sistemas parásito de cría-hospedador han investigado las adaptaciones y contra-adaptaciones asociadas a esta interacción biológica, pero sólo recientemente los investigadores han entendido que el reconocimiento y expulsión de los huevos parásitos son parte de un proceso complejo, en el que la decisión de rechazar depende principalmente de los costos asociados a esa acción, de los estímulos externos percibidos por el hospedador o de su estado de motivación influenciado principalmente por el riesgo de parasitismo. Por lo tanto, la plasticidad fenotípica del comportamiento de expulsión del huevo podría desempeñar una función importante en la evolución del comportamiento de expulsión. La depredación, afectando particularmente a la relación costes-beneficios entre expulsar o mantenerse inactivo por el riesgo de depredación, podría ser una de las presiones ambientales que afectaría el proceso de expulsión de los huevos parásitos. Sin embargo, los estudios que investigan la interacción entre la depredación y el parasitismo de la cría son muy limitados. Nosotros encontramos que los mirlos expuestos al riesgo de un depredador de adultos mostraron un aumento parcial del reconocimiento de los huevos y una tasa de expulsión significativamente más baja, mientras que el riesgo de un depredador de nidos (huevos o pollos) no modificó su comportamiento de expulsión de huevos. Curiosamente, este efecto aumentó hacia el final de la temporada de cría. Estos descubrimientos abren una nueva línea de investigación en el estudio del parasitismo de la cría y proporcionan nuevos conocimientos en la comprensión de la regulación del proceso de expulsión de huevos.

La **tercera parte** de esta tesis se centró en la depredación del nido. Los estudios sobre este tema han explorado las adaptaciones de las presas frente a la presión de los depredadores (es decir, las estrategias anti-depredadoras), centrándose principalmente en las defensas morfológicas y comportamentales. Sin embargo, los investigadores solo se han dado cuenta recientemente, de que también la fisiología tendría un papel crítico en las estrategias anti-depredadoras. Sabemos que la depredación, y en particular el riesgo asociado a la depredación, afecta a algunas funciones fisiológicas de las presas, como por ejemplo sus respuestas hormonales. Sin embargo, se sabe todavía muy poco sobre su efecto sobre otros procesos fisiológicos. El sistema inmunológico es, de hecho, un importante componente fisiológico, que promueve la supervivencia de un organismo defendiéndolo de patógenos externos, enfermedades e infecciones. A pesar de la reciente evidencia de que el riesgo de depredación puede alterar la respuesta inmune de las presas, esta asociación ha sido por mucho tiempo ignorada. En el primer capítulo de esta parte se explora si un riesgo de depredación de nidos a corto plazo, típico de un encuentro con depredadores, puede provocar cambios en el sistema inmunológico de los pollos (**Capítulo 4**). Utilizando de nuevo el mirlo como especie modelo, testamos experimentalmente varios tipos de riesgo de depredación de nidos y medimos un conjunto de variables inmunológicas para obtener un cuadro más detallado de esta potencial relación. Encontramos que el riesgo de depredación de nidos provocó un aumento de la ovotransferrina, de los niveles de inmunoglobulina y del número de linfocitos y eosinófilos, lo que sugiere una activación general de la respuesta inmune que prepararía a los pollos para responder a la posible inflamación o infección que un ataque de depredadores podría causar. Curiosamente, sólo los niveles altos y extremos del riesgo simulado causaron cambios inmunológicos, lo que indica que los pollos modularían sus respuestas inmunes según el nivel de amenaza percibido.

Por último, hemos estudiado si un aumento a largo plazo del riesgo de depredación del nido, más típicamente asociado al concepto de la "ecología del miedo", podría también implicar cambios inmunológicos en los pollos (**Capítulo 5**). Los cambios del sistema inmunológico pueden provocar importantes costes en los organismos, por lo tanto, es posible que las respuestas al riesgo de depredación a corto o largo plazo sean diferentes. Además, el período de estancia de los pollos en el nido es particularmente crítico para las aves, ya que, durante esta fase, invierten la mayor parte de sus recursos en el desarrollo y esto podría interferir con la respuesta inmunológica al riesgo de depredación. Esto es lo que de hecho encontramos. Nuestra manipulación del riesgo de depredación durante todo el período de estancia en el nido causó en los pollos un aumento de las inmunoglobulinas, pero una reducción de los linfocitos, lo que sugiere que la duración de la amenaza y los costos asociados con los cambios inmunológicos podrían provocar diferentes efectos. Cabe destacar que, en los dos últimos estudios (Capítulos 4 y 5), encontramos que la condición física de los pollos (es decir, su estado de salud o condición corporal) afectó a las respuestas inmunológicas frente a la depredación de nidos. Por ejemplo, en el caso de la manipulación a corto plazo, sólo aquellos pollos sin endoparásitos o que mostraban buenas condiciones corporales fueron capaces de aumentar su respuesta inmune, mientras que la condición corporal y la tasa de crecimiento mediaron las respuestas inmunológicas en la manipulación a largo plazo.

Esta tesis corrobora que, tanto el parasitismo de la cría como la depredación de nidos son dos presiones selectivas decisivas que afectan considerablemente la evolución de las aves altriciales mediante los mecanismos comportamental y fisiológico que estas interacciones provocan. El riesgo de depredación es capaz de desencadenar una respuesta inmune en el organismo y esto puede tener importantes consecuencias en los organismos en desarrollo, como los pollos de las aves altriciales, ya que alterarían los compromisos entre el sistema inmune por un lado y los procesos fisiológicos del desarrollo por otro. Además, demostramos la existencia de la interacción entre la depredación y el parasitismo de cría, ofreciendo una nueva perspectiva de las fuerzas que puede modelar la evolución de las defensas de los hospedadores frente a los parásitos.

GENERAL INTRODUCTION

1.1. Two important selective pressures driving evolution in birds: brood parasitism and predation.

Natural selection is one of the basic mechanism of evolution and can be defined as the process in which individuals possessing certain genotypic characteristics tend to survive and reproduce and therefore transmit their genotypic qualities to future generations (Endler 1986). Natural selection can operate only if three premises occur: 1) variation in phenotypical traits, 2) a direct link between these traits and the capacity of organism of reproducing (variation in fitness) and 3) the hereditability of these traits (Endler 1986).

Selective pressures are those factors which alter fitness of living organisms within a given environment and, through the action of natural selection, drive the evolution (Fisher 1930, Endler 1986). The immediate consequence of the evolutionary process is the increase in genotype frequencies that better counteract selective pressures in a given population. Selective pressures can be divided in abiotic and biotic factors; abiotic pressures deriving from non-living factors being present in the environment in which the organism lives, whereas biotic pressures are living organisms that interact with the affected organism within the same ecosystem (Soler 2002a).

Biological interactions between species are important selective pressures driving evolution since they are able to produce evolutionary changes in the organisms involved (Maynard Smith 1998). Among them, predation and brood parasitism have a decisive impact on selection mechanisms (Møller, Dufva & Allender 1993, Abrams 2000). Predation involves an organism (the predator) killing and consuming another (the prey; Caro 2005), while parasitism is the interaction in which an organism (the parasite) consumes resources from another organism (the host), resulting in a decrease in fitness to the host (Bush *et al.* 2001). Brood parasitism is a form of parasitism in which certain individuals, the parasites, use parental care of unrelated individuals, the hosts, to grow their progeny (Rothstein 1990). These two antagonistic interactions evolved in several different ways, but the most frequent form is known as “arms race” where the “victim” species (i.e. prey or host) develops adaptations that allows it to avoid the interaction, while the other species (i.e. predator or parasite) evolves adaptive counter-adaptations that tend to overcome defensive strategies (Soler 2002b).

A crucial component of biological interactions on which natural selection is able to operate is behaviour (Wolf *et al.* 2007). The ability of organisms to adapt their behaviour in response to selective pressures represent an important factor affecting the relationship between predator and prey or parasite and host. In fact, adaptations and counter-adaptations can involve behavioural characters, just like occurs with morphological oners (Wcislo 1989). Nowadays it is assumed that also behavioural characters are hereditable, can vary between individuals and may be adaptive by allowing an animal to increase its reproductive success (Soler 2016). According to these assumptions, also behaviour can be considered as a promoter of evolutionary changes and therefore, investigations that examine its adaptive significance are of great interest in evolutionary biology.

1.2. Avian brood parasites and host's defences: a model system for coevolution

Brood parasitism has been frequently studied in birds; avian brood parasites lay their eggs in the nest of another individual, the host, which incubates and takes care of parasitic offspring (Rothstein 1990, Soler & Soler 2000, Soler 2014). Brood parasitism can take two form: intraspecific, when parasites lay eggs in nests of their own species (Yom-Tov 2001, Eadie & Lyon 2011) or interspecific, when parasites lay their egg in nests of a different species (Davies 2000, Payne 2005). Intraspecific brood parasitism has been found in 236 species (Yom-Tov 2001) and is expected to be more frequent in precocial species, since they have larger clutches (in which is more difficult to detect the parasitic egg) and their offspring require fewer parental care, or in colonial species, where nests are crowded in small spaces (Yom-Tov 2001). Interspecific brood parasitism is less common and has evolved in about a hundred species, which corresponds approximately to 1% of the bird species existing in the world (Davies 2000). It was found in 5 families: Indicatoridae (17 species), Anatidae (1 species), Cuculidae (57 species), Icteridae (5 species), and Estrildidae (20 species; Davies 2000).

Only two brood parasitic species, which belong to Cuculidae, live in Europe: the common cuckoo *Cuculus canorus* and the great spotted cuckoo *Clamator glandarius* (Payne 1997). Their reproductive strategies are different; while the female of the common cuckoo lays one egg per host nest and the newly hatched cuckoo nestling ejects all host eggs or chick in the parasitized nest, the great spotted cuckoo can lay more eggs per nest and the hatched cuckoo does not evict its nest mates, but it shares parental care with them (Davies 2000). Moreover, the great spotted cuckoo is a specialist parasite since females parasitize regularly only two corvid species (Soler & Møller 1990, Soler & Soler 2000), while the common cuckoo parasites a wide variety of hosts, even though individual females belong to separate races, named gentes (Gibb *et al.* 2000), specialized in a single host species.

The common cuckoo-host system has been used to develop the second part of this thesis (**Chapters 2 and 3**), which investigates the possible factors affecting the defensive mechanisms of hosts against brood parasitism in the frame of a coevolutionary process. The eggs of the common cuckoo have been found in more than 100 passerine species in Europe (Moksnes, Røskaft & Braa 1991a). The main host species are: reed warbler *Acrocephalus scirpaceus* in marshlands, meadow pipit *Anthus pratensis* in moorlands, dunnock *Prunella modularis* and robin *Erithacus rubecula* in woodlands and pied wagtail *Motacilla alba yarrelli* in open farmlands (Davies 2000). In addition to these, other several species are frequent or rarely parasitized, such as garden warbler *Sylvia borin* or blackcap *Sylvia atricapilla* (Moksnes *et al.* 1991b). Two of these potential hosts, Western Bonelli's warbler *Phylloscopus bonelli* (hereafter Bonelli's Warbler) and common blakbird *Turdus merula* (hereafter blackbird), were used to carry out our experimental studies.

Bonelli's warbler (**Chapter 2**) was chosen because the information about its relationship with common cuckoo is limited despite the fact that: 1) Campobello and Sealy (2009) documented some parasitized nests in Italy, 2) two closely related species (i.e. willow warbler *P. trochilus* and chiffchaff *P. collybita)* are commonly parasitized in the woodlands of Northern Europe (Perrin de Brichambaut 1997) and 3) it is the most frequent breeding *Phylloscopus* species in many woodlands of Southern Europe, where shares the habitat with the common cuckoo. On the other hand, we used the blackbird as our second model species (**Chapter 3**) because 1) it is commonly used for egg-rejection experiments (Moskát, Karcza & Csörgo 2003, Hale & Briskie 2007, Polačiková & Grim 2010, Samaš *et al.* 2011, Ruiz-Raya *et al.* 2016, Soler *et al.* 2017), thus offering detail information of this anti-parasitic strategy and because 2) it usually suffer extremely high nest predation pressures (Ibáñez-Álamo & Soler 2010), which makes it an excellent model to investigate the interaction between brood parasitism and predation risk.

Avian brood parasitism is considered one of the clearest example of coevolution because the adaptations and counter-adaptations of both parasite and host species result in the arm race coevolutionary process (Dawkins & Krebs 1979). Coevolution, intended as those interactions that provoke reciprocal evolutionary adaptations driven by natural selection, is considered today, one of the main processes that have led to the enormous biodiversity present on Earth, thanks to the speciation resulted from the reciprocal interactions between species (Thompson 1994). Additionally, it is thought to be responsible for some of the most important life-history events ever happened, such as the origin of eukaryotic cells (Thompson 1994). The avian brood parasite-host system presents a series of characteristics that make this interaction an ideal model to study coevolution: 1) the interactions usually occur between few species, 2) they are easily detected by humans, since they involve visual and acoustic cues, 3) most of the parasite's adaptations and hosts' counter-defences are manifested in or close to a specific place, the nest and 4) the costs and benefits of brood parasite-host interaction are easily recognizable since the brood parasite's fitness is maximized when the entire brood of the host is lost (Rothstein 1990).

Avian brood parasitism generates high costs in term of fitness reduction given that usually only the cuckoo's offspring survives in parasitized nests (Rothstein 1990). Therefore, host species are selected to evolve defensive mechanisms against parasites, being the most widespread and important the recognition and rejection of the foreign egg (Davies & Brooke 1989). As the coevolutionary arms race hypothesis predicts, the defensive measures adopted by the hosts will favour the development of new mechanisms in the parasites that will allow them to counteract the effectiveness of these defences, for example, by producing eggs more and more similar (mimetics) to those of their hosts (Davies & Brooke 1989), or by forcing the hosts to accept its eggs (mafia behaviour; Zahavi 1979, Soler *et al.* 1995). Defensive mechanisms of egg-rejection adopted by hosts also incur costs: 1) hosts can mistakenly recognize their own eggs as parasitic and eject them (recognitions error; Davies 1996) or 2) or they can accidentally break some of their own eggs when trying to eject the parasitic one (ejection costs; Lotem & Nakamura 1998, Røskaft & Moksnes 1998). The relative size of host and parasitic eggs has a determinant role in the choice of egg-rejection mechanism, since the costs associated to the ejection are generally more severe for small hosts with respect to medium or large hosts, given their small size which limits or prevents the egg ejection (Moksnes *et al.* 1991a). Blackbird is a medium-sized bird which commonly ejects experimental parasitic eggs by easily grasping them and without incurring in ejection costs (Soler *et al.* 2015), whereas small species, like Bonelli's warbler, are known to suffer mechanic difficulties to hold the cuckoo egg because of their relative small bill and, therefore, they are constrained to puncture it in order to hold it or to abandon the nest, incurring in higher ejection costs (Moksnes *et al.* 1991a, Antonov *et al.* 2006, Antonov *et al.* 2008). Knowledge on the responses against brood parasitism in Bonelli's warbler are very scarce, and there is no information regarding its egg-rejection behaviour in relation to the size of parasitic eggs (**Chapter 2**).

Another key factor that may affect host's rejection of parasitic eggs and of critical importance from a methodological point of view is the material whereby model eggs are made of (**Chapter 2**). Most of the studies on hosts' defences against avian brood parasitism have been carried out by using artificial model eggs, usually made by hard material (i.e. hard plastic or clay; (Davies & Brooke 1989, Antonov *et al.* 2009, Lindholm & Thomas 2000, Moskát *et al.* 2003). Despite of their extended use, some authors have pointed out that hard model eggs may not provide a truthful ejection rate estimate (Moksnes *et al.* 1991a, Martín-Vivaldi, Møller & Soler 2002, Prather *et al.* 2007). On the contrary, soft material have been used in fewer studies (Marchetti 1992, Marchetti 2000, Moskát & Fuisz 1999, Moskát, Szentpéteri & Barta 2002), but there is no information about its effect on rejection rates. Soft materials might increase the ease of egg ejection, just as hard material can do the opposite (Moksnes *et al.* 1991a).

The mechanism by which hosts reject the parasitic egg is a complex behavioural process because hosts have to evaluate several factors in order to decide whether to eject or accept the foreign egg (Hauber & Sherman 2001, Soler *et al.* 2012): the degree of mimicry and the size of parasitic eggs, their ability to eject eggs without incurring in costs, but also external stimuli, like the abundance of parasites in the breeding areas and the associated risk of being parasitized and, fineally, the internal state of motivation (Moksnes & Røskaft 1989, Antonov *et al.* 2008, Antonov *et al.* 2009, Moskát & Hauber 2007, Lindholm & Thomas 2000, Soler *et al.* 2012). In fact, egg-rejection is a behavioural process in which the ejection is only the last step of the sequence. The first step entails the recognition of the foreign egg and the second one involves the decision whether to reject or not the egg (Soler *et al.* 2012). This complex mechanism shows therefore an important degree of phenotypic plasticity that may vary depending on the external pressures mentioned above. One of these pressures could be predation, but only few studies have explored the interaction between these two important selective pressures, all of them from the opposite perspective. This is whether brood parasitism has an effect on nest predation (i.e. brood parasitism increases the detection of host nests by predators (Hannon, Wilson & Mc Callum 2009, Ibáñez-Álamo, Arco & Soler 2012). But the contrary, this is whether nest predator risk might affect host defences against brood parasites, has not been investigated so far (**Chapter 3**).

1.3. Predation and its effects on prey

Predation is a biological interaction resulting in the death of the prey and represents an intense selective pressure by exerting a decisive impact on the fitness of prey

(Sih 1987), which are selected therefore to evolve anti-predator strategies in the frame of an arms race process. Anti-predator strategies involve morphological (Lima & Dill 1990), behavioural (Wirsing & Ripple 2010) and physiological (Sapolsky, Romero & Munck 2000) defences. Morphological changes induced by predation pressure have been observed in several vertebrate such as fishes (Heynen, Rentrop & Borcherding 2014), amphibians (Maher, Werner & Denver 2013) and birds (Swaddle & Lockwood 1998). With respect to anti-predator behaviour, prey may reduce their activities (Jones & Dornhaus 2011), increase grouping and vigilance (Creel, Schuette & Christianson 2014), or escape and find protection in the closest refuge (Martín & López 2005). In addition, it was observed that animals are able to adjust their anti-predator behaviours depending on the level of perceived predation risk (Lima & Dill 1990), given that prey usually respond more strongly to a more dangerous predator or to situations of elevated vulnerability (Owings *et al.* 2001). Finally, the physiological effects induced by predation pressure can produce an increase in glucocorticoids and heat shock protein (HSPs), the intensification of cardiovascular and respiratory activities and the alteration of immune system (Sapolsky *et al.* 2000, Clinchy *et al.* 2004, Hawlena & Schmitz 2010, Zanette, Clinchy & Suraci 2014). The purpose of these mechanisms is to enhance the likelihood of survival during threatened conditions, ultimately increasing prey's fitness (Wingfield & Ramenofsky 1999).

Nest predation

During the breeding season, an organism is potentially more vulnerable to predation, both directly, as reproduction is costly and exposes the organism to a higher predation risk and indirectly, since the predation can also affect the outcome of a reproductive event (Magnhagen 1991). In fact, during the early stages of development, offspring are generally vulnerable and can easily fall prey of a predator's attack. In birds, the reproductive cycle and the rearing of offspring are associated with a specific place, the nest. Nest predation represents therefore a decisive selective pressure both for adult and young individuals because implies the loss of fitness associated with the reproductive event for the former, and the total loss of fitness for the latter. For instance, predation is the primary cause of reproductive failure in many bird species (Martin 1995), particularly in altricial species, whose offspring totally depend on parental care to survive and to develop during a relative long period. Nest predation therefore drives the evolution of important avian anti-predatory strategies, both in adults and offspring, to reduce their vulnerability to predators (Martin 1995, Lima 2009, Ibáñez-Álamo *et al.* 2015).

Nestlings are clearly more vulnerable to a nest predator's attack than their parents, but in a certain way, they are able to manage predation risk by deciding to adopt different anti-predator defences (reviewed in Lima 2009, Martin & Briskie 2009, Magrath *et al.* 2010, Ibáñez-Álamo *et al.* 2015). Anti-predatory strategies are present in older nestlings, which have already developed their capacity to perceive danger and can actively respond to predators (Kleindorfer, Hoi & Fessl 1996, Tilgar *et al.* 2010, Ibáñez-Álamo, Chastel & Soler 2011). Behavioural strategies adopted by nestlings during a risky situation, such as that imposed by a nest predator depend on the level of the perceived danger and usually involved staying silent and hidden in the nest or leaving it suddenly (Ibáñez-Álamo *et al.* 2015). Older nestlings can assess the risk independently of their parents by both visual (Kleindorfer *et al.* 1996) and acoustic cues (Magrath, Pitcher & Dalziell 2007). Importantly, the adoption of anti-predator strategies could be also triggered by parents; there are several piece of evidence showing that parental alarm calls are used to alert nestlings about the kind of predator that is threating the nest and to inform them about the level of risk (Platzen & Magrath 2005, Suzuki 2011, Suzuki 2014). This information is assessed by nestlings in order to decide which defence to adopt. The ability of parents to communicate their offspring is particularly important for altricial nestlings, which are particularly exposed to nest predation given that some of their activities (i.e. begging) can attract predators (Haff & Magrath 2011, Ibáñez-Álamo *et al.* 2012). Parental alarm calls can help to modulate nestlings' behaviour, for example by stopping their begging in order to avoid detection (Davies, Madden & Butchart 2004, Magrath *et al.* 2010). Therefore, the different acoustic sources are able to provoke different anti-predatory defences in nestlings and thus, it is an important factor to be considered in studies concerning response to nest predation (**Chapter 4**).

Physiological effects of nest predation risk: the possible role of immune function

Because vertebrates cope with unpredictable and deleterious ecological factors by mounting a stressor response (Romero 2004), integrating physiology in behavioural ecology has becoming a major issue (Hau & Goymann 2015). Stress response implies an alteration of physiological and behavioural homeostasis in an individual (Sapolsky *et al.* 2000) by which an increase of survival during life-threating situations is promoted. Physiological stress response to predation risk has been proposed to be a common and important anti-predator strategy, which integrates and interacts with the morphological and behavioural defensive mechanisms (Hawlena & Schmitz 2010). Physiological processes include metabolic rate, endocrine and immune system, which are intrinsically linked (Dhabhar & McEwen 1997, Lochmiller & Deerenberg 2000, Lee 2006, Crespi *et al.* 2013 but see Versteegh *et al.* 2012) and exert an important role in the balance between individual conditions and behaviour.

The evidence by which nestlings would respond to nest predation risk by anti-predatory physiological defences is still scarce and shows opposing results (Ibáñez-Álamo *et al.* 2015). Regarding endocrine responses, for example, nestling distress calls elevate corticosterone levels but only in older nestlings of pied flycatcher *Ficedula hypoleuca* (Tilgar *et al.* 2010), whereas direct acoustic cues of predators increase testosterone but decrease corticosterone in blackbird nestlings (Ibáñez-Álamo *et al.* 2011).These findings suggest that the effect of nest predation risk on the physiology of nestlings would be associated with the cues they perceived and with the age of nestlings.

Information about changes in response to nest predation risk is even more scarce for the immune system (Ibáñez-Álamo *et al.* 2015), which is the most important self-maintenance system (Roitt *et al.* 2001), promoting survival by defending the organism against pathogens and infections (Lee 2006). Besides starvation and predation, disease is the most important cause of mortality (Roitt *et al.* 2001). In a context of predation, the immune system could play an important role to prevent the deleterious effects that a predator's attack can produce in the prey even after escaping from it (Dhabhar & McEwen 1999, Martin 2009). Wounds inflicted by predators to prey can be easily infected or transmit external pathogens (Butler 2015) and consequently could bear important costs for prey. Regarding nestlings, the probabilities that at least one chick successfully escapes from a nest predation event are high and not uncommon (Halupka 1998, Robinson & Robinson 2001) and therefore, the immunological responses in nestlings can be an important adaptive anti-predator response **(Chapter 4).** In addition to this direct effect, predation could indirectly affect immune function through the physiological stress response triggered by the exposure to predation risk (Sapolsky *et al.* 2000). Actually, the activation of stress response is mediated by the regulation of hormonal levels (i.e. glucocorticoids), which are able to modulate other physiological components (Wingfield & Ramenofsky 1999). Among them, the allocation of energetic resources to muscles and the down-regulation of the immune system may be especially important in a context of predation (Sapolsky *et al.* 2000). Despite the potential interaction between immunity and predation, very few studies have examined the potential role of the immune system in response to predation risk, especially for young individuals (Ibáñez-Álamo *et al.* 2015). Tilgar et al. (2010) observed changes in leukocyte profiles of pied flycatcher nestlings exposed to a chronic increase of predation risk. In another species, nestlings that were captured by a potential predator (a human) showed changes in cell-mediate immune responses (Goedert, Dias & Macedo 2014) and, finally, a reduction in innate immune investment was found in ring-billed gull *Larus delawarensis*, a semi-precocial bird in response to an acute and short-term stress exposition (Chin, Quinn & Burness 2013). These studies, despite their contrasting results, highlight the potential importance of the immune system in anti-predator strategies of nestlings (**Chapters 4 and 5**).

The indirect effects of predation

Although an efficient immune system is clearly beneficial for organisms, its production, maintenance and activation incur in several costs deriving from the nutritional and energetic resources required in these processes (Lochmiller & Deerenberg 2000). Consequently, an organism faces trade-offs between immunity and other functions or activities that share common resources. In birds, such trade-offs were found with migration (van Gils *et al.* 2007), parental effort (Bonneaud *et al.* 2003), territorial behaviour (Owen-Ashley & Wingfield 2006) and growth (Soler *et al.* 2003). It is possible that also the selective pressure exerted by nest predation might affect some of these trade-offs (Hawlena & Schmitz 2010; **Chapters 4 and 5**). As a matter of fact, the existence of these trade-offs is crucial during the early stages of life, where young individuals are more vulnerable and allocate most of their resources to development (O' Connor 1984). In nestlings, resources availability, sibling competition and the high growth rates have been showed to constrain many life-history traits, affecting ultimately adult fitness (Metcalfe & Monaghan 2001, Uller 2008, Love & Williams 2008).

From a more general point of view, the effects of predation on physiology are part of those indirect costs, deriving from the adoption of the anti-predator defences in response to the predation risk. This indirect effect are known as "fear effects" (Brown, Laundre & Gurung 1999), "non-lethal effects" (Lima 1998) or "predation risk effects" (Zanette *et al.* 2014) and have received an increasing attention recently (Laundré, Hernandez & Ripple 2010, Cresswell, Lind & Quinn 2010, McCauley, Rowe & Fortin 2011, Clinchy, Sheriff & Zanette 2013). These researchers have pointed out that, even if anti-predator strategies limit prey's vulnerability to predators, the associated consequences of such strategies may expose prey to indirect costs which ultimately result in a fitness reduction. The limited access to food resources is a suitable example: the presence of predators induce preys to invest more in vigilance than in foraging, causing a deterioration of body condition or fecundity (Creel, Winnie & Christianson 2009). With regard to the physiological anti-predator strategies, the consequences of endocrine or immune responses imply a series of costs for which individuals are obligate to optimize trade-offs between ecological and physiological requirements. The integration of these indirect costs in studies concerning predation risk (i.e. nest predation risk) is necessary and might lead, therefore, to discover new evolutionary roles of this selective pressure.

The immune system

The immune system of Vertebrates is commonly divided in an innate and acquired component (Janeway *et al.* 1997), whose functions interact in a coordinate manner in the recognition and defence of pathogens by limiting their circulation in the organism. Both the innate and acquired immunity include a cellular component (i.e. leukocytes) and circulating proteins which constitute the humoral component (Roitt *et al.* 2001).

The innate immunity constitutes the first line of defence against external perturbation. This component recognizes and responds to pathogens in a generic way and provide immediate defence against infections (Beutler 2004). Innate immunity consists of: 1) physical and chemical barriers, such as the skin, which prevent the entry of microorganisms; 2) the phagocytic cells, such as neutrophils (heterophils in birds), macrophages and natural killer cells, which participate in the first steps of infection by phagocyting microbes and 3) the complement system, composed of several blood plasma proteins which lysis pathogens and promote phagocytosis (Beutler 2004). Also the acute-phase proteins, whose production increases during inflammatory process in order to eliminate or inhibit microbes, form part of innate immune system (Cem Gabay & Irving Kushner 1999).

The acquired immunity is composed of highly specialized cells that eliminate pathogens and is based on the long-term immunological memory to a specific pathogen after an initial response (Flajnik & Kasahara 2010). The activation of acquired immunity is induced by lymphocytes. T lymphocytes are designated to cell-mediated immunity by the activation of antigen-specific cells, phagocytes and cytokines, whereas B lymphocytes produce specific antibodies (immunoglobulins), which recognize the specific antigen and mark it in order to destroy it (Flajnik & Kasahara 2010).

OBJECTIVES

1) To investigate in detail the breeding biology of a poor known species, the Bonelli's warbler, at the core of its distribution range, focusing especially on potential reproductive problems. (**CHAPTER 1)**

2) To explore the potential altitude effect on the parameters of the Bonelli's warbler breeding biology. (**CHAPTER 1)**

3) To study how the size of the parasitic egg used in egg-rejection experiments could affect the egg-rejection behaviour (i.e. ejection and nest desertion rates) in the small hosts. (**CHAPTER 2**)

4) To explore the potential effect of soft material (i.e. plasticine) of artificial model eggs used in egg-rejection experiments, in particular investigating changes on ejection efficiency. (**CHAPTER 2**)

5) To investigate whether predation risk affect blackbird defensive behaviour against brood parasitism (i.e. egg-rejection process), particularly focusing on egg recognition and the decision of ejecting. (**CHAPTER 3**)

6) To test if the effects on the egg-rejection process induced by predation change according to the type of predators (adult or nest predator). (**CHAPTER 3**)

7) To explore the impact of a short-term increase of nest predation risk on the immune system in blackbird nestlings. (**CHAPTER 4**)

8) To determine if different levels of nest predation risk can provoke different levels of intensity in immunological response. (**CHAPTER 4**)

9) To analyse the effect of an increase of nest predation risk during the whole nestling period on nestlings' immune system. (**CHAPTER 5**)

10) To explore the potential impact of nest predation risk in altering the normal trade-offs between the immune system and the developmental traits (i.e. growth rate and body condition) in nestlings. (**CHAPTER 4 AND 5)**

GENERAL METHODOLOGY

1.1. Model species and study areas

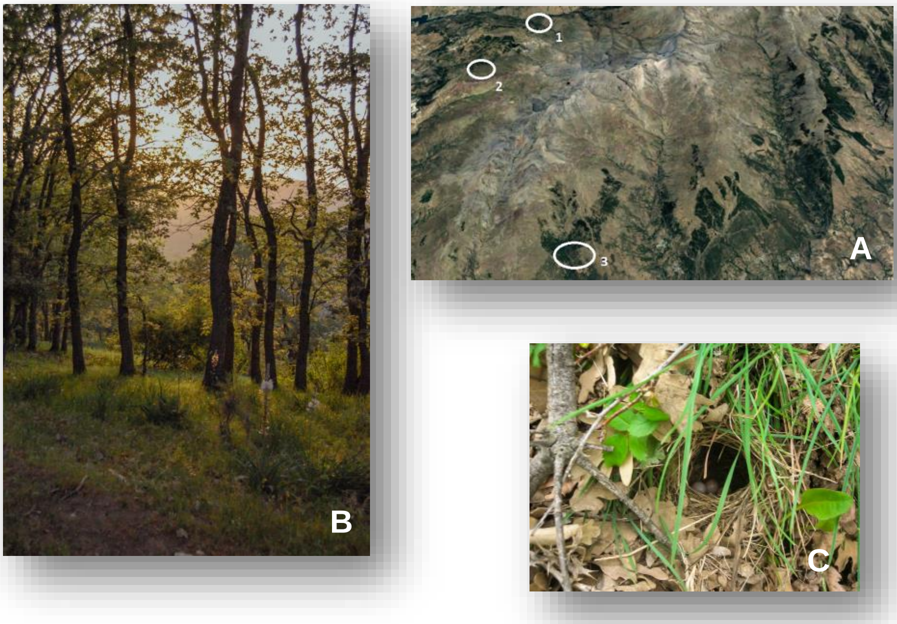
1.1.1. Bonelli's Warbler in Sierra Nevada.

(Western) Bonelli's warbler is a small passerine (7 – 11.5 g) of the Passeriformes order belonging to the genus *Phylloscopus* (Fig. 1). It was considered as conspecific with Eastern Bonelli's warbler (*P. orientalis)*, but differences in mitochondrial DNA (Helbig *et al.* 1995) suggested to treat the species separately (Sangster *et al.* 2002). Bonelli's warblers breed in South West Europe and North Africa. All population are migratory wintering in a narrow belt from Senegal to Cameroon and western Chad (Clement & Christie 2013). More than 60% of the Bonelli's warblers inhabit the Iberian Peninsula, where they breed in mountain deciduous woodlands principally composed of Pyrenean oak *Quercus pirenaica*, located in the supramediterranean climatic belt (Costa-Tenorio, Morla & Sainz-Ollero 1998). Bonelli's warbler are apparently monogamous with a reproductive period varying according to the latitude; in Southern Spain females start laying during the second week of May (Cramp 1985). Previous knowledge on their breeding biology was scarce and refers principally to a population living in the marginal areas of their distribution (south of Germany; Riedinger 1974). This study reported that females lay on average 5-6 white eggs, the incubation period last 12-13 days and, once the eggs hatch, nestlings stay in the nest for other 12-13 days (Riedinger 1974). By contrast, no information on its breeding biology is available for its core area of distribution, the Mediterranean woodlands.

Figure 1: Ringed adult of Bonelli's Warbler. The ringing was performed at the beginning of the season in order to differ males and females during the reproductive cycle.

Among all common cuckoo host species, the *Phylloscopus* genus plays an important role in relation to woodland habitats, especially the willow warbler, the chiffchaff and the wood warbler (*P. sibilatrix*; Moksnes, Røskaft & Tysse 1995). It has been documented that in the north of Europe (i.e. Finland) both the willow warbler and the chiffchaff are two of the five most parasitized hosts of the common cuckoo (Perrin de Brichambaut 1997). Willow warblers respond to egg-recognition experiments by rejecting approximately 90% of the model eggs (Moksnes & Røskaft 1992). In more southern latitudes like Mediterranean mountain woodlands, both the wood warbler and the Bonelli's warbler seems to act as host for common cuckoos (Campobello & Sealy 2009, Martínez *et* *al.* 2010). Nevertheless only few summaries of the frequency of parasitism exist for passerine species in Mediterranean countries (Perrin de Brichambaut 1997). This is particularly important for the Bonelli's warbler because, firstly, it represents the only *Phylloscopus* species breeder in some Mediterranean areas, and secondly, it has been only studied once in relation to egg recognition experiments (Martín-Vivaldi *et al.* 2013).

We monitored Bonelli’s warbler during the breeding season of 2012 and 2013 in the Sierra Nevada high massif in Southeast Spain (37º 56’ N, 3º 23’ W). The climate of this study area is characterized by annual temperatures that vary between 8º C and 13º C, descending below 0ºC in winter-early spring and reaching up to 20º C in July and August. Rainfall are irregular, oscillating between 650 and 1200 mm per year, depending on the slope and altitude (Rivas-Martinez 1987). The study area included three sub-areas ranging from 1200 to 2000 m a.s.l.: two areas were located on the south-facing slope of the massif, whereas the other was on the north face (Fig. 2A). The three areas are characterized by steep slopes and open warm deciduous woodlands composed principally of Pyrenean Oak, an endemic species of South West Europe and North Africa and an undergrowth layer, dominated by bushy and grassy vegetation (Fig. 2B; Costa-Tenorio *et al.* 1998) where Bonelli’s warblers built their domed nests (Fig. 2C). Pyrenean Oak forests represent important areas in the Mediterranean region since they have high values of species richness and density (Martínez *et al.* 2010). The common cuckoo is also well distributed in this



Figures 2: A) Three study areas of Bonelli's warbler: 1) Serpentina area, 2) Monachil area, 3) Soportujar area. B) Typical habitat of Bonelli's warbler in Sierra Nevada. C) A domed nest of Bonelli's warbler with eggs

habitats, even if its density is relative low (0.42 cuckoo per 10 ha; Martínez *et al.* 2010). In this region, their main potential hosts are the European stonechat (*Saxicola torquata*) and the European robin (*Erithacus rubecula*), but Bonelli's warbler and Sub-Alpine warbler (*Sylvia cantillans*) are also considered as two potential common cuckoo hosts (Martínez *et al.* 2010). Woodlands of Sierra Nevada are also characterized by extremely high levels of predation rate for certain species, like the blackbird (Ibáñez-Álamo & Soler 2010), but there is no similar information for Bonelli's warbler available.

1.1.2. Blackbird in the Valley of Lecrín

The common blackbird is a medium-sized bird belonging to the Passeriformes order (Fig. 3A). It is considered a polytypic species, consist of 6-8 geographical subspecies distributed in the west Palearctic region and it was introduced in Australia and New Zealand, where is considered an invasive species. Blackbird are a sedentary, migratory or partial migratory species according to latitude (Collar 2005). In Central and Western Europe it is resident or partially migratory. The breeding period starts in February and finishes at the end of August, changing according to the latitude. The nest, built only by the female, is situated in bushes or trees. Females of northern population lay 5-6 eggs, while only 3 eggs were laid in southern populations. Eggs are incubated during 11-15 days and the nestling period lasts 10-14 days. Nestlings continue being fed by their parents during the post-fledgling period until they are 20 days old (Collar 2005).

Figure 3: A) Incubating blackbird female. B) Orange groves in Valley of Lecrín where blackbirds build their nest.

The area in which we studied blackbird during the breeding period of 2014 and 2015 is a rural area situated in the Valley of Lecrín (37º 17' N, 3º 59' W, 580 m a.s.l.), in orange and lemon groves spaced out by olives (Fig. 3B). This area is situated in the mesomediterranean climatic belt, with temperature varying between 12ºC and 18ºC degree and low rainfall (350 – 600 mm per years; Costa-Tenorio *et al.* 1998). Blackbird populations of this area are monogamous and territorial. Females start building the nest early in March and the last eggs are laid approximately at the end of June. During this period blackbirds can exceptionally raise up to three broods. The nest is usually situated in orange or lemon trees and, more rarely, in olive trees. The clutch is composed of 2.88 ± 0.12 eggs on average, which are incubated only by the female during 13-14 days. Nestling stay in the nest for 11-13 days approximately (Ibáñez-Álamo & Soler 2010). Nest predation rate in the study area is intermediate, 48.9 % (Ibáñez-Álamo & Soler 2010), and the most common predators consist of mammals, avian species, and snakes. The common cuckoo is not currently present in this study area, but it was until recently (thirty years ago; Soler *et al.* 2017). Earlier studies on blackbird revealed its egg recognition and rejection abilities (Davies & Brooke 1989, Grim & Honza 2001), but cases of parasitism by cuckoo are rare nowadays (Grim & Honza 2001). Nevertheless, the blackbird is a medium-sized passerine, which build open nests, and has short nestling period; all these traits make this *Turdidae* an ideal potential host of common cuckoo (Soler, Møller & Soler 1999). The egg-discrimination abilities, its aggressive behaviour against cuckoo dummies (Grim *et al.* 2011, Ruiz-Raya *et al.* 2016) and its reluctance to feed cuckoo nestlings experimentally introduced in their nests (Grim *et al.* 2011, Grim 2006) strongly suggest that blackbird should have been parasitized by common cuckoo in the past, and currently, it would be at the head of the "arm race" coevolutionary process.

1.2. General field procedures

1.2.1. Nests search and visits

The first step of the field work corresponds to the active search of the nests of both species in order to carry out the experimental manipulations used to answer to our hypotheses. The approach by which we searched the nests was different depending on the biology of the species and the complexity of the study area. To locate nests of Bonelli's warblers (**Chapters 1 and** 2) we used parental activity, in particular by firstly looking for the male in order to detect the territory and secondly, by monitoring female's activity in order to find the nest. The location of blackbird nests (**Chapters 3, 4 and 5**) was carried out by walking throughout the orange tree line and systematically looking for nests. For both species we recorded all the nests found during egg laying, incubation or nestling periods. Nests were marked with a Global Positioning System device (Garmin Gecko 201) to know their exact location. Nests of both species were visited every 2 – 3 days in order to determine the exact laying and hatching date and know when exactly starting our manipulations.

1.2.2. Brood parasitism manipulation

The experimental designs relative to the second part of this thesis (**Chapters 2 and 3**) required the use of artificial eggs in order to parasite nests of both species and to study their egg-recognition behaviour. To do this, we used real eggs as well as model eggs (Fig. 4).

Real eggs of both species were collected from abandoned nests of the same populations (nests were considered abandoned when clutch remained cold for three consecutive visits).

Figure 4: A) Large non-mimetic model egg of plasticine used with Bonelli's warbler. B) Mimetic model egg obtained by painting a real blackbird egg.

For the experiment with Bonelli's warblers we used both non-mimetic model eggs and real eggs (**Chapter 2**). We use three different size of model eggs made of red plasticine: small model eggs, 50% smaller than Bonelli's warbler eggs, medium-sized model eggs, similar to Bonelli's warbler natural eggs and large model eggs (Fig. 4A), 50% larger than Bonelli's warbler eggs and similar in size to common cuckoo eggs (Martín-Vivaldi *et al.* 2002). The model eggs allowed us to investigate egg-rejection behaviour with respect to the size of parasitic egg. The real Bonelli's warbler eggs were painted red and used in the manipulation control group in order to test the potential impact of plasticine material on egg-rejection responses. Finally, we created a control group (where nests were not parasitized) to control for the potential effect of the cameras on Bonelli's warbler responses.

To parasite blackbird nests (**Chapter 3**), we utilized only real eggs, which were painted using acrylic paint to obtain mimetic-model eggs (Fig. 4B), following the methodology of a previous study (Soler *et al.* 2015). These models elicit intermediate ejection responses in blackbird females (Soler et al. 2015), therefore allowing to detect both an increase and reduction in its ejection rate according to the predation risk (see predation risk manipulation section).

In order to study the females' behaviour towards the introduced model egg (**Chapters 2 and 3**) we placed a video camera (Panosonic HDC-SD40, Osaka, Japon) close to the focal nest of both species (Fig. 5), following the methodology described in other similar studies analysing brood parasitism or nest predation in these species (Martín-Vivaldi *et al.* 2013, Ruiz-Raya *et al.* 2015 and 2016, Ibáñez-Álamo & Soler 2016, Vivaldi *et al.* 2013, Ruiz-Raya *et al.* 2015 and 2016, Ibáñez-Álamo & Soler 2016, Soler *et al.* 2017)**.**

1.2.3. Predation risk manipulation

The manipulation of nest predation risk was done only for blackbirds (**Chapters 3, 4 and 5**). It was carried out by using acoustic cues that simulated an increase in predation risk at the nest. The use of acoustic cues in order to manipulate nest predation is a common procedure in this kind of studies and has been successfully used for several studies, including blackbird (Eggers *et al.* 2006, Magrath *et al.* 2007, Peluc *et al.* 2008, Zanette *et al.* 2011, Suzuki 2014, Ibáñez-Álamo *et al.* 2011, Ibáñez-Álamo & Soler 2016).

To simulate predation risk we used predator acoustic cues, parental alarm calls or nestlings distress calls depending on the aim of the specific study. Control treatment included sounds from other passerines from the area. We collected these sounds from an online database (www.xeno-canto.org), except for nestlings' distress calls that were directly recorded (Sony ICD-PX333 Digital Voice Recorder) from blackbird nestlings of our population. We converted all recordings to digital audio files by using the software Audacity. Audio files were broadcasted by speakers which were hidden under a camouflaged cloth, connected to an MP3 player and positioned near the nest (Fig. 6)

Figure 5: Video camera placed near to a blackbird nest during the experimental parasitism in order to film female's egg-rejection behaviour.

Figure 6: One of the speakers used to broadcast the acustic cues in the predation risk manipulations.

1.3. Laboratory analyses

Immunological assays

To investigate the effect of nest predation risk on blackbird nestlings' immune system during both acute short-term (**Chapter 4**) and chronic long-term exposures (**Chapter 5**), we performed several different assays on their plasma and counted leukocyte cells from the blood smears collected during the experimental manipulations (see below).

All blood samples (250-300 µl) were collected between 10:00 and 14:00 h when nestlings were 10-11 days. They were stored at 4°C (maximum 5 hours after collection) until its centrifugation (13000 rpm for 10 min) in the laboratory. Plasma samples were stored at -25ºC. We quantified 12 immunological parameters to consider the complexity of the immune system (Matson *et al.* 2006), collecting data from both humoral and cellular components, as well as from innate and acquired immunity.

Humoral innate immune system

(i) Haemolysis/Haemagglutination titres (HL-HA)

Both agglutination and lysis quantify the levels of innate immunity. Agglutination process is indicative of the levels of the circulating natural antibodies (NAbs) while lysis titres estimate the action of complement and other lytic enzymes (Carroll & Prodeus 1998) Quantification of agglutination and lysis was achieved by a serial dilution of plasma samples and the assessment of the dilution step at which both reactions stopped. Specifically, we placed 25 µl of plasma in the first two rows of a 96-well round-bottomed plate. From rows 2–11, we performed ten 1:2 dilutions using Dulbecco's PBS. Plates were pre-treated overnight by 60 µl milk solution (5 g powdered milk/l Dulbecco's PBS) and successively washed by 70 µl of 0.5mL Tween 20 in 1 L PBS for three times. We added 25 µl of 1% rabbit red blood cell suspension to the plasma dilutions and placed the plates in a water bath at 37°C for 90 min. Subsequently, we tilted the plates 45° and scanned them after 20 min for the agglutination scores. The plate was additionally incubated at room temperature for another 70 minutes (not tilted) and then scanned again to score lysis. Scoring of all scans was done by a single person (GR; see Matson *et al.* 2005 for more details).

(ii) Haptoglobin (HP)

Haptoglobin is an acute phase protein whose increase usually indicates a response to infection, inflammation or trauma (Millet *et al.* 2007, Matson *et al*. 2012). We measured Hp concentration in plasma following the method provided with a commercial available assay kit (TP801; Tridelta Development Ltd., Maynooth, Ireland), which coulorimetrically quantified the haemoglobin binding capacity of plasma (Matson *et al.* 2006, Matson *et al.* 2012).We measured absorbance at two wavelength (450 and 630 nm) prior to the addition of final reagent that initiated the colour-change reaction. The pre-scan of 630 nm allowed us to correct for the differences in plasma colour by subtracting the pre-scan absorbance value from the final absorbance value. The 450 nm pre-scan measure corrects for potential differences in redness of the plasma samples. Absorbance was read by using a spectrophotometric microplate reader (VersaMax, Molecular Devices, Sunnyvale, California, US).

(iii) Ovotransferrin (OVT)

Ovotransferrin (OVT) is an acute phase protein which exerts its antibacterial, antiviral (Giansanti *et al*., 2002 and 2007) and antifungal activities (Valenti et al., 1985).

We measured OVT concentration following Horrocks et al. (2011b) procedure. Briefly, the assay measures the total iron-binding capacity, which correlates with OVT concentration. Firstly, ferric iron is added (Fe^{3+}) under alkaline conditions. In the second step, the unbound excess iron is reduced to Fe^{2+} by adding ascorbic acid. The Fe^{2+} is inactivated by binding with chromogen FerroZine, a blue complex. In the last step, an acid is added which releases the ovotransferrin-bound Fe^{3+}. The solution becomes dark due to an increase in Fe^{2+}-FerroZine complex. OVT final concentrations were calculated using absorbance values read by spectrophotometric microplate reader (VersaMax, Molecular Devices, Sunnyvale, California, US).

(iv) Nitric oxide (NOx)

Nitric oxide is a small molecule highly reactive and diffusible. Its presence increased when induced by inflammatory cytokines, microorganisms or endotoxins (Sild & Hõrak 2009). The production of NOx in biological tissue is assessed on the basis of its oxidation end-products, nitrate and nitrite. The method to quantify nitrite/nitrate concentration has three main step. In the first one a deproteinization of plasma occurs by adding 40 µl of 75 mmol/L $ZnSO_4$ solution to 10 µl of plasma. Subsequently, we added 50 µl of 55 mmol/L NaOH solution to the previous mixture and centrifuged (16000 rpm); we transferred 80 µl of supernatant to a new tube to which 80 µl of glycine buffer (0.2 mol/L, pH 9.7) was added. In the second step, we activated cadmium granules stored in 0.1 M sulphuric acid and then the granules were rinsed and swirled in $CuSO_4$ solution for 2 min. We added the cadmium pellets to the previous tubes with the mixture of plasma and we shook 15 min with maximum force. In this process nitrate is reduced to nitrite. In the last step we transferred 100 µl of the samples to a new tube and we added 50 µl of Griess reagent sulpanilamide. Tubes are shaken 15 min and 200 µl were transferred into the wells of a microplate. We measured the absorbance at 542nm (final scan) by using microplate reader (VersaMax, Molecular Devices, Sunnyvale, California, US).

Acquired immune system

(v) Immonuglobulins (IgY)

Immunoglobulins are glycoproteins of acquired immunity which neutralize pathogens, induce the activation of the complement system and promote cell migration to the sites of infection (Hartle *et al.* 2014). Total immunoglobulin concentrations (IgY) were measured from plasma samples with a direct ELISA (enzyme-linked immunosorbent assay), following the procedure developed by Martinez et al. (2003). In brief, plates were coated with a serial dilution (1:9000) of plasma (90 µl) in carbonate-bicarbonate buffer (0.1M, pH 9.4) and incubated overnight at + 4°C. Afterwards, plates were blocked with 300 µl 2% defatted milk diluted in PBS-Tw buffer for 1h at + 37°C. In each well, 100 µl of anti-chicken antibodies (Antichicken IgG-HRP Peroxidase antibody produced in rabbit, Sigma A9046) diluted 1:30000 in PBS buffer were added and the wells were incubated for 2h at + 37°C. Successively, plates were washed (4x) and incubated for 20 minutes at + 37°C in the dark with a solution containing OPD (o-Phenylenediaminedihydrochloride

Sigma P8287-50TAB) and H_2O_2 diluted in citrate buffer (pH 5.0). The reaction was stopped by adding 50 µlof HCl 3M and absorbance was immediately measured at λ=492 nm using a plate spectrophotometer (Thermo Scientific Multiskan Spectrum and SkanIt software v2.4.2). Data obtained with this procedure are expressed in optical density units, therefore the content of total IgY is proportional to the optical density. For each sample, mean absorbance value was calculated from replicates (3) and "corrected" by subtracting the mean value of "blank" absorbance to account for non-specific binding related to background activity.

Cellular component

(vi) White blood cells count

In order to quantify white blood cells, all blood smears collected were fixed in absolute methanol and stained with Giemsa (GS500-500ml SIGMA-ALDRICH Giemsa stain, modified) for 45 min and. All smears were scanned with an optical microscope (1000x magnifications with oil immersion). We counted a minimum of 100 leucocytes in each slide (Fig. 7A). Each cell was classified as heterophil, lymphocyte, eosinophil, basophil or monocyte, following the description of Campbell and Ellis (2007). Leukocyte count allowed us to calculate H/L ratio, which is an haematological variable that provides information about stress (Clinchy *et al.* 2004, Tilgar *et al.* 2010, Nazar & Marin 2011). Smears of 2015 were also examined to evaluate the presence of haematozoan parasites (genera *Leucocytozoon*, Fig. 7B). While we was counting the leukocytic cells we also inspected the infected blood cells, which develop into gametocytes to complete its reproductive cycle (Forrester & Greiner 2009).

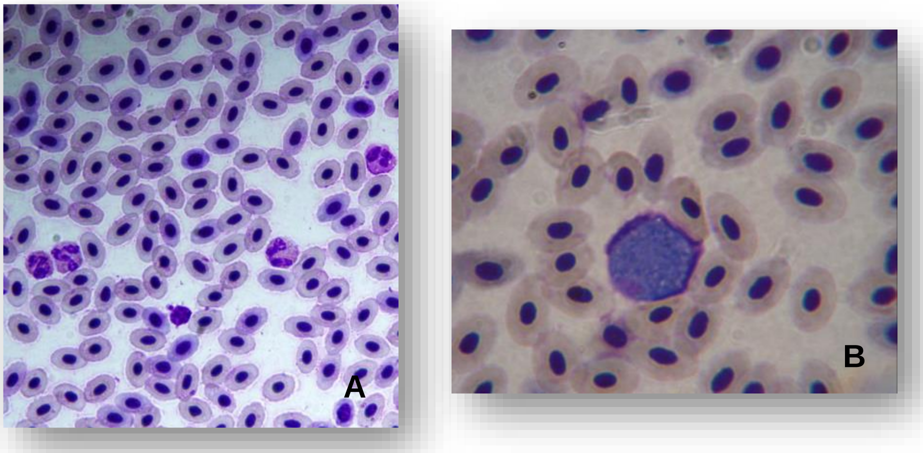


Figure 5: A) The hemogram from a blackbird nestling. Four granulocytes (heterophils and eosinphils) are positioned on the both side of the smear; a lymphocyte is positioned in the center. B) A *Leucocytozoon* sp. gametocyte

PART I

BREEDING BIOLOGY

CHAPTER 1

BREEDING BIOLOGY OF WESTERN BONEELI'S WARBLER *PHYLLOSCOPUS BONELLI* IN THE MEDITERRANEAN REGION

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Capsule The Western Bonelli's Warbler *Phylloscopus bonelli* has a nest success of only 25% in the core of its range in western Europe.

Aims To investigate the breeding biology of Western Bonelli's Warbler *P. bonelli*, focusing on possible altitude effects and potential reproductive problems.

Methods Three Western Bonelli's Warbler populations were monitored during 2012 and 2013 breeding seasons in the massif range of Sierra Nevada, Spain. We determined all the breeding parameters and calculated daily survival and success rates for each reproductive period.

Results The three studied populations did not differ in any breeding parameters. Altitude showed a positive relationship with clutch size and duration of incubation period, but negative relationship with nestling tarsus growth and body mass gain. Daily survival rates during incubation and nestling period were similar to those of common warblers, but the species presented a low breeding success of 25%.

Conclusion The absence of differences among the three populations suggests that the information provided here could be representative of its distribution in the woodlands of Sierra Nevada. The novel and detailed information reported is crucial not only for expanding our understanding of this species but also to draw attention to the potential risks that it might face in the near future, considering the reduction that this species has suffered in Sierra Nevada during recent decades.

Key words: Western Bonelli's Warbler, breeding parameters, Iberian Peninsula, incubation stage, nestling stage, altitude

Introduction

The study of the breeding parameters of a species is essential for the understanding of its biology and ecology, but this information is even more important for those species which are poorly known or threatened (Green *et al.* 2004). The Western Bonelli's Warbler (*Phylloscopus bonelli*; hereafter Bonelli's Warbler) is one such poorly known species (Clement & Christie 2013). To date, the few studies about this species focused on other topics (i.e. genetics, acoustics or migration/distribution range) rather than on the breeding biology itself (Helbig *et al.* 1995, Bremond 1976, Prince 1997, Dietzen *et al.* 2007, Pilastro *et al.* 2008). In contrast to other European *Phylloscopus* species whose breeding biology has been well studied (e.g. Wood Warbler *Phylloscopus sibilatrix*, Chiffchaff *Phylloscopus collybita* and Willow Warbler *Phylloscopus trochilus*; see Clement & Christie 2013), there is only a single detailed study about the breeding biology of Bonelli's Warblers (Riedinger 1974). This study was carried out more than 40 years ago and investigated a lowland population in southern Germany. Bonelli's Warblers typically inhabit more southern regions and higher altitudes, such as the mountain range of the western Mediterranean (Clement & Christie 2013). Thus, it is likely that the reproductive parameters reported in by Riedinger (1974), based on a peripheral population and different latitudinal and altitudinal ranges, do not fully represent the breeding biology of this species. In fact, animal populations living on the edge of their distribution ranges usually present variations in some life-history traits in order to cope with environmental challenges of such areas (Diaz *et al*. 2007). Latitude and altitude can also affect life-history traits (Badyaev 1997, Badyaev & Ghalambor 2001, Cardillo 2002). For example, several within and between species studies have demonstrated that clutch size increases with latitude because of the seasonality of resources (i.e. high food availability per individual bird in spring; Bell 1996, Yom-Tov & Geffen 2011) or a shorter duration of the breeding season in temperate areas compared to the tropics (Skutch 1985; Schaefer *et al.* 2004). On the other hand, the ecological conditions at high altitudes, such as colder temperatures, shorter breeding seasons and greater fluctuations in food availability, favour an increased investment per offspring (i.e. larger eggs; Badyaev 1997, Lu *et al.* 2009, Li & Lu 2012). Riedinger's (1974) supplied basic information regarding some aspects of the breeding biology of Bonelli's Warbler, such as clutch size, laying date and egg parameters, but did not provide other key data, such as daily survival rates or hatching/fledging success rates, nestling growth rates or parental activity. To our knowledge, no previous studies have provided detailed information on the breeding biology of Bonelli's Warblers in its core habitat, the mountain woodlands of the Mediterranean.

The conservation status of Bonelli's Warblers is of "Least Concern" according to the Internation Union for Conservation of Nature (BirdLife International 2014), however it holds the "SPEC 2" category according to the directive of the European Union on Birdlife, which categorized this species with an unfavourable conservation status in Europe (Birdlife International 2004). This matter raises some concerns about the viability of its populations considering that the majority of Bonelli's Warblers breed in Europe. For instance, populations from southern Germany (Del Hoyo *et al.* 2006) and France (Dubois *et al.* 2000; Eionet 2014) have been in decline both in individuals and breeding range. In Spain, where 65% of all individuals are present (Del Hoyo *et al.* 2006), Bonelli’s Warblers have been experiencing a moderate increase (Sociedad Española de Ornitología; SEO/BirdLife 2010), but in some areas, such as the southern parts of the Iberian Peninsula, they are in serious decline (Zamora & Barea-Azcón 2015). In fact, Bonelli’s Warblers are considered of "special interest" in Spain, where the loss of habitat due to frequent fires, human land-use changes and the impact of grazing by livestock could explain their local population decline (Urios *et al.* 1991, Valle 2003).

The main aim of this study is to investigate the breeding biology of the Bonelli's Warbler in the core of its distribution range; the woodlands of Sierra Nevada (southeast Spain). This species has been one of the most abundant passerine birds in that area (Martinez *et al.* 2010), but has suffered an important reduction in breeding population size (from 1.36 birds per hectare to 0.34 birds per hectare) in the last 30 years (Zamora & Barea-Azcón 2015). In addition, we provide new key data such as hatching and fledging success, and breeding behavioural such as nest building and incubation from video recordings. It is important to know this information as it is so far undocumented, but it would also to help to diagnose and address the drivers of the population declines.

Material and Methods

Study area and population

Bonelli’s Warblers were monitored during 2012 and 2013 breeding seasons, from the end of April to mid-July, in the National Park located in the Sierra Nevada high massif in southeast Spain (37° N, 3° W). Bonelli’s Warbler breeds in mountain woodlands, principally composed of Pyrenean oak *Quercus pirenaica*, located in the supramediterranean climatic belt. These areas have high values of species richness, diversity and density in the massifs of south Spain (Martinez *et al.* 2010). The climate is characterized by an irregular annual rainfall, ranging between 650 and 1200 mm per year, depending mostly on altitude. The average temperatures vary between 8° and 13° C, descending frequently below 0° during winter and reaching up to 20° in summer (Rivas-Martínez 1987). During the first year, three areas with similar altitudinal ranges were investigated: the Serpentina area (37° 08’N, 3° 24’ W), between 1000 and 1600 mabove sea level (a.s.l.), the Monachil area (36° 05’N, 3° 26’ W), between 1200 and 1900 m a.s.l. and the Soportújar area, (36° 56’N, 3° 23’ W), between 1200 and 2000 m a.s.l. We chose these three areas on the basis of a previous study (Martínez *et al.* 2010) in which distribution and density of Bonelli’s Warblers and other species living in Sierra Nevada were monitored. Because of the high density of breeding pairs reached in these three areas (1.10, 0.93 and 0.92 pairs per hectare for Serpentina, Monachil, and Soportújar, respectively; Martínez *et al.* 2010), we think that the three studied populations should show typical reproductive parameters for the woodlands of the Mediterranean region at the core of its distribution. The studied populations also offered a large altitudinal gradient (around 1000 m) allowing us to test the potential effect of this factor on the breeding biology of this species. The study populations were on average 13.6 km from each other (range: 6 km – 20 km) and were located on both north (Serpentina and Monachil) and south (Soportújar) facing slopes. During 2013 we concentrated our research effort only the: Soportújar area.

The three areas have steep slopes and open, warm deciduous woodland composed of Pyrenean Oak, a deciduous tree which is distributed from southwest France through to north Morocco. In the southern Iberian Peninsula it grows between 1000 and 1800 m a.s.l, occasionally as high as 1900 m a.s.l.on south facing slopes, in siliceous soils, mostly in the supramediterranean climatic belt (Costa-Tenorio *et al.* 1998). Holm Oak *Quercus ilex* and different species of pines, *Pinus pinaster, P. Nigra* and *P. sylvestris* are the other common tree species widespread in the most elevated part of the study area. The undergrowth is dominated by bushy areas with legumes, such as brooms (*Citysus scoparius*, *Adenoarpus decorticans*) and some open grassy areas (Costa-Tenorio *et al.* 1998). In this habitat Bonelli's Warblers forage principally insects, such as small flies and mosquitoes (Diptera), aphids (Aphidoidea) or small caterpillars (Lepidoptera), but also small spiders (Araneae) and other invertebrates (Cramp 1985).

Data collection

We located nests by following parental activity. Initially we concentrated on males' songs in order to detect their territory, and subsequently we looked for females for periods of 30 minutes per day. If the female was not detected on the first day, we visited the territory on subsequent days to find her and detect the nest location (always limited to 30 minutes period of searching to reduce the risk of desertion). When we found a nest we marked its exact location and altitude with a Global Position System device (Garmin Geko 201). Each nest was visited every 2-3 days in order to obtain the following biological parameters: clutch size, laying date, hatching date, incubation period, brood size, hatching success, nestling period, number of nestlings that leave the nest, fledging success and breeding success. See below for definition of each parameter. Given that the Bonelli's Warbler is a single brooded species (Cramp 1985; own data), we calculated each parameter for each pair.

Laying and incubation stages

Laying date was the day on which female laid the first egg (day 1= 1 April). Since Bonelli's Warblers usually start incubating with the last egg (Cramp 1985), the incubation period was calculated as the period between the laying of the last egg and the date on which the first egg hatched (hatching date).

In order to study incubation behaviour, we captured the adults (at least one of the pair) using mist nets, and filmed the nests to collect data on adults' activity during this period. All individuals were measured and ringed with different colour ring combinations that allowed for individual recognition from the distance (by using binoculars) or in the video recordings. We captured 33 individuals: 23 males, 7 females and 3 individuals of which we could not determinate precisely the sex. We determined the sex by using brood patch or body measures (i.e. weight, tarsus and wing length) as in *Phylloscopus* genus males are slightly larger than females (Tiainen 1982). We confirmed all the data by analyzing the video recordings in which we could recognize the banded birds and the different breeding behaviour patterns between males, which never entered into the nest, and females. Nests were recorded for two hours between 8:00 and 13:00 hours, one or two days after the start of incubation using a Panasonic HDC-SD40 video camera placed within 3 m of the nest. We analyzed the videos by using VLC software and extracted the following variables from them: (i) females' incubating time (time spent incubating by females per hour), (ii) nest visits (number of female visits to the nest for incubating) and (iii) off-bouts (time in which female was away from the nest per hour). The placement of cameras did not seem to affect the behaviour of birds (pers. obs.) and no nest was deserted because of the presence of the camera.

We calculated the daily survival rate for the incubation period (Johnson 1979). This method considers both the fate of the nest and the known activity period, correcting for the increased probability of a nest to survive to later stages. Therefore, in this procedure the daily survival rate indicates the probability that a nest with eggs will survive to the next day. In addition, we provided the proportion of nests in which at least one egg hatched (hatching success).

Brood size refers to the mean number of eggs that successfully hatched. When the clutch was completed, we measured length and width of all eggs with a digital caliper (accuracy 0.01 mm), and weighed them with a digital balance (accuracy 0.1g) just after clutch completion. The volume of the eggs was calculated with the following formula: 0.51 x length x wide^2 (Hoyt 1979).

Nestling period

Nestling period was calculated as the period between the hatching of the first egg and the date in which nestlings fledged (i.e. the first day we found the nest empty). Chicks already have the capacity to leave the nest when 11 days old (Del Hoyo *et al.* 2006), so all nests found empty before time were considered to have had their contents depredated. We calculated the daily survival rate for the nestling period and the complete breeding period using the same procedure described above. We also calculated the proportion of nests in which at least one chick fledged from successfully hatched nests (fledging success). The number of fledglings was calculated as the number of nestlings per nest that successfully left the nest. Finally, we calculated the daily survival rate considering the whole the breeding period, from laying to the date of the fledging. We also calculated the proportion of nests in which at least one chick fledged considering all the nests found during the breeding season (breeding success). Nests in which at least one chick was found dead or disappeared during the nestling period were considered to have suffered starvation, because potential nest predators in the study area, like Jay *Garrulus glandarius*, Montpellier snake *Malpolon monspessulanus*, rats *Rattus* sp. and domestic cats *Felis silvestris catus* are large enough to ingest all the nestlings at the same time. Body mass, tarsus and wing length of every chick were measured three times along the nestling period: when they were 2-4, 5-7 and 8-10 days old. In every visit we marked each chick of the brood with a different colour by means of non-toxic markers (FaberCastel Multimark) to allow for individual differentiation in subsequent visits.

Statistical analysis

All analyses were performed using R 2.15.3 for Windows (R Development Core Team 2012).

We used ANOVA and Kruskall Wallis tests look for differences in breeding parameters among the three study areas during 2012. We used ANOVA to analyze laying date, while Kruskall-Wallis test was used to analyze clutch size, incubation, number of eggs hatched, duration of nestling period and number of chicks fledged because of the non-normality distribution of the dependent variable. Finally, we used Fisher exact test to analyze the frequency of hatching and nestling success nest. The study area was considered as a factor. After confirming that there were no significant differences between the three areas for any of the variables considered (see results), we decided to use a single database combining all data collected as representative of the population of Sierra Nevada.

We fitted linear models (LM, package stats in R) considering all the predictors that could be biologically relevant to each of the breeding parameters (laying date, clutch size, incubation period, brood size, nestling period, altitude and year). When two predictors presented collinearity we decided to drop the one which could be linearly predicted from the other. For instance, between brood size and clutch size, we considered only brood size which indirectly predicted clutch size. Using this procedure we presented the models with all possible biological predictors for each dependent variable, showing both significant and non significant ones. For all LM models we considered altitude as a covariate and year as a fixed effect because we were interested in looking for an altitudinal effect and the potential differences between those two years. Additionally we had only two years of data and therefore we could not consider year as a random factor (Zuur *et al.* 2009).

To calculate if the proportions of hatched eggs with respect to clutch size, fledged chicks with respect to the brood size and fledged chicks with respect to the clutch size were related to year or to altitude we fitted χ^2 test and a generalized linear model (GLM) respectively.

In order to analyze egg size and mass, we used a linear mixed model (LMM, package nlme in R) in which we included nest as random effect to control for non-independence of eggs from the same nest.

We calculated nestling growth in term of mean daily gain in body mass, tarsus length and wings length by assuming that the growth was linear for most of the nestling period and by fitting a linear slope through the parameters against age for each nestling. We fitted a LMM model in which we considered the slope of each variable as the response variables and brood size, nestling period, altitude and year as predictors, while nest was considered as a random factor in order to control for non-independence of chicks from the same nest.

At the end of all model analyses we visually inspected residuals and residual *versus* fitted values to check for deviations from normality using qqplot. All means are given with ± se.

Results

We found 40 and 76 nests in 2012 and 2013 respectively, located between 1283 and 1888 m a.s.l (average 1654 ± 13 m, N = 93).The nests were found during nest building and egg

Table 1: Results obtained for the Linear Mixed Models run to assess the predictors influencing breeding parameters. * denote significant differences at P < 0.05 level. Coefficients of the models (**β**) show positive or negative relationship between predictor and dependent variable in those cases in which we found the statistical significance.

	β	**df**	**F**	**P value**	
Laying date					
altitude		1	0.45	0.50	
year	-1.145	1	13.45	0.001	*
residuals		58			
Clutch size					
laying date	-0.039	1	46.82	< 0.001	*
altitude	+0.0032	1	11.79	0.001	*
year		1	0.08	0.77	
residuals		47			
Incubation period					
clutch size		1	0.21	0.65	
laying date		1	3.19	0.09	
altitude	+0.004	1	8.24	0.007	*
year		1	2.61	0.11	
residuals		28			
Brood size					
incubation period	-0.636	1	21.06	< 0.001	*
clutch size	+1.488	1	18.99	< 0.001	*
laying date		1	1.87	0.18	
altitude		1	0.26	0.61	
year		1	0.13	0.71	
residuals		21			
Nestling period					
brood size		1	0.19	0.73	
laying date		1	0.42	0.52	
altitude		1	0.17	0.68	
year		1	0.32	0.57	
residuals		13			
Number of chick fledged					
nestling period		1	0.44	0.52	
brood size	+2.220	1	14.80	0.005	*
laying date		1	0.07	0.79	
altitude		1	0.38	0.55	
year		1	0.21	0.65	
residuals		10			

Table 2: Breeding parameters of the three study areas in 2012. We used ANOVA (laying date), Kruskall-Wallis (Clutch size, incubation, eggs hatched, nestling period and chicks fledged) and Fishers exact test (frequency of hatching and nestling success).

Parameters				**Soportujar**			**Monachil**			**Serpentina**		
Incubation period	**Test Statistic (F or H)**	**df**	**P value**	**Mean**	**SE**	**N**	**Mean**	**SE**	**N**	**Mean**	**SE**	**N**
Laying Date	0.22	2	0.81	***30 May***	4.24	11	***27 May***	2.56	13	***27 May***	5.34	9
Clutch size	2.39	2	0.30	***4.62***	0.26	7	***4.75***	0.13	12	***4.29***	0.29	7
Incubation	2.45	2	0.29	***12.25***	0.16	8	***12.57***	0.43	7	***11.75***	0.25	4
Eggs hatched	3.07	2	0.22	***4.50***	0.5	6	***4.57***	0.2	8	***3.50***	0.5	4
Hatching success			0.58	***62%***		8	***64%***		11	***33%***		6
Nestling period												
Nestling period	0.37	2	0.83	***12.0***	0.23	6	***12.5***	0.64	4	***13.0***	2.0	2
Chicks fledged	0.14	1	0.71	***4.0***	0.58	4	***4.3***	0.33	3	**-**	-	-
Nestling success			0.75	***50%***		8	***33%***		9	***33%***		6

laying period, when females typically give excitement calls when coming to the nest (Cramp 1985). We did not detect significant differences for any of the breeding parameters considered among the three different study areas in 2012 (all P values > 0.05, Table 1).

Nest building stage

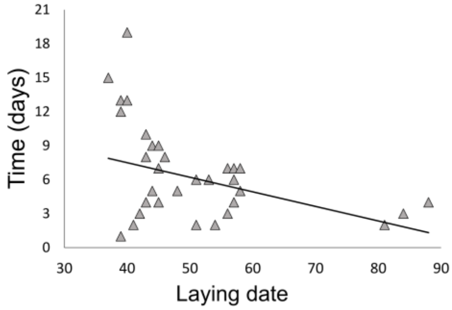

Figure 1: Regression of the time between the end of nest building and the laying of the first egg, and the laying date of each nest. We calculated dates of breeding season counting from the 1st of April as date 0. As the season progressed the time between nest building and laying was reduced.

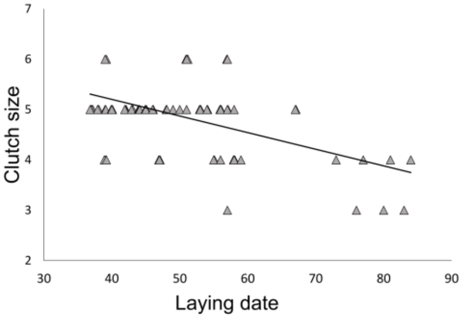

Figure 2: Regression between clutch size and laying period. We calculated dates of breeding season counting from the 1st of April as date 0 Females laid fewer eggs as the breeding season advanced.

The time between the end of nest building and the laying of the first egg decreased as the breeding season advanced (LM, $F_{1,36} = 6.32$, $P = 0.02$, Fig. 1). Two out of 14 nests and 12 out of 27 found during the nest building period in 2012 and in 2013, respectively, were either not finished or finally used by Bonelli's warblers. Of these 12 nests, 10 were abandoned in the first part of the breeding season (before June). All nests found were built exclusively by females. Once the place was chosen she started to set the natural hollow (sometimes made by the bird) and later she positioned some leaves in it. Finally, she placed grass to cover the nest. Both leaves and grass were collected from the surroundings of the nest, sometimes more than 20-30 m away from it.

Laying and Incubation stages

Laying date

Eggs were laid earlier in 2013 (mean = 20 May ± 2 days; *n* = 42, range = 7 May – 27 June) than in 2012 (mean = 29 May ± 2 days, *n* = 26, range = 15 May – 22 June; $F_{1,58} = 13.45$, $P < 0.01$), while we did not find significant differences depending on altitude considering both years together ($F_{1,58} = 0.45$, $P = 0.50$). Bonelli's Warbler females in our populations laid a mean of 4.90 ± 0.08 eggs (*n* = 71, median = 5, range = 3-6, see Table 3).

Table 3: Distribution and comparison of clutch sizes in Bonelli's warbler populations from Southern Spain and Southern Germany ($F_{1,125} = 12.33$, $p < 0.001$, $N = 127$).

Clutch size	3	4	5	6	7	**Mean**	
Spain	4 %	17 %	63 %	15 %	0 %	**4.9**	N = 71
Germany	0 %	5 %	64 %	25 %	5 %	**5.3**	N = 56

Clutch size

A significant negative relationship between clutch size and laying date (Fig. 2) was found in the model (LM, $F_{1,\,47} = 46.82$, $P < 0.01$, $y = -0.038x + 6.80$, Table 2). This implies that females breeding earlier laid more eggs than females breeding later in the breeding season. Moreover, we found that clutch size increased with altitude (LM, $F_{1,\,47} = 11.79$, $P < 0.01$, Table 2).

Incubation period

Incubation period was 12.62 ± 0.16 days, (range = 11-15; N =37) and it was not correlated with clutch size (LM, $F_{1,\,28} = 0.21$, $P = 0.65$) but increased with altitude (LM, $F_{1,\,28} = 8.24$, $P < 0.01$, Table 2). The mean time spent incubating per hour was 45.39 ± 4.15 minutes ($n = 9$). Females left the nest a mean of 1.28 ± 0.12 times per hour ($n = 9$) and spent a mean of 10.67 ± 0.83 minuntes out of the nest per hour ($n = 9$). We observed only once a visit of a male warbler that arrived to the nest with a grasshopper in the bill and fed it to the incubating female.

Eggs size

Egg size averaged 15.16 ± 0.08 mm in length (range = 13.95 – 17.01 mm, $n = 65$) and 12.22 ± 0.06 mm in width (range = 10.93 – 15.81 mm, $n = 65$). The mean of egg volume and mass were 1158.86 ± 13.52 mm^3 and 1.11 ± 0.01 g ($n = 65$), respectively. Both egg mass and volume decreased in larger clutches (LMM, $F_{1,44} = 11.17$, $P < 0.01$, $F_{1,44} = 15.26$, $P < 0.01$ respectively; Fig. 3A and 3B). Although the relationship did not reach the statistically significant threshold, egg volume tend to decrease with altitude (LMM, $F_{1,44} = 3.60$, $P = 0.06$) independently of the clutch size (LMM, $F_{1,44} = 0.42$, $P = 0.52$). Egg mass was not correlated with altitude (LMM, $F_{1,44} = 0.45$, $P = 0.49$).

Daily survival rate for incubation period and hatching success

Daily survival rate for incubation period was 0.958 ($n = 70$). The proportion of nests in which at least one egg hatched did not vary between years or altitude ($\chi^2 = 1.52$, df = 1, $P = 0.22$, and GLM $z = 1.17$, $P = 0.24$, respectively). In our study population a mean of 4.5 ± 0.17 ($n = 34$) eggs hatched per nest (brood size). A greater proportion of eggs hatched in larger clutches than in smaller clutches (LM model, $F_{1,21} = 18.99$, $P < 0.01$, Table 2). Moreover, we found a negative relationship between brood size and incubation period (LM model, $F_{1,21} = 21.06$, $P < 0.01$, Table 2).

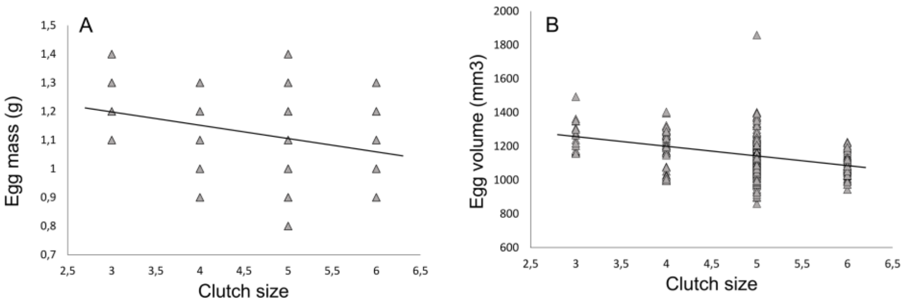

Figure 3: Relationship between egg parameters (A, mass, and B, volume) and clutch size. In the larger clutch size eggs were smaller and lighter than those of the smaller ones.

Nestling stage

Nestling period

Chicks remained at the nest for 12.5 ± 0.22 days (range = 11-15; *n* = 24). The nestling period was not associated with any parameters in the model (Table 2).

Daily survival rate for nestling period and fledging success

The daily survival rate for nestling period was 0.945 (*n* =45). The proportion of nests in which at least one chicks fledged over successfully hatched nests did not differ between years ($\chi^2 = 1.44$, df = 1, P = 0.23) or along altitude (GLM z = 1.63, P = 0.09). Successful pairs managed to fledge a mean of 4.00 ± 0.24 chicks (*n* = 18). This value was significantly correlated with brood size (LM, $F_{1, 8} = 14.80$, P < 0.01, Table 2).

Nestling growth

Nestling growth was estimated from the linear slope of body mass, tarsus length and wing length against age foe each nestling. Growth of tarsus was significantly and negatively correlated with the duration of the nestling period ($F_{1, 26} = 9.36$, P = 0.02); body mass gain was only marginally non-significantly related to the duration of nestling period ($F_{1, 26} = 4.98$, P = 0.06) and wing length growth did not show any relationship with the length of the nestling period ($F_{1, 26} = 0.17$, P = 0.68). Nestling growth was not correlated with brood size (mass: $F_{1, 26} = 0.14$, P = 0.72; tarsus length: $F_{1, 26} = 3.86$, P = 0.10; wing length: $F_{1, 26} = 0.44$, P = 0.53). Nestling development was significantly and negatively correlated with altitude for growth of body mass and tarsus length ($F_{1, 26} = 12.34$, P = 0.01; $F_{1, 26} = 28.10$, P < 0.01 respectively) but not for wing length ($F_{1, 26} = 0.02$, P = 0.92).

Daily survival rate for breeding period and breeding success

Nestling starvation was found in four nests (17%, *n* = 23) in 2012 and in six nests in 2013 (12%, *n* = 51) and did not vary between years ($\chi^2 = 0.43$ df = 1, P = 0.51). The daily survival rate for the breeding period was 0.955 (*n* =79). The proportion of nests in which at least one chick fledged did not differ significantly between 2012 and 2013 ($\chi^2 = 1.60$ df = 1, P = 0.21) nor in relation to altitude (GLM z = 0.38, P = 0.70).

Discussion

The absence of differences in breeding parameters among Bonelli's Warblers from all three study areas for 2012 suggests that our findings could be considered as representative of its distribution in the mountain woodlands of Sierra Nevada.

Nest building stage

All nests in our study were located above 1200 m a.s.l., which is much higher than other European populations, where nests can be found as low as 300 m a.s.l. (Riedinger's study 1974,Cramp 1985). This can be explained because the environmental requirements for breeding in this species (i.e. humidity and temperature) at these altitudes in Mediterranean areas correspond to those of lower altitudes further north (Waught 2002). Furthermore, our data suggests that Bonelli's Warbler is the highest breeding *Phylloscopus* species in European (Cramp 1992).

All nests were located on the ground, a trait common to all species of the genus *Phylloscupus* (Cramp 1985). However, for some species there is variation in this trait. For example, in the Willow Warbler about 7% of the nests were recorded as being more than 30 cm above the ground (Cramp 1955) and in the Chiffchaff more than 10% of the nests were found at 50 cm above the ground (Rodrigues & Crick 1997). In contrast, for the Bonelli's Warbler, we found that all (100%) nests were placed on the ground as observed in Riedinger's population (1974), which suggests that this is a fixed trait for this species throughout its distribution area. Nests were a domed structure located in small hollows. They were usually located under overhanging tussock or at the base of Spanish Broom *Spartium junceum* plants and sometimes under overhanging banks.

Constant monitoring of nests allowed us to get information about nest building behaviour. As occurs in other populations of Central Europe (Cramp 1985), we found that Bonelli's warbler nests were built exclusively by females. During the first part of the breeding period females built the nest and waited for more than 10 days before laying; while as the season progressed the time between nest building and laying was reduced (Fig. 1). In contrast, in a Germany population (Riedinger 1974) no more than 3-4 days elapsed between the end of the nest building stage and the start of laying. This long delay in laying has also been found for other *Phylloscopus* species (e.g. Wood Warbler *P. sibilatrix*; Wesolowski & Maziarz 2009). Probably the females that arrived first built their nests earlier, but delayed the date of laying because food abundance could be relatively low at the beginning of the breeding season, when overnight temperatures are still frequently below zero (Stutchbury & Robertson 1987, Wesolowski & Maziarz 2009). It has also been suggested that early setting females delay the laying of their eggs in order to benefit of breeding synchronously with other conspecifics through the dilution of predation risk (Stutchbury &Robertson 1987, Maddox &Weatherhead 2006, Wesolowki 2013).

We recorded 14% and 21% of nest abandonment during nest building in 2012 and 2013 respectively. Several reasons could be behind this result. It is possible that our presence in the field provoked these abandonments, however, we think that it is highly unlikely given that our activity at the nest was not intensive (one visit every 2-3 days, and only 30 min of nest searching per day from a considerable distance before finding the nest location). Nest desertion could also be due to mate switching or predation of egg laid between nests visits (Westneat 1992, Maddox & Weatherhead 2006). Mate switching could be the main reason of nest desertion in our populations given that most of the unfinished nests were found in the first part of the breeding season (i.e. before June).

Laying and incubation stages

In our population, females started laying at the end of May, while in southwest Germany laying begins in the second week of May (Riedinger 1974). It is likely that climatic conditions could affect this parameter since, in Mediterranean region, Bonelli's Warblers live at higher altitudes where warmer temperatures are reached later than in the lower woodlands of central Europe (Sanz *et al.* 1997, Fargallo *et al.* 1997). We found nine days difference between mean laying date in 2013 (20 May) and 2012 (29 May). The weather conditions during the pre-laying period could affect the start of reproductive activities (Novoa *et al*. 2008) and, in fact, April in 2012 was almost 2ºC colder than the 1970-2000 average, whereas it was 1ºC warmer in 2013 (Spanish Meteorological Agency, AEMET). Thus, it seems likely that this is the reason for the delay of laying in 2012.

Bonelli's Warblers in Sierra Nevada had a significant lower clutch size compared with birds from a German population (Riedinger'1974, Table 3). The increase of clutch size with increasing latitude has been documented both between and within species (e.g. Ojanen *et al.* 1978, Perrins & Birkhead 1983, Martin 1996, Cardillo 2002, Rose *et al.* 2013), and exceptions are very rare (Soler &Soler 1992). Most studies reported a negative relationship between clutch size and altitude, both at species and population levels (Badyaev 1997, Lu 2005, Lu 2008, Lu *et al*. 2009, Badyaev & Ghalambor 2001 but for exception see Weathers *et al.*2002, Johnson *et al.* 2007). In general they argued that bird fecundity declines in response to the harsher environmental conditions at higher altitudes which constrained the production of eggs. However, we found the opposite pattern. We do not think that the altitude difference in clutch size was driven by laying date because this parameter did not vary with altitude. Lu (2008) found no evidence of reduction in clutch size with altitude in a related species, the Tickell's Leaf Warblers *Phylloscopus affinis*, or among other Asiatic warbler species. It could be that the same environmental pressures, such as altitude in this case, may not act in a consistent manner among or within species, and we can attribute this finding to the differences in local condition or environmental variation, such as differences in food availability (Martin 1996) or predation pressure (Slagsvold 1982). In particular, smaller clutches at higher altitudes could be a consequence of the increase in predation along altitude (Slagsvold 1982) although other studies showed a general decrease of predation pressure at higher altitude (Caulson 1956, Badyaev 1997). A lower predation risk in the upper part of the studied areas, possibly mediated by a higher brood replacement rate or a change in the predator community, might be responsible for the increase in clutch size of Bonelli's Warblers along the altitudinal gradient of Sierra Nevada.

Females laid fewer eggs as the breeding season advanced (Fig. 2). This is a common biological trait that has also been reported in other species of the genus, both in Europe (Wood Warbler, Wesołowski & Maziarz 2009 and Chiffchaff, Rodrigues & Crick 1997) and Asia (Tickell's Leaf Warblers, Lu 2008). Smaller clutches usually occur at the end of the breeding period when condition are less favourable and food becomes less readily availability (Garamszegi *et al.* 2004). In addition, we found a negative relationship between clutch size and egg volume, consistent with previous findings (Blackburn 1991, Soler & Soler 1992, De Neve & Soler. 2002) and usually explained by the tradeoff between these two parameters in terms of parental investment (Perrins & Birkhead 1983). In our population, egg volume decreased with increasing altitude but this relationship was not driven by a larger clutch size at higher altitudes given that the interaction between these predictors was not significant. Johnson *et al.* (2006) argued that smaller eggs at high altitudes could be favoured by selection to facilitate an extra energy allocation strategy, sacrificing egg size before clutch size.

Through the use of cameras, we were able to confirm information regarding important aspects of incubation behaviour. Females incubated more than 45 minutes per hour. We observed that while females incubated, males normally stayed in the proximity of the nest calling, often from regular perches. Males frequently visited the net when incubating females were not present. Since we observed just once a male feeding a female at the nest, we can conclude that in this species males do not generally feed females during incubation. Thus, the most probable reason for interrupting incubation would be that females need to feed themselves (Ibáñez-Álamo & Soler 2012). Females living at higher altitudes spend more time incubating and this relationship was not caused by the increase of clutch size in altitude because clutch size was not correlated with the duration of the incubation period. The colder climate at high altitudes may increase thermoregulatory needs during embryonic development and this factor could protract the duration of the incubation. Generally, high altitude birds spend more time in all parental care activities (Badyaev 1997, Badyaev & Ghalambor 2001).

The daily survival rate of nest (0.958) was similar, or slightly lower, to that found in other *Phylloscopus* species, such as Willow Warblers *P. trochilus*, Chiffchaffs or Wood Warblers (0.958, 0.978 and 0.989 respectively; Tiainen 1983, Rodrigues & Crick 1997, Mallord *et al*. 2012). Almost all eggs of completed clutches hatched (4.5 of 5 eggs), which is similar hatching success to that recorded in the Wood Warbler (5.5 off 6 eggs; Wesołowski & Maziarz 2009). The number of eggs hatched is directly related to clutch size, while a negative relationship was found between brood size and incubation period, probably because females could start incubating with the penultimate egg (pers. obs.).

Nestling stage

Fledging success, that is, the proportion of the nests in which at least one chick fledged from successfully hatched eggs, was 0.29. The daily survival rate during nestling period (0.945) was similar to that found for Willow Warbler in Southern Finland (0.946;

Tianinen 1983) but relatively lower compared with another population in Britain (0.981; Peachet *et al.* 1995) or with other related species as Chiffchaff (0.982; Rodrigues & Crick 1997) or Wood Warbler (0.969; Mallord *et al*. 2012). On average, four young fledged from every successful nest following a mean nestling period of 12.5 days; a period which is common to other warbler species (Cramp 1985). Even if both nestling and incubation periods usually increase with altitude (Badyaev & Ghalambor 2001, but see Badyaev 1997), we found this relationship only for the incubation period (see above).

Table 4: Biometric body measures for adults and chicks of Bonelli's warblers depending on age.

	Body mass (g.)			**Wings (mm.)**			**Tarsus (mm.)**		
Chicks age (days)	Mean	S.E.	N	Mean	S.E.	N	Mean	S.E.	N
2-4	3.20	0.24	28	11.61	0.75	26	8.98	0.42	20
5-7	6.12	0.27	28	23.77	1.19	26	13.97	0.41	20
8-10	8.08	0.13	28	35.70	0.83	26	17.50	0.83	19
Adults	7.79	0.11	31	63.27	0.41	33	18.97	0.07	33

We found an extremely low breeding success in our population (25%). This value is lower than that found in two related species: 39% in a Finnish Willow Warbler population (Tiainen 1983) and 57% in a Wood Warbler population located in Wales, UK (Mallord *et al.* 2012). As the 67% of all active nests failed well before the nestlings were able to leave the nest, we are confident that most of the nest failure occurred because of nest predation. This is also supported by the fact that the majority of all unsuccessful nests found were damaged or completely destroyed, indicating the action of nest predators. Nevertheless, a more detailed study about the effect of nest predation for this species, including the identification of nest predators, will be very interesting to assist any future conservation actions.

When nestlings were about to leave the nest, they had already reached adult body mass and tarsus lengths (Table 4), but not wing length; a feature that reaches adult size after fledging (O’Connor 1984). In accordance to this, we found that nestlings that spent more time in the nest were those which grew tarsus length and gained body mass more slowly, but these patterns were not found for wing length. Furthermore, body mass and tarsus length grew more slowly at higher altitudes, while we did not found relationship for the length of the wings. This body size pattern may be attributed to constraints on individual growth due to climate severity, food scarcity and/or hypoxia at higher altitude (Lu *at al*. 2009). Low temperatures at high altitude impose greater energetic requirements for self-maintenance, which may limit the resources that can be allocated to growth. Low food availability could favour a decrease in nestling provisioning which could also affect growth rate and even nestling survival. According to Dillon *et al.* (2006) hypoxia may reduce metabolic activities. Since the duration of nestling period was not correlated with the altitude, nestlings raised at high altitudes fledged at the same time as those raised at lower altitudes, but they experienced slower growth rates.

Conservation aspects

Considering that the distribution of Western Bonelli's Warblers is essentially limited to western Europe, with more than 65% of all breeding pairs in Spain, the low breeding success reported here raises an important conservation concern. It is even more relevant because the species has declined in the last few years in some parts of Europe (Dubois *et al.* 2000, Del Hoyo *et al.* 2006) and, particularly in the mountain range of Sierra Nevada (Zamora & Barea-Azcón 2015). In this area, Bonelli's Warbler still represent one of the most abundant breeding passerine species (Martínez *et al.* 2010), but the population size has declined to a quarter of what it was 30 years ago (Zamora & Barea-Azcón 2015). Bonelli's Warblers live in well-defined habitats, mainly in Mediterranean mountain regions which involve many fragile and threatened ecosystems (Santos *et al.* 2002). There is no information about breeding site fidelity of Bonelli's Warblers, but three birds that were ringed in 2012 were seen in the same breed areas the following year. This suggests that Bonelli's Warblers could be relatively faithful to their breeding sites, but more data are needed. Therefore, any environmental or stochastic change could severely affect Bonelli's Warbler populations given the low breeding success found in this study, and the impact could be effective over the whole Mediterranean range. In fact, fires seem to have an important impact in the demography of this species in certain regions (Urios *et al.* 1991). Both the low breeding success in this population and the threatened habitat used by Bonelli's Warblers suggest that the status of this species may easily worsen and will need close monitoring. Therefore, the novel and detailed information reported in our study about the breeding biology of Bonelli's Warblers could be crucial not only for expanding our understanding of this interesting species but also to call attention to the potential risks that it face now, or might face in the near future (Zamora & Barea-Azcón 2015) due to a low breeding success. We suggest that we should monitor more closely its population trends and apply conservation measures if needed.

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PART II

BROOD PARASITISM

CHAPTER 2

SIZE AND MATERIAL OF MODEL PARASITIC EGGS AFFECT THE REJECTION RESPONSE OF WESTERN BONEELI'S WARBLER

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Abstract

Given the high costs imposed by avian brood parasitism, hosts have adopted different defenses to counteract parasites by ejecting the foreign egg or by directly deserting the parasitized nest. These responses depend mainly on the relative size of the host compared to the parasitic egg. Small hosts must deal with an egg considerably larger than their own, thus, nest desertion becomes the only possible method of egg rejection in these cases. The use of artificial model eggs made of hard material in egg-recognition experiments has been criticized because hard eggs underestimate the frequency of egg ejection. However, no available studies have investigated the effect of softer material even if it is likely to overestimate this response. Here, we investigate the potential effect of size of experimental parasitic eggs in relation to egg-rejection behaviour (egg ejection and nest desertion rates) in the Western Bonelli's Warbler *Phylloscopus bonelli*, a small host, by using plasticine non-mimetic eggs of three different sizes. In addition, we tested the potential effect of material, comparing ejection and desertion responses between real and plasticine eggs. As predicted, small eggs were always ejected, while nest desertion occurred more frequently with large eggs, thus suggesting that nest desertion would occur because of the constraints imposed by the large eggs. We found that plasticine may misrepresent the responses to experimental parasitism, at least in small host species, because this material facilitates egg ejection, provoking a decrease in nest desertion rate. Thus, a particular caution is needed in the interpretation of the results in egg-rejection experiments performed using dummy eggs made of soft materials.

Key words: artificial eggs, brood parasitism, ejection rate, nest desertion, small host.

Introduction

Avian brood parasites impose high costs on their hosts, causing a general decrease in breeding success (Davies & Brooke 1989). A total loss of breeding success occurs in those hosts parasitized by species such as the Common Cuckoo *Cuculus canorus*, an early-hatching parasite that evicts all host eggs or chicks (Davies 2000, Kilner 2005). To counteract these costs, host have evolved different defensive strategies, which in turn select for new adaptations in brood parasites in a process that gives rise to a coevolutionary arm-race between hosts and parasites (Dawskin & Krebs 1979, Davies & Brooke 1989, Moknes *et al.* 1991, Soler 2014). The most effective and widespread defensive response by hosts is the discrimination and rejection of foreign eggs, either by ejecting the parasitic egg or by deserting the parasitized nest (Davies 2000). Ejection occurs in two ways: by grasping the egg in the bill (grasp ejection) or by piercing the eggshell to make a hole and gripping the egg (puncture ejection; Davies 2000). The ejection of the parasitic egg presents costs associated with recognition, including recognition errors when the host's own eggs are ejected (Davies & Brooke 1989), and rejection costs related to the physical difficulties of ejecting the foreign egg, which may accidentally damage their own eggs (i.e. rejection costs; Davies & Brooke 1989, Soler *et al.* 2002, Stokke *et al.* 2015). The desertion of parasitized nests usually involves higher costs due to the complete loss of the clutch, the subsequent search for a new nest-site, building of a new nest, the production of a replacement clutch and breeding later in the season (Hauber 2002, Hoover 2003).

The relative size of the host and parasite is important in relation to the costs faced by each species. Soler *et al.* (2015) found that in Common Blackbirds *Turdus merula*, a medium-sized host considered to be a grasp ejector (Davies & Brooke 1989, Honza *et al.* 2005, Soler *et al.* 2015), the size of introduced eggs had a significant effect by itself because the larger the egg, the lower the probability of ejecting it. Moreover, it has been shown in the same species that egg rejection was negatively affected by egg mass, given that heavy eggs were more frequently accepted than lighter ones of the same size (Ruiz-Raya *et al.* 2015). Small hosts or hosts with relatively small bills have more difficulties in ejecting the parasitic egg. For species that present a grasp index (a measure of the ability to hold an egg; Rohwer & Spaw 1988) smaller than 200 mm^2, it could be extremely difficult or even impossible to eject the parasitic egg. In small host species, the parasitic egg is considerably larger than their own eggs and, given that the host cannot physically grasp the egg, they commonly eject it by puncturing it or deserting the nest (Moksnes *et al.* 1991, Antonov *et al.* 2009, Soler *et al.* 2015). Furthermore, the smallest host species cannot puncture eject the parasitic egg either (Hosoi & Rothstein 2000) and therefore, nest desertion represents a key defense mechanism. For example, Antonov *et al.* (2006) found that the Marsh Warbler *Acrocephalus palustris*, a small host which commonly responds by puncture ejection, more frequently deserted nests that had been experimentally parasitized with cuckoo eggs (which are larger than their own) because of ejection difficulty. Antonov *et al.* (2006) associated this result with the strength of the cuckoo egg shell rather than egg size. However, it is likely that the size of parasitic eggs could itself also affect ejection and desertion rates. The size of parasitic eggs in small hosts might play a critical role not only determining whether to eject or desert, but also in affecting female incubation behaviour, for example, if the clutch cannot be completely covered by the incubation bird or if the normal incubation time pattern is altered. Nevertheless, to our knowledge, this questions have not been investigated in small hosts.

Artificial model eggs made of harder materials than the eggshell of real eggs, such as plaster, hard plastic or clay, have been frequently used in egg-recognition experiments (Rothstein 1982**,** Davies & Brooke 1989, Soler & Møller 1990, Moksnes *et al.* 1992, Lotem *et al.* 1995, Davies *et al.* 1996, Peer *et al.* 2000). Nevertheless, different authors have pointed out that the hardness of artificial eggs may underestimate the frequency of rejection because the difficulty of puncturing these eggs would be much higher than that of puncturing a real egg (Moksnes *et al.* 1991, Martín-Vivaldi *et al.* 2002, Prather *et al.* 2007). There are fewer studies in which soft material was used (Marchetti 1992, 2000, Moskát *et al.* 1999, 2002), but it seems likely that the use of soft materials could reduce the difficulty of rejection, thus overestimating the ejection response. For instance, two studies carried out to analyse the ejection rates in two small hosts belonging to the genus *Phylloscopus* showed contrasting results. Martín-Vivaldi et al. (2012) investigated egg-rejection behaviour of the Western Bonelli's Warbler *Phylloscopus bonelli*, a small passerine parasitized by the Common Cuckoo in Mediterranean woodlands (Campobello *et al.* 2009; Martínez *et al.* 2010), by introducing mimetic and non-mimetic model eggs made of plaster, a hard material difficult to puncture. Bonelli's Warblers rejected these eggs, which were of the same size of Common Cuckoo eggs (Martín-Vivaldi *et al.* 2002; c. 50% larger than Bonelli's Warblers eggs) three times more often by deserting the nest than by ejecting the parasitic egg. By contrast, no case of desertion occurred in a similar Asian warbler species, the Yellow-Browed Leaf Warbler *Phylloscopus humei* studied by Marchetti (2000) where model parasitic eggs used were aproximately 75% larger than host eggs but made of plasticine, a soft material that does not completely harden. This study found that the most females (82%) ejected the large experimental egg, whereas only a few accepted it after pecking several times.

In this study, we investigate the potential effect of size and material of experimental parasitic eggs in relation to egg-rejection behaviour in the Bonelli's Warbler, a very small host of Cuckoos. Fisrt, we seek to determine whether the size of the parasitic egg influences ejection and desertion rates. By using non-mimetic model eggs made of plasticine of three different sizes (smaller, same size and larger than Bonelli's Warbler eggs), we predict that nests parasitized with large model eggs should be deserted more frequently than nests parasitized with small or medium-sized model eggs, whereas in nests parasitized with smaller and medium-sized model eggs, Bonelli's Warbler should be able to eject them. Secondly, we study the potential effect of soft material used to create model eggs in egg-recognition experiments, by comparing the responses of Bonelli's Warblers, a puncture ejector species (Martín-Vivaldi *et al.* 2002), to the introduction of real and plasticine eggs. We predict that this soft material overestimates egg ejection rate. Using the information from video recordings, we also seek to identify any negative effects on incubation pattern of the presence of parasitic eggs.

Methods

Study area and population

The study was conducted in 2013, between the end of April and mid-July, in the National Park of Sierra Nevada, SE of Spain (36° 56’N, 3° 23’ W). The area is a steep slope, located between 1200 and 2000 m asl in the supra-Mediterranean climatic belt, formed by an open deciduous woodland composed of Pyrenean Oak *Quercus pirenaica*, Holm oak *Quercus ilex* and three pine species *Pinus pinaster, P. nigra, P. sylvestris*.

Western Bonelli’s Warbler is a small (7 - 11.5 g) passerine which breeds in Western Europe and Western North Africa, and nests on the ground in mountain woodlands. In winter, all populations moved to the south edge of the Sahara, from Mauritania and Senegal to Chad (Cramp 1985). Males and females are visually identical, but male are slightly larger than females, as is the case in congeneric species (Tiainen 1982). Males and females also differ behaviourally, particularly during the breeding period. Females spend most of their time near the ground and typically give excitement calls when coming to the nest, whereas males are usually higher in the canopy of trees and sing from perch sites in the tree crown. In our study area, Bonelli’s Warblers started to breed by the second half of May, laying on average 4.9 eggs, with both the incubation and nestling periods of 12-13 days (Roncalli *et. al.* 2016).

Experimental procedures

We located Warbler's nests by following parental activity. Once we located a nest, we visited it every other day to establish the exact laying date. Because female Bonelli’s Warblers lay one egg per day (Cramp 1985, Roncalli *et. al.* 2016), we could accurately determine the date on which clutch size was completed before conducting egg-addition experiments 1 or 2 days after clutch completion.

To test the effect of egg size on rejection behaviour, we experimentally parasitized Bonelli's Warbler nests using non-mimetic eggs of different sizes made of red plasticine (Fig. 1). We carried out three different experimental treatments (Table 1): (1) small model eggs, 50% smaller than Bonelli’s warbler eggs; (2) medium-sized model eggs, similar in size and weight to real Bonelli’s Warbler eggs (egg length, $t = -0.71$, $df = 14$, $P = 0.49$; egg width, $t = 1.24$, $df = 14$, $P = 0.23$; egg weight, $t = -1.27$, $df = 14$, $P = 0.22$); and (3) large model eggs, 50% larger than Bonelli’s warbler eggs and very similar in size to real Common Cuckoo’s eggs (see Martín-Vivaldi *et al.* 2002 and Álvarez 2003 for detailed measurements). In addition, we created two control groups: (4) manipulation control treatment, in which we introduced a real Bonelli’s Warbler egg painted red (Fig. 1), which allowed us to test the potential effect of plasticine material in egg-rejection behaviour (second objective), by comparing ejection and desertion rates between this group and the group with medium-sized model eggs (see above); and (5) the control treatment, in which we followed the same procedures as for the other, except that the nests were not experimentally parasitized. This last group allowed us to control for the potential effect of cameras over host responses.

The experiment was made in 42 nests including 10 nests for each of the experimental groups (large, medium, and small) and 6 for each of the two control groups. We could use only 5 manipulation control treatment nests because one of them was depredated before the end of the experiment. For ethical reasons, we reduced our sample sizes as much as possible without compromising our ability to detect significant differences. This is because we predicted that nest desertion could be a frequent response to our experimental parasitism, which has important implications for this species, given that its populations have been severely reduced in the area for the last 30 years (Zamora & Barea-Azcón 2015).

Table 1: Measures of real Bonelli's Warbler eggs and non-mimetic eggs of different sizes. We used five Bonelli's Warbler eggs and 10 eggs for each size treatment.

	Bonelli's Warbler egg	Small model egg	Medium model egg	Large model egg
Size (mm.)				
Length	15.68 ± 0.08	12.11 ± 0.35	15.28 ± 0.28	19.42 ± 0.51
Width	12.22 ± 0.06	9.29 ± 0.35	11.95 ± 0.34	14.70 ± 0.42
Weight (gr.)	1.11 ± 0.01	0.60 ± 0.01	1.19 ± 0.04	2.36 ± 0.11

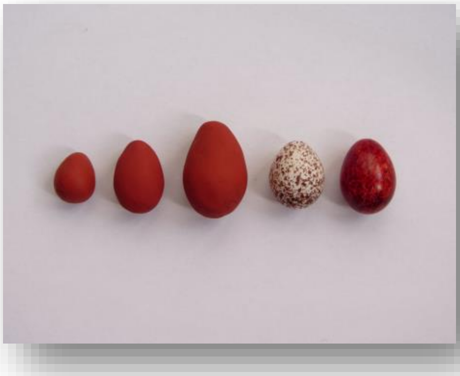

Figure 1: From left to right, non-mimetic eggs of different sizes made of plasticine, Bonelli's Warbler egg and real egg painted red, as used in the experiments

After the introduction of the egg, we placed a video camera (Panasonic HDC-SD40, Osaka; Japon) close to the focal nest (1-3 m) for 2 h to record female behaviour at the nest, with special interest in determining the possibility of ejection events. Each nest was checked every 24 or 48 h over the following 5 days. Five days was considered an adequate time interval in these experiments because this period has commonly been used in several egg-rejection experiments (e.g. Davies & Brooke 1988, Grim et al. 2011, Soler et al. 2015). We considered the model egg to has been accepted when it remained warm in the nest for 5 days, even if it had marks left by the bill (one case). In this case, on the fifth day, we removed it and considered the trial finished. Each model egg was used only once. We considered the model egg to be ejected if it disappeared from the nest during this five-day interval. On the other hand, we noted nest desertion as rejection behaviour when we found the clutch, including the model egg, cold for two consecutive visits.

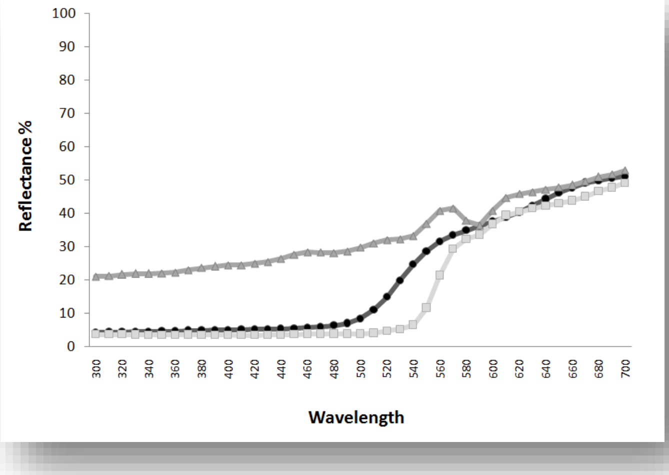

Figure 2: Reflectance spectra from natural Bonelli’s Warbler egg (grey triangles), real red painted egg (white squares) and plasticine model egg (black circles).

To measure the colours of each egg type (the natural Bonelli’s Warbler egg, the real painted egg and the plasticine model egg), we used a MINOLTA CM-2600d/2500d spectrophotometre (Konica Minolta, Japon). We obtained the curve of reflectance in the range of 300-700 nm. Real painted and plasticine eggs presented very similar colours based on the major overlap of their reflectance curves (Fig. 2). In contrast, natural Bonelli's Warbler eggs, which are white-cream coloured, with tiny, brownish and blackish spots (Fig.1), showed a very different curve compared to the other two red eggs (Fig.2). Subsequently, we estimated the difference in colour among eggs with respect to the spectral sensitivity of each of the four avian photoreceptors (ultraviolet, short, medium and long wavelength). Data for detailed spectral sensitivity were not available for our species. Therefore, we extracted spectral sensitivity by using the average avian UV system provided in the vismodel function in the ‘pavo’ package for R software (Maia *et al.* 2013). Applying the visual models of Vorobyev and Osorio (1998) we calculated quantum catches at each photoreceptor and determined the colour distance corrected for the receptor noise based on relative photoreceptor densities. Colour difference is expressed as $\mathrm{JND_S}$ (just-noticeable differences). The higher the contrast values the higher the difference in colour as perceived by birds (Šulc *et al.* 2016). These results confirm the differences among egg types observed in the photo (Fig. 1) and the curves of reflectance (Fig. 2). The difference between plasticine model eggs and real painted eggs was smaller (7.64 JNDs) compared to the differences between natural eggs and plasticine (18.87 JNDs) or real painted eggs (23.90 JNDs).

Female behaviour and ejection events

To minimize observer bias, blinded methods were used when behavioural data were extracted from video recording by using VLC software. We collected the following variables for the 2-h observation period at each nest: (1) Incubation latency (time spent between female arrival to the nest and the start of incubation), (2) nest visits (number of female visits to the nest per hour), (3) off-bouts (time in which females were away from the nest per hour), (4) incubation time, expressed as the percentage of time spent incubating during all the time that female stayed in the nest (on-bouts),and (5) number of touches directed at the model egg. Female touches have been used in several egg-recognition experiments as an indication of recognition of the parasitic egg (Antonov *et al.* 2009, Ruiz-Raya *et al.* 2015, Soler *et al.* 2015). Egg-ejection events filmed ($n = 9$) were also carefully checked to determine the method of ejection (grasping or puncturing) and latency time until ejection of the parasitic egg. To establish the method of ejection, we also confirmed the information from recordings by examining the marks left on the model eggs recovered both within (when deserted) and outside (when eggs were ejected) nests. In all cases of ejection, we carefully searched the surroundings of the nest (4 m diameter around the nest) after each detected ejection to try to find the model egg. The marks of puncture ejections appeared as a clear hole produced by the tip of the bill, whereas grasp ejections left one or two lines produced by the edges of the bill on the plasticine eggs (Fig. 3).

Figure 3: Two examples of marks left on ejected plasticine model eggs by Bonelli's Warbler females. The egg on the left shows several holes indicating attempts at puncture ejection of the egg, while the egg on the right shows several lines typical of grasp ejection

Statistical analysis

To determine the effect of the size and material of the introduced egg, we used Chi-square goodness-of-fit tests for frequency tables, correcting with the Monte Carlo approach in the cases in which the frequency was l< 5. To explore the effects of laying date and clutch size on desertion and ejection rates, we calculated two independent GLMs (Generalized Linear Model) fitted to a binomial distribution. All variables from the video recordings were analysed by an ANOVA or Kruskal-Wallis test, depending on the characteristics of the variable. We included treatment as a factor for all analyses. We used R 2.15.3 for Windows (R Development Core Team 2012) and Statistica 8.0 (Statsoft Tulsa, OK, USA) to perform the analyses. All data are in the form of mean ± 1 se.

Results

Female responses according to egg size and material

We found a significant effect of the egg size on the response against parasitic eggs (χ^2= 10.9, df = 4, P = 0.03). As we predicted, nests parasitized with large model eggs were more frequently deserted than those with medium and smaller model eggs (χ^2 = 8.4, df = 2, P = 0.01, Fig. 4). Similarly, the ejection rate was lower in nests parasitized with large model eggs than in the other two experimental groups (χ^2 = 10.95, df = 2, P = 0.01, Fig. 4). Neither laying date nor clutch size affected desertion (z = - 0.59, p = 0.55; z = 0.89, P = 0.37) or ejection rates (z = - 1.22, p = 0.22; z = 0.005, P = 0.99).

The material of the model egg also significantly affected rejection behaviour (χ^2 = 7.5, df = 1, P = 0.017). Plasticine eggs were ejected in all cases while medium-sized real eggs were ejected significantly less often (χ^2 = 4.2, df = 1, P = 0.04, Fig. 5). Neither the laying date nor the clutch size affected desertion (z = - 0.86, P = 0.39; z = - 0.22, P = 0.82) or ejection rates (z = 0.68, P = 0.49; z = 1.58, P = 0.11).

Female behaviour when confronted with the experimental egg

We analysed female behaviour in 30 nests. Another two cases were not recorded because of logistic problems and 10 records were lost because of technical problems. In no case did we detect a male entering a nest during recordings, so all rejection behaviour related to females. Incubation latency did not significantly differ among experimental groups ($F_{(4,24)}$ = 0.803 P = 0.53), even when take into account the fact that in control nests females began incubating immediately on arrival at the nest. Neither the nest visit rate nor time spent off the nest differed significantly between experimental groups (Kruskal-Wallis χ^2= 7.39, df = 4, P = 0.11 and $F_{(4,23)}$ = 2.12 P = 0.11, respectively). Egg size affected female touches ($F_{(2,9)}$ = 4.70, P = 0.04). Touches directed at the large model eggs were more numerous (81 ± 18, n = 5) than those directed to medium-sized or small eggs (10 ± 20, n = 4; 2 ± 24, n = 3, respectively). Moreover, the presence of a large model egg margnially affected incubation time ($F_{(2,17)}$ = 3.41, P = 0.05) by reducing it (77 ± 5 %, n = 6) in comparison with those parasitized with medium-sized or small eggs (94 ± 5 %, n = 9; 95 ± 5 %, n = 3, respectively). The total touches directed to real experimental eggs (109 ± 21, *n* = 5) were more numerous than those directed at medium-sized plasticine eggs ($F_{(1,7)}$ = 9.98, P = 0.02). Nevertheless, we found no effect of material on incubation time ($F_{(1,12)}$ = 0.42, P = 0.53).

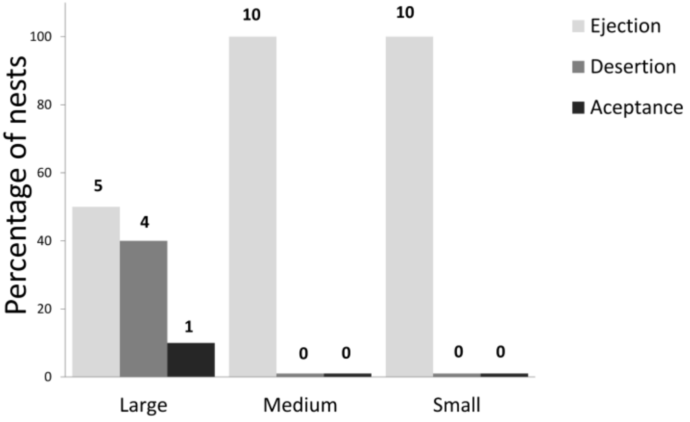

Figure 4: Acceptance, desertion and ejection rates for the egg-size experimental treatment (large, medium and small). Sample sizes for each treatment are shown at the top of each column.

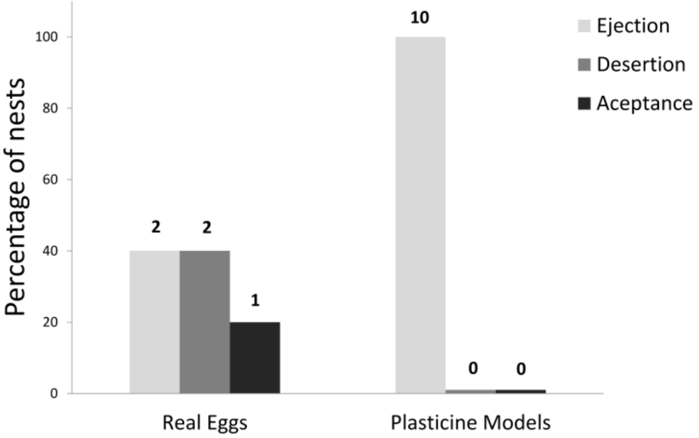

Figure 5: Acceptance, desertion and ejection rates for nests 'parasitized' with real Bonelli's Warbler eggs and plasticine model eggs (medium-sized model eggs similar in size and weight to real eggs). Sample sizes for each treatment are shown at the top of each column

Ejection events

We filmed nine ejection events: four of small model eggs, four of medium-sized model eggs, and one of large model egg. All ejections were by females. Males commonly perched in the surroundings of the nest, checking it when females were absent. Female warblers spent more time ejecting the larger egg (2117 s; more than 35 min) than medium or small eggs (41.7 ± 105.8 s and 218 ± 81.9 s, respectively; $F_{(2,6)} = 57.79$, $P < 0.001$). When females ejected small and medium-sized eggs they flew away from the nest with the egg grasped with their bills (Video S1). In the only case of ejection of a large model egg, the female was able to move the egg only a few centimetres outside the nest by grasping the narrow pole of the egg, and only after pecking it repeatedly and consequently deforming it before grasping (Video S2). These data are confirmed by the fact that two medium-sized model eggs found after ejection showed grasping marks homogeneously distributed on their surface, in contrast to the six large plasticine eggs recovered, which presented grasping marks preferentially on their poles. The size of the parasitic egg seemed to affect the ejection distance also, as suggested by the fact that around the parasitize nests no small eggs were found, and 20% ($n = 10$) of medium-sized eggs and 40% ($n = 5$) of large eggs.

Discussion

Egg size

We found a strong effect of the size of the parasitic egg on the ejection rate. In the nests in which we introduced the large model egg (similar in size to those of the Common Cuckoo) female Bonelli's Warblers ejected significantly fewer eggs than in nests with either small or medium-sized eggs (Fig. 4), where in all cases females were able to eject it. Moreover, Bonelli's Warblers responded by deserting only nests parasitized with large eggs (Fig. 4). Our results are as predicted, and are similar to those found in Common Blackbird, which more frequently ejected small parasitic eggs than medium-sized or large ones and more often deserted the nest when parasitized with large eggs (Soler *et al.* 2015). The Common Blackbird is a medium-sized grasp ejector species that can easily eject the parasitic egg. In contrast, our species is a small host, had the highest frequency of desertion in nests with larger eggs, presumably because its small bill prevented ejection. In agreement with this, desertion has been considered the main response in several other *Phylloscopus* hosts (Moksnes *et al.*1991, Stokke *et al.* 2010, Martín-Vivaldi *et al.* 2012). For instance, 95% of Willow Warbler *P Phylloscopus Trochilus* and 83% of Chiff Chaff *P Phylloscopus collybita* nests were deserted as a response to artificial parasitism (Moksnes *et al.* 1991).

The presence of a large model egg also affected the time spent incubating. During the 2 h of filming, females incubated approximately 15% less in these experimental nests than in control possibly to the detriment of egg viability. However, we could not test this hypothesis because of the high nest predation pressure in our study area which impeded us to calculate hatching success for many nests. The time in which females were in the nest without incubating was used to inspect and touch the model egg, as well as to try to eject it. Large eggs were touched more frequently than were medium and small ones.

Further, the time spent ejecting larger eggs was significantly longer than when ejecting medium and small eggs, in accordance with the result found in blackbirds (Soler *et al.* 2015). Female Bonelli’s Warblers spent more time ejecting large eggs probably because of the physical constraints, given that the female that ejected the large egg spent more than half an hour trying continuously to peck the egg, pushing it to the border and grasping it by the narrow pole (Video S2). The distance at which the ejected eggs were found also significantly depended on the size of the artificial egg, supporting the existence of these physical constraints. The larger the egg, the closer it was to the nest.

Egg material

In the studies on avian brood parasitism researchers often have been used model eggs to simulate real brood parasitism. Previous studies have pointed out the different results found in egg-rejection experiments depending on the hardness of the model eggs used; if the egg is too hard, small hosts cannot puncture and eject it (Moksnes *et al.* 1991, Martín-Vivaldi *et al.* 2002, Prather *et al.* 2007). However, no previous study has examined the effect of using model eggs made of soft material such as plasticine. We found that plasticine has a clear effect on the response to experimental parasitism. Female Bonelli's warblers consistently ejected the parasitic egg in those nests in which we introduced medium-sized plasticine eggs, while ejection and desertion occurred at similar rates in the nests parasitized with real red-painted eggs (Fig. 5). The differences in egg-rejection method could be attributed to differences in egg appearance between the two models used (Fig. 1) suggesting that plasticine eggs would have been recognized more easily than real painted ones, and consequently more frequently ejected. However, our spectrophotometric results (curves of reflectance and JND_S) indicate that the two types of model eggs were very similar, and most importantly, both models were very different from natural Bonelli’s Warbler eggs. In addition, real painted eggs, which differed most from Bonelli’s Warbler eggs, were also more frequently accepted by the birds (Fig. 5), strongly suggesting that the material of experimental eggs (and not the appearance) is the main factor affecting the observed differences in egg-rejection method.

We also found that real painted eggs were touched more frequently than plasticine eggs, indicating the manipulation difficulties in ejection efforts.Female Bonelli’s Warblers could easily peck and grasp plasticine eggs, ejecting them very quickly, even within a few seconds. This effect cannot be associated with differences in size or weight between real and model eggs because they were similar, but rather with the different material. Clearly, a plasticine egg was easier to eject than a real egg, which has a stronger and slick eggshell; in fact, not all the ejection attempts were successful because of the difficulties of handling the egg. Surprisingly, and contrasting with our findings for nests parasitized with large model eggs, incubation time was not affected by the presence of real eggs despite the high frequency at which the real egg was touched. One possible explanation is that the real egg, being harder and shinier than a plasticine model egg, increases the risk of damaging their female's own eggs and discourages her from trying to eject it.

The use of plasticine, which is considerably less strong than the shell of a real egg, might suggest that plasticine eggs are mistaken with faeces, and therefore that the ejection could be the result of nest sanitation instead of egg-recognition behaviour. Recent studies have suggested that nest sanitation play an important role in the evolution of the ejection of parasitic eggs and should be a prerequisite for anti-parasite defenses (Guigueno & Sealy 2012, Poláček *et al.* 2013, Yang *et al.* 2014). For example, Poláček *et al.* (2013) found that white model eggs were removed significantly faster than dark-brown eggs by Tree Sparrow *Passer montanus*, ad ascribed this to the fact that birds may perceive white eggs as faecal sacs. However, in our study we used model eggs painted red, which are very different from white faecal sacs. Moreover, our experiment was carried out at the end of the laying period, when the clutch was completed, while faeces removal behaviour is expected to reach the peak once the eggs hatch (Poláček *et al.* 2013).

The effect of plasticine found in our study may explain the different results obtained compared with those reported by Martín Vivaldi *et al.* (2012). In both cases the egg size used was similar (House Sparrow *Passer domesticus* eggs), but while we used plasticine they used model eggs made of harder materials (plaster). In their study, Bonelli's warblers rejected experimental eggs mainly by deserting the nest and only in one case by ejecting. Our results showed an opposite pattern. Therefore, the hardness of plaster eggs used by Martín Vivaldi *et al.* (2012) may have underestimated ejection rate. Moreover, in nests where we introduced a real non-mimetic egg of Bonelli's Warbler, females deserted 40% more often than in nests in which we placed plasticine eggs of the same size.

Our results also may offer an explanation for the high ejection rate (>80%) that Marchetti (2000) found in the Hume's Leaf Warbler. In this case, females rejected all the larger eggs introduced, because of their aberrant size, but it is likely that the high ejection rate found was the consequence of using plasticine models because, as we have shown in our experiment, plasticine facilitated pecking and grasping of the model egg. Because the two studies worked with two different species, it might also be possible that the differences in the ejection rate between Bonelli's Warbler and Hume's Leaf Warbler has species-specific explanations. Hume's Leaf Warbler is not currently parasitized by Cuckoos but shows strong discrimination abilities as well as highly specific aggressive behaviour against Cuckoos (Marchetti 1992), suggesting that it may have won the coevolutionary arm-race against them (Soler 2014). On the contrary, Bonelli's Warbler still seems to be parasitized in some areas of its distribution range (Campobello *et al.* 2009; Martínez *et al.* 2010) indicating that it is in an earlier stage of such arm-race.

Ejection events and female behaviour

In all the experiments in which we managed to record ejection, all the plasticine eggs were ejected by females, this being the expected result for species in which only the females incubate (Soler *et al.* 2002), such as Bonelli's Warblers (Roncalli *et. al.* 2016). Females invariably ejected by grasping (including large eggs). Moreover, most of the ejection marks found on the eggs recovered were one or two lines, the typical marks left by the edges of the bill during grasp-ejecting attempts. These results would seem to contradict previous findings indicating that small hosts eject parasitic eggs mainly by puncturing (e.g. Rohwer & Spaw 1988, Moksnes *et al.* 1991, Soler *et al.* 2002). Unfortunately, neither of the two real eggs ejected was filmed during the 2 h of recordings and we cannot exluded the possibility that the soft material of egg models used could have altered the method of ejection in comparison with a natural situation. Notably, Underwood and Sealy (2006) found that in Warbling Vireos *Vireo gilvus*, a species classified as the smallest puncture-ejecting host of Brown-Headed-Cowbird *Molotrus ater* eggs, 91% of plaster model eggs were ejected by grasping. The costs associated to puncture-ejecting may be higher than the costs of grasping because hosts have to peck the parasitic egg repeatedly and vigorously in order to pierce it (Soler *et al.* 2002). In the puncture ejectors of Brown-Headed Cowbird eggs, an average 0.25 host eggs were lost per ejection attempt, while in grasp ejectors the average was 0.06 (Lorenzana & Sealy 2001). On the basis of this evidence, it would not be unexpected for Bonelli’s Warblers to be capable of ejecting model eggs by grasping. It has also been suggested that some small hosts could be capable of both grasp and puncture ejection (Underwood & Sealy 2006). In support of this argument, the video recordings of the nests belonging to the manipulation control treatment (real Bonelli’s Warbler eggs painted red) showed that females vigorously touched the models both by pecking and trying to grasp them.

Conclusion

We have shown that the size of the parasitic egg significantly affected the rejection response. Large model eggs hampered ejection, favoring desertion of the nest as a rejection response, probably as a consequence of a physical constraint to manipulate the parasitic egg. Moreover, as has been reported to occur with hard materials, the plasticine model eggs do not provide real ejection and nest desertion rates, because this soft material overestimated egg ejection. In the light of these findings the conclusions obtained from egg-recognition experiments made using plasticine egg models should be considered with caution. The use of real eggs is strongly recommended whenever possible.

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CHAPTER 3

ADULT BUT NOT OFFSPRING PREDATION RISK MODULATES THE EGG-REJECTION PROCESS IN BLACKBIRDS

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Abstract

Predation and brood parasitism have critical effects on the fitness and demography of animals, but few studies have focused on the potential interactions between these two important selective forces. For instance, egg rejection, the most important defensive response of hosts against brood parasites, may be affected by variations in predation risk which might divert their focus from the nest (present reproduction) to self-maintenance (future reproduction). In this study we explore if the risk of predation affects the recognition and rejection of parasitic eggs and if this potential effect changes according to the type of predator (adults vs offspring). To do so, we experimentally parasitized nests of common blackbirds (*Turdus merula*) with model eggs simultaneously exposing them to different predation risk situations by simulating the presence of a sparrowhawk (adult predator), a magpie (egg predator) or a turtle dove (control). We found that blackbirds exposed to the adult predation risk showed a partial increase in egg recognition and ejected a significant low number of eggs. This effect increases towards the end of the breeding season. We discuss our results in relation to the life-history theory. Our findings suggest that a predation risk directed to the adult of a parasitized nest, but not to their offspring, can play an important role in the egg-rejection process, consequently affecting the outcome of the evolutionary relationship between brood parasites and their hosts.

Keywords: egg rejection, egg recognition, nest predation, adult predation, brood parasitism

Introduction

Brood parasitism and nest predation are two important biological interactions representing decisive selective forces in nature, capable of driving evolutionarily several traits (Lima 2009, Soler 2014, Ibáñez-Álamo et al. 2015). During the breeding period individuals invest the majority of resources in reproduction and offspring care (Harshman & Zera 2007), and during this period both brood parasitism and predation undertake an even more important pressure, exerting a greater influence on life-history traits by shaping the trade-offs between self-maintenance and the production of viable offspring (Roff 2002, Cox et al. 2010).

Since avian brood parasites impose high cost on their hosts (Rothstein 1990, Davies 2000, Soler 2014) host species have evolved several defensive mechanisms of which the rejection of the parasitic egg is the most important (Davies 2000, Soler 2014). Egg rejection is a complex behavioral process composed by three different phases: first, hosts have to recognize the parasitic eggs; second, they have to evaluate the situation in order to decide whether to eject or not the experimental egg; and third, the action of ejection itself (Hauber & Sherman 2001, Soler et al. 2012). Interspecific variations in host resistance to brood parasitism seem to be genetically fixed (Stokke et al. 2008), while the differences within species suggest that in addition to the genetic component, there is a certain degree of phenotypic plasticity (Hauber et al. 2006, Stokke et al. 2008, Soler et al. 2012) deriving from trade-offs between the costs and the benefits of egg rejection under different ecological conditions. Hosts have to evaluate the possibility of incurring in recognition errors (ejecting one of their own eggs instead of the parasitic one; Davies 2000), and rejection costs (the risk of damaging one or more of their own eggs while trying to eject the parasitic egg; Davies 2000). Likewise, the perception of the risk of being parasitized is another important factor that influences the decision to reject, because the presence of the brood parasite near the nest increases the probability of rejecting the parasitic egg (Moksnes 1993, Lindholm & Thomas 2000). Therefore, although hosts recognize parasitic eggs, they can decide not to reject it depending on different external stimuli and their internal state of motivation (Underwood & Sealy 2006, Moskát et al. 2007, Antonov et al. 2008, 2009, Soler et al. 2012, Ruiz-Raya et al. 2015).

Predation could act as one of such external stimuli affecting the egg-rejection process. Predation can profoundly modulate several components of the breeding biology of birds (Caro 2005), particularly those associated to the nest (Martin 1995, Martin & Briskie 2009, Ibáñez-Álamo et al. 2015), and thus it is expected that predation and brood parasitism may condition each other and interact simultaneously in relation to the nest environment. In fact, some studies have explored part of such relationship investigating how brood parasitism can affect predation of hosts. These studies have been mainly focused on the possibility that the activity of brood parasites could increase the probability of detection of host nests by predators (Hannon et al. 2009). The causes for such increase in nest predation seem to rely on the higher frequency of foster parents' feeding visits (Hoover & Retz 2006) and the loud begging calls produced by parasitic nestlings (Ibáñez-Álamo et al. 2012). In contrast, very few studies have investigated the opposite perspective, that is, whether predation can affect hosts' activities related to brood parasitism. This aspect was indirectly considered by studying the physical resemblance between adult common cuckoos (*Cuculus canorus*; hereafter cuckoo) and Eurasian sparrowhawks (*Accipiter nisus*; hereafter sparrowhawks) (Welbergen & Davies 2011). This study demonstrated that cuckoos mimic hawks in order to frighten adult hosts so that they can safely parasitize their nests. Another possibility, to our knowledge not explored so far, is that predation risk could influence host defenses against brood parasites, particularly egg recognition and rejection. For example, an increased adult predation risk could distract parents from their clutches making more difficult to recognize parasitic eggs. Alternatively, an elevated nest predation risk could increase the rate at which parents check their nests and potentially favor the detection of foreign eggs. Despite these probable effects during the first phase of the egg-rejection process, predation could also affect the second phase by altering the trade-off between costs and benefits associated to the ejection of a parasitic egg. Even after the detection of a foreign egg in the clutch, adults might be deterred to eject it if the risk to be preyed upon (or to a lesser extent that of its clutch) is high.

Table 1: Summary of predictions according to APR and NPR treatments. The up arrow indicates an increase, the down arrow indicates a decrease.

Predictions	**APR** (adult predator treatment)	**NPR** (nest predator treatment)
Recognition	↓ **(1a)**	↑ **(1b)**
Ejection rate	↓ **(2b)**	↑ **(2b)**
Incubation activity		
on-bouts	↑ **(3a)**	↓ **(3b)**
nest attentivness	↑ **(3a)**	↓ **(3b)**

Here, we explore these hypotheses by means of experimentally parasitizing common blackbird (*Turdus merula*; hereafter blackbird) nests with model eggs while simultaneously exposing adults to three different predation risk situations: (1) an exclusive threat for the parents (adult-predation risk, APR), (2) a threat only directed towards offspring (nest-predator risk, NPR), and (3) a control situation with no increased predation risk (CON). We make several predictions based on the above-mentioned hypotheses (Table 1). In particular, we can predict (1a) that blackbirds exposed to an adult predator should recognize foreign eggs less often because their attention will be mainly placed in protecting themselves from predation. On the other hand, (1b) parents exposed to a nest predator should recognize a higher proportion of parasitic eggs because they will increase the checking of the clutch in order to look for potential missing eggs (Doligez & Clobert 2003). We also expect (2a) a reduction in egg-rejection rate under the adult-predator treatment given the lower motivation to eject caused by the important cost of being preyed upon. In contrast, (2b) egg-rejection rate should increase in the nest-predator treatment due to the predicted increase in egg recognition. Finally, adult blackbirds exposed to an increased predation risk should also change their incubation behavior in both treatments based on previous studies with the same species (Ibáñez-Álamo & Soler 2012). We expect (3a) that adult blackbirds facing a risk to themselves (APR experimental group) should have longer on-bouts in order to avoid being detected without sacrificing the incubation of their eggs (Martin & Briskie 2009). In this scenario the incubation attentiveness should increase because females should spend more time hidden in the nest and sat on the clutch. Regarding the NPR experimental group, we hypothesize a reduction in on-bouts (3b), as parents will probably spend more time looking for the nest predator in order to expel it from the area (Ibáñez-Álamo & Soler 2012). A reduction in incubation attentiveness could also be expected because of the increased time spent by females to inspecting the clutch in order to detect missing (preyed upon) eggs.

Materials and Methods

The study was conducted in a population of common blackbirds located in the Valley of Lecrín (Southern Spain, 36º56' N, 3º 33' W) during the breeding season of 2014. This area is dominated by orange groves in which blackbirds built their nests. The density of blackbird nests is 2.9 ha^{-1} and nest predation rate is 48.9 % (Ibáñez-Álamo & Soler 2010). The site presents both typical adult predators such as sparrowhawks or booted eagles (*Hiaraaetus pennatus*) and offspring predators such as mammals (stone marten *Martas foina*, genet *Genetta*, weasel *Mustela nivalis*, domestic cats *Felis catus*, rats *Rattus spp.*), birds (principally corvids) and snakes (ladder snake *Elephe escalaris*, Montpellier snake *Malpolon monspessulanus*). The blackbird is considered a potential host species of the common cuckoo and has been frequently used in egg-recognition experiments (Polačiková & Grim 2010, Samaš et al. 2011, Soler et al. 2015, Ruiz-Raya et al. 2015, 2016) because it exhibits the cognitive capacities to recognize and reject the experimental eggs introduced into its nests, despite the fact that it is not currently parasitized. This species is a medium-sized host that rejects eggs by grasping ejection (Soler et al. 2015). We actively searched for blackbird nests throughout the breeding period; once the nest was located we visited it every two days to determine the exact laying date and clutch size. The experiment was initiated only when the clutch size was completed. In this way we prevented the habituation to the sounds during the laying period (Zanette 2011; see below). We used only nests with a complete clutch of three eggs, which represent the median in our population (Ibañez-Álamo & Soler 2010), to standardize our study at this respect.

Experimental procedure

We manipulated predation risk in experimentally parasitized blackbird nests. To do this, we introduced a parasitic egg into the nest while exposing blackbirds to different predation risk situations using playbacks (see below). Once the clutch was completed, we placed a video camera (Panasonic HDS-SD40) near the nest (approximately 2m) and filmed female behavior during 1.5h ("previous session") in order to check the usual female behavior at the nest, without the experimental egg. This "previous session" was used as the control. The "egg session" started just immediately after the "previous session", when we parasitized blackbird nests by introducing the parasitic egg and the speakers started to play sounds. We finished this session after 2 h of recording, when we removed the camera. We left the speakers playing for the following five days in order to explore the potential effects on ejection behavior (see below). All video recordings were carried out in the morning, between 08:30 to 13:00. To parasitize nests, we used mimetic-model eggs, obtained by collecting natural blackbird eggs from abandoned nests of the same population. We painted the eggs following the procedure used by Soler et al. (2015). Basically, we painted them using two different acrylic paints: blue-green (background) and light brown (spots; Fig 1). We decided to use mimetic eggs because they elicit intermediate ejection responses (Soler et al. 2015), thus, allowing blackbirds to increase or reduce their responses depending on the predation risk situation. Each egg was used only once. We checked the nest contents every day during five days to assess the response of blackbirds against the parasitic egg. This five days interval is frequently used in other egg-rejection experiments to assess host responses towards parasitic eggs (e.g. Davies & Brooke 1988, Grim 2011, Soler 2015). We considered the model egg to have been accepted when it remained warm in the nest for this period. In this case, on the fifth day, we removed it and considered the trial finished. We considered the model egg to be ejected if it disappeared from the nest during this five-day interval but hosts' eggs were still warm in the nest. On the other hand, we noted nest desertion when we found the clutch, including the model egg, cold for two consecutive visits.

In order to manipulate the predation risk we exposed incubating females to the calls of two different diurnal predators during the “egg session”: the sparrowhawk, a typical predator of adult blackbirds (Adult-predator treatment, APR; Newton 1986) that is known to affect blackbird’s antipredator behavior (Møller & Ibáñez-Álamo 2012); and the Eurasian magpie (*Pica pica*; hereafter magpie), which is an important nest predator for blackbirds (Nest-predator treatment, NPR; Collar 2005) that alters different behaviors related to the nest in this species including incubation patterns (Ibáñez-Álamo & Soler 2012, 2016). As a control group (CON) we used calls of turtle dove (*Streptopelia turtur*),

Figure 1: Picture of the experimental treatment. The arrow marks the model egg used.

which is also present in the area but does not present a threat to either adults or offspring. We used calls from 8-10 individuals per species collected from an online database (www.xeno-canto.org). Recordings were converted to digital audio files by using the software Audacity. Each playback consisted of a 4 minutes presence period (20 seconds of calls interspersed with 40 seconds of silence) followed by an 8 minutes absence period (silence). The playbacks were joined together in a single 3 hours long audio file. The order of playbacks within the audio files as well as the order of audio files was changed randomly to avoid habituation of blackbirds to the sounds. The speakers were hidden under a camouflaged cloth, connected to an MP3 player and broadcasted near the nest (8 m) from dawn until dusk for five days at the mean of 70 dB.

Video recording variables

To obtain information on female behavior in relation to the nest we analyzed different variables from the video recordings. First, we quantified two variables associated to incubation behavior: (1) “on-bouts” (the mean time that females spend visiting their nests) and (2) “incubation attentiveness”, measured as the time spent incubating divided by all the time at the nest. Second, we compiled several variables associated to egg recognition: (1) “first-contact touches first visit” (the number of times that females touched the eggs since their first arrival to the nest until they sat to incubate) and (2) “egg-inspection time first visit”, as the time spent by females checking and touching the eggs during the first visit. Moreover, we quantify (3) “incubation touches”, as the number of times that females touched the eggs during the incubation divided by the time spent at the nest, and (4) “egg-inspection time”, considering the sum of the time spent by females checking and touching the eggs divided by the time spent at the nest. Previous studies suggest that egg-touching behavior performed by females at the nest can be considered a clear indication of foreign egg recognition, even if ejection does not occur (Soler et al. 2002, 2012, Underwood & Sealy 2006, Antonov et al. 2008, 2009). The relationship between these variables and egg recognition has been also demonstrated in blackbirds (Ruiz-Raya et al. 2015, 2016, Soler et al. in press).

Statistical analysis

All analyzes were performed using R version 3.1.1 for Windows (R Core Team 2014). Variables of egg recognition and incubation behavior were analyzed by general linear mixed models (*lme* function in the 'nlme' package; Pinheiro et al. 2016) and using the square root transformation when the residuals of our data were not normally distributed and their variances were not homogeneous. Because of the presence of many zeros in the variable “first-contact touches first visit” we used zero inflate mixed models and analyzed the data with the *glmmadbm* function in the 'glmmADBM' package (Skaug et al. 2006). All the models referred to the video recording variables included the interaction between session and treatment in order to determine if the differences between “previous session” and “egg session” were affected by the predation-risk treatment. Moreover, we added laying date as covariate and female identity was considered as random factor because the data of the two sessions belonged to the same nest. In order to verify whether the treatment by itself could have an effect, we performed an additional analysis in which we selected only the “egg session” where the speakers were playing, and discard the “previous session”, since during this control session, the speakers were not playing yet. To analyze this sub selection we included the same predictors used before for video recording variables, but without introducing the random factor, as we used only the data from the “egg session”. Thus, we run general lineal models using the *lm* function in the 'stats' package using the same square root transformations used before and zero inflate models using the *hurdle* function in the 'pscl' package (Zeileis et al. 2008).

Using the *glm* function in the 'stats' package we run a generalized linear model with binomial error and logit link function to assess the effect of the treatment on ejection rate. The model included the predation-risk treatment (APR, NPR or CON) as factor and laying date as covariate.

The differences among the levels of significant factors were compared by Tukey post hoc tests, using the 'multcomp' package (Bretz el al. 2015) for general linear mixed models while we used the *interactionMeans* function in the 'phia' package for the differences found in generalized linear model. This function creates a data frame with the adjusted means of a fitted model or the slopes associated to its covariates, plus the standard error of those values, for all the interactions of given factors. These interactions are plotted by pairs of factors (De Rosario-Martinez 2015). Values provided in the manuscript are mean ± SE.

Results

We managed to carry out the experiment in 16, 16, and 13 nests for the turtel dove, magpies and sparrowhawks group respectively.

Egg recognition

Predation risk did not affect the recognition of the foreign egg in the first visit. In fact, all females seemed to recognize the foreign egg independently of the predation risk-treatment when first arriving to the nest. In this regard, egg-inspection time in the first visit was higher during the “egg session” compared to the “previous session” (Fig 2a; $F_{1, 43} = 13.36$, $p < 0.001$; Table 2), independently of the experimental treatment (Fig 2a; treatment*session $F_{2, 43} = 0.36$, $p = 0.70$; Table 2). We found no effect of laying date ($F_{1, 42} = 0.70$, $p = 0.41$; Table 2) or predation-risk treatment by itself on this variable ($F_{2, 42} = 0.77$, $p = 0.41$; Table 2). These results are in agreement with “first-contact touches first visit”, because the number of touches increased in the “egg session” (Fig 2b; $\chi2 = 5.69$, $p = 0.02$; Table 2) independently of the interaction with the predation-risk treatment (Fig 2b; treatment*session $\chi^2 = 1.47$, $p = 0.48$; Table 2). Laying date was not significant ($\chi^2 = 0.15$, $p = 0.70$; Table 2) and neither predation risk-treatment by itself ($\chi^2 = 6.92$, $p = 0.14$; Table 2). However, when we consider the 2 hours that females spent at the nest and not just the first visit, we find a partial effect of predator treatment regarding the variable “incubation touches”. This variable did not differ between sessions by itself ($F_{1, 43} = 1.95$, $p = 0.17$;

Table 2: General linear mixed models and zero inflate mixed models. All the models referred to the video recording variables and included session (before and after the introduction of the egg), treatment (APR, NPR, CON), the interaction between session and treatment (to determine if the differences between "previous session" and "egg session" were affected by the predation risk treatment) and laying date

Incubation behavior	**F or $\chi 2$**	**P-value**
Duration of on-bouts		
session	11.94	**0.001**
treatment	0.84	0.43
session*treatment	2.97	0.06
laying date	0.20	0.66
Nest attentiveness		
session	14.01	**<0.001**
treatment	0.85	0.44
session*treatment	2.50	0.09
laying date	0.07	0.79
Egg recognition		
First-contact touches first visit		
session	5.69	**0.02**
treatment	6.92	0.14
session*treatment	1.47	0.48
laying date	0.15	0.70
Egg-inspection time first visit		
session	13.36	< **0.001**
treatment	0.77	0.41
session*treatment	0.36	0.70
laying date	0.70	0.41
Incubation touches		
session	1.95	0.17
treatment	0.14	0.87
session*treatment	3.28	**0.04**
laying date	4.17	0.05
Egg-inspection time		
session	15.62	< **0.001**
treatment	0.42	0.66
session*treatment	1.32	0.27
laying date	0.73	0.40

Table 2), but changed significantly in relation to the interaction with the predation-risk treatment (Fig 2c; treatment*session $F_{2, 43} = 3.28$, $p = 0.04$; Table 2). Only females exposed to the APR treatment touched significantly more their eggs in the "egg session" than in the "previous session". Females exposed to the NPR treatment showed similar patterns between sessions (Fig 2c). We found a marginal effect of laying date ($F_{1, 42} = 4.17$, $p = 0.05$; Table 2), because the number of incubation touches decreased along the breeding season, while predation-risk treatment alone did not show any influence ($F_{2, 42} = 0.14$, $p = 0.87$; Table 2). On the contrary, "egg-inspection time" is higher during the "egg session"

in comparison with the "previous session" (Fig 2d; $F_{1,43} = 15.62$, $p < 0.001$; Table 2) but in this case, this relationship was not associated with the risk of predation (Fig 2d; treatment*session $F_{2,43} = 1.32$, $p = 0.27$; Table 2). Laying date ($F_{1,42} = 0.73$, $p = 0.40$; Table 2) and the predation-risk treatment did not affect this variable either ($F_{2,42} = 0.42$, $p = 0.66$; Table 2).

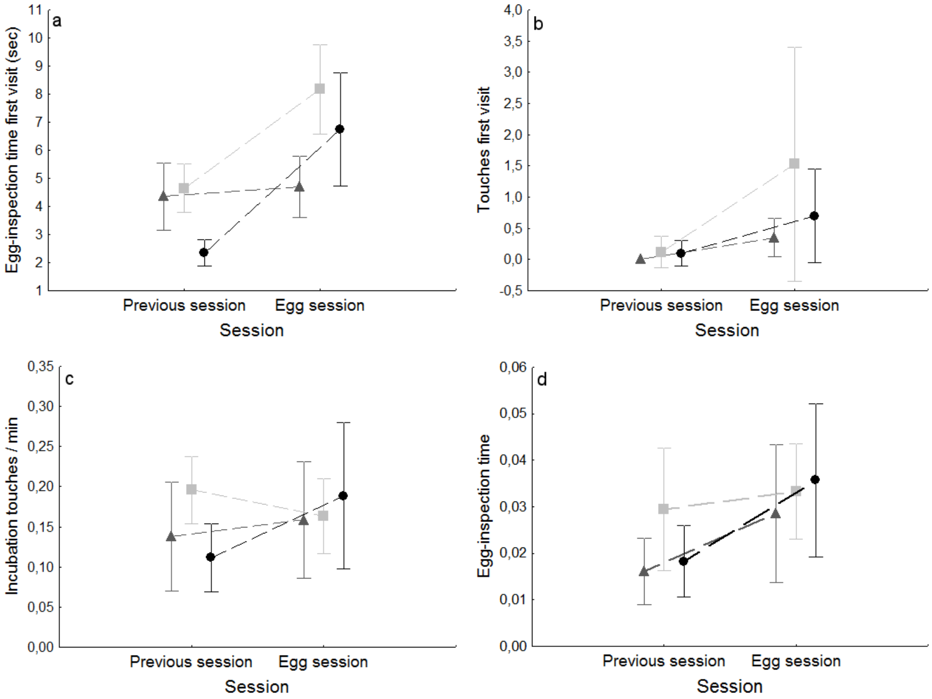

Figure 2: Recognition of mimetic eggs according to the predation risk treatment. Symbols represent the mean and associated bars ± SE. APR is represented by black circles, NPR by dark grey triangles and CON dove by light grey squares. Differences between the "Previous session" (control) and "Egg session" (after introducing the parasitic egg) regarding: a) egg-inspection time for the first visit, b) first-contact touches first visit, c) egg-inspection time for all visit combined and d) incubation touches. For more detailed explanation of each variable see Method and Material section.

Egg ejection

Blackbird females exposed to the APR treatment significantly ejected lower eggs compared with females of other groups, whereas females under the NPR treatment did not modify their ejection rate (Fig 3). However, this effect was associated to date (treatment*laying date; $F_{2,39} = 6.07$, $p = 0.02$). The females that ejected lower eggs were those that bred later in the season (post hoc test, $p = 0.03$), whereas the ejection rate remained constant during all the breeding period for the females exposed to the NPR treatment (post hoc test, $p = 0.14$).

Incubation behavior

The experimental increase of predation risk changed marginally the patterns of blackbirds' incubation behavior. "On-bouts" increased in the presence of the introduced egg ($F_{1, 43} = 11.94$, $p = 0.001$; Table 2); but this relationship seemed to be affected by the type of predation risk as we found a marginally significant effect for the interaction session*treatment ($F_{2, 43} = 2.97$, $p = 0.06$; Table 2). In particular, females facing an elevated APR almost doubled their time at the nest during the "egg session" (40 min 42 sec) compared to the "previous session" (20 min 37 sec; Tukey test, $p = 0.001$), while females of the NPR or the CON situations did not show significant changes (Tukey test, $p = 0.46$ and $p = 0.99$ respectively). The predation-risk treatment alone did not produce significant differences ($F_{2, 42} = 0.84$, $p = 0.43$; Table 2). We did not found a relationship between this variable and laying date ($F_{1, 42} = 0.20$, $p = 0.66$; Table 2). The "incubation attentiveness" was significantly lower in the "egg session" compared to the "previous session" ($F_{1, 43} = 14.01$, $p < 0.001$; Table 2) indicating an effect of the parasitic egg which seemed not to be mediated by the predation-risk treatment (treatment*session $F_{2, 43} = 2.50$, $p = 0.09$; Table 2). On the contrary, neither treatment by itself nor laying date did affect this variable ($F_{2, 42} = 0.85$, $p = 0.44$ and $F_{1, 42} = 0.07$, $p = 0.79$ respectively; Table 2).

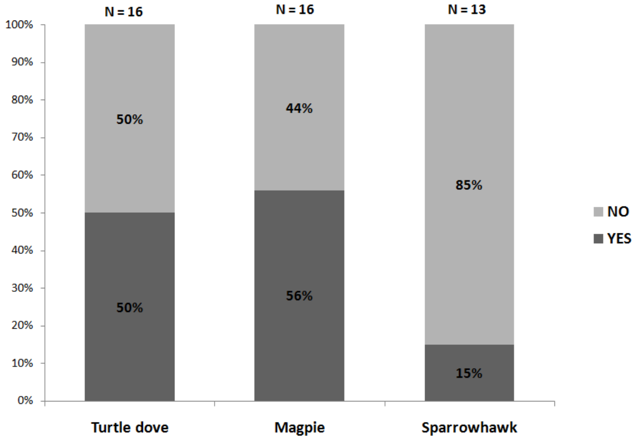

Figure 3: Ejection rate according to the predation risk treatment. Sample size (N) is showed on the top of the hsitograms.

Discussion

Our findings show that predation risk affects host defenses against brood parasites and highlight the existence of a relatively important interplay between these two relevant selective pressures. In particular, our results support that an elevated adult predation risk induce females to eject low eggs, by affecting therefore the egg-rejection process, mainly the decision of ejecting (second phase) and partially the egg recognition (first phase). In contrast, nest predation risk did not affect the egg-rejection process indicating that not all threats posed by predators affect similarly antiparasitic host defenses.

Egg recognition

According to our results, the first phase of the egg-rejection process (egg recognition) was affected only partially by APR, but not by NPR, contrasting with our first predictions (1a, 1b). During the first visit, both the number of touches that females gave to the eggs and the "egg-inspection time" increased significantly in the "egg session" compared to the "previous session", when the foreign egg had not been introduced yet. This implies that the model egg was recognized immediately after females returned to their nests. These results are in agreement with those found in previous studies on the same species (Ruiz-Raya et al. 2015, 2016). Nevertheless, this effect was not affected by the sound presented during the "egg session" and was thus independent of the environmental predation risk. Discrimination of parasitic eggs was also evidenced by the "egg-inspection time" over the two hours of video recordings; females spent more time checking the eggs in the nest during the "egg session" compared to the "previous session" and this is in accordance to previous egg-recognition studies realized with other species (Antonov et al. 2008, 2009). And in this case, predation risk does not seem to be playing an important role again. Interesting and contrary to what we predicted for APR (1a), predation risk did affect the "incubation touches", another variable related to egg recognition. In particular, female blackbirds exposed to the APR touched more often their eggs in the "egg session" than in the "previous session" (Fig 2c) suggesting that APR might alter at least some aspects of the egg-recognition behavior. This is an interesting result that matches with what we have found in relation to the effect of the APR on incubation behavior. Females exposed to sparrowhawk calls were those that increased twice the duration of "on-bouts" and remained hidden in the nest consequently avoiding their detection by potential predators. But at the same time, female blackbirds that remained longer in the nest also have more time available for looking, rolling and inspecting their own nest. Thus, an adult predator will increase the probabilities of detecting a parasitic egg likely as a by-product of an antipredator defense that makes females to spend more time in the nest, even though ultimately these blackbirds will accept parasitic eggs more easily (Fig 3).

Egg ejection

Females exposed to the APR situation showed a lower ejection rate, fitting with prediction 2a, whereas NPR did not show any effect, consequently, not matching with prediction 2b (Fig 3). Interestingly, this effect depended on the breeding season, since the females that ejected lower eggs as the breeding season advanced only for the APR situation. This suggests that the effect on egg ejection associated with the potential presence of the sparrowhawk was more intense during late spring. The effect of the APR manipulation in ejection rate seems even more relevant as any of the previous egg-rejection experiments conducted in the same blackbird population (which did not manipulated predation risk) found changes in egg-rejection rates over the breeding season (Soler et al. 2015, 2016). A potential explanation for this temporal effect might be the existence of other environmental cues towards the end of the breeding season (i.e. encounters with real sparrowhawks) that reinforced our experimental manipulation. It is possible that these uncontrolled cues increased during the sparrowhawk's breeding period (i.e. due to the presence of sparrowhawk fledglings), which also matches with the end of the blackbird's breeding period (Newton 1976). In fact, the reproductive period of several hawk species seems to have evolved to coincide with the maximum abundance of passerine fledglings allowing to cover the higher food demands of hawks' families while reproducing (Newton 1986). In the circumstance in which an event of brood parasitism occurs and the egg is recognized, the pressure derived from the potential presence of a predator would induce the parasitized females to evaluate between two options: deciding to eject the egg by assuming all the risk that this activity involves when a potential threat to themselves is present, or keeping the egg with all the costs associated to rear a possible cuckoo offspring. In the first case, females may risk their life because the predator could detect them when they fly away from the nest with the egg. Moreover, their mobility and flight capacity might be reduced due to the egg (Ercit et al. 2014), because it represents an extra weight very displaced from the gravity center of the bird. In the second case, if the egg was really parasitic, females would lose the entire current brood because the cuckoo chick would evict all the content of the nest. Our results clearly indicate that the APR affected the decision of ejecting the foreign egg suggesting that this is not a stimulus strong enough to induce female blackbirds to assume the very high (deadly) potential cost of ejecting it in the presence of an adult predator; females are more careful to the sparrowhawk in order to protect themselves from the threat of being captured and preyed upon. In this way they save the possibility to invest in future reproductive events, given that the blackbird is a multiple-brooded species and in the Iberian Peninsula it usually raises three broods per season (Aparicio 2008). Moreover some authors have calculated that its maximum longevity is on average 10-13 years, being the highest in the Order Passeriformes, after the Family Corvidae (Wasser & Sherman 2010). Therefore, blackbirds generally have many opportunities of breeding during its lifetime and the potential loss of a single reproductive event should be less costly than losing all future reproductions. This could also explain why we found that female which ejected lower were those breeding at the end of the season, exactly when the activities of sparrowhawks are probably more intense and consequently when the possibilities to be preyed upon should be higher too.

The effect of predation risk on hosts' defenses against brood parasitism offers a new insight into the interplay between these two important selective forces. Given that our results indicate that the presence of adult predators discorages egg ejection, we could predict that, when parasites select their potential hosts, they could evaluate to choose those species or populations that suffer high levels of adult predation risk. In this way, the parasitic offspring should have more opportunities to avoid the rejection by their foster parents allowing them to survive until they leave the nest. This hypothesis however could only be accepted if the benefits obtained due to the reduction in egg rejection overcompensate the costs associated to the higher probability of predation of parasitic fledglings. The low number (more than twice) of ejection rate found under APR would suggest that this possibility is not unlikely. However, host adults in such areas might also be more depredated and parasites should also compensate this fact, for example, by selecting high quality foster parents (Soler et al. 1995) with a lower probability of being preyed upon (Bortolotti et al. 2002, Møller et al. 2009).

Our results also offer an additional benefit associated to the coloration of the cuckoos mimicking sparrowhawks. The most common explanation for this adaptation is to scare the hosts helping the parasite to lay their egg undisturbed (Welbergen & Davies 2011). However, our findings imply that this adaptation may also favor the cuckoo by reducing the probability of ejection of their eggs. Despite this is an intriguing possibility, more studies will be needed to test if the patterns observed here using acoustic cues and long periods of increased APR could also be applied to visual cues (cuckoos cannot mimic sparrowhawk sounds) and punctual encounters.

Incubation behavior

The effect of the increase in APR seemed to be partially relevant for female's behavior during incubation, by increasing the duration of "on-bouts" (affecting indirectly egg recognition; see above), while the "incubation attentiveness" is not affected. This fits in part with our prediction 3a. On the contrary, prediction 3b was not fitted as the magpie did not affect the incubation behavior. The results obtained for the NPR contrast with those previously published for another blackbird population from Spain, in which females exposed to magpie calls reduced their activity at the nest during both the incubation (Ibáñez-Álamo & Soler 2012) and nestling stages (Ibáñez-Álamo & Soler 2016). The different results obtained by our study in comparison with those of Ibañez-Álamo & Soler (2012) might be explained by the important differences between these two blackbird populations. Blackbirds in our study population invest less in a single reproductive event than those in the population used by Ibáñez-Álamo & Soler (2012), probably due to the higher nest predation pressure in the former, which was twice compared with that of our study area (Ibañez-Álamo & Soler 2010). These differences would suggest that the nest predation pressure in our study area might be not intense enough to provoke consistent changes in incubation behavior. A number of studies with different species have shown that prey exposed to spatially or temporally changes in predation pressure present high degree of phenotypic plasticity, modifying the antipredatory defenses and their intensity (Tollrian & Harven 1998, Åbjörnsson et al. 2004, Takatsu & Kishida 2013, Shaffery & Relyea 2016). In fact, anti-predator behavior may be lost or modified when predation intensity is relaxed (Vervust et al. 2007). Then, blackbird females in our population could respond less intensely to the experimental increase of nest predation risk because of the general lower predation pressure in the study area.

To sum up, predation risk seems to modulate host responses to brood parasitism. In particular, while nest predation risk does not seem to be important at this respect, adult predation risk affects the egg-rejection process mainly by inducing lower ejection rates of parasitic eggs. Furthermore, this effect is affected by time and increased towards the end of the breeding season, which is probably associated to a higher activity of sparrowhawk too. Adult predation risk only modified some aspects of the first phase of the egg-rejection process, namely “incubation touches” (i.e. egg recognition), probably influenced by a self-protection behavior (staying for longer periods in the nest to avoid being depredated). Our findings offer a new perspective on the interplay between predation risk and brood parasitism demonstrating that not all threats posed by predators are equal in relation to host defenses. Our results open a new research line in the study of brood parasitism and particularly advance our understanding of the regulation of the egg-rejection process.

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PART III

NEST PREDATION RISK

CHAPTER 4

SHORT-TERM INCREASE IN PREDATION RISK MODIFIES NESTLLINGS' IMMUNE SYSTEM

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Abstract

1. Predation risk is thought to modify prey's physiology mainly through stress response. However, little is known about the potential effects of predation risk on the immunity of animals, particularly in young individuals, despite its possible importance to successfully overcome wounding and pathogen aggression following a predator attack.

2. We investigated the effect of three progressive levels of nest predation risk on several components of the immune system (11 immunological parameters) in common blackbird nestlings and analyse the potentially complex relationships between these two factors.

3. We altered nest predation risk by manipulating the acoustic cues perceived by blackbird nestlings during 1h. We used sounds of predators, parental alarm calls and conspecific distress calls to reproduce a moderate, high and extreme level of risk respsctively.

4. Nest predation risk induced an increase in ovotransferrin, immunoglobulins levels and the number of lymphocytes and eosinophils, suggesting a general activation of the immune response. Thus, the perception of a potential predator per se could stimulate the immune function of blackbird nestlings and prepare the organism to cope with the possible inflammation or infection provoked by a predator attack. Interestingly, only high and extreme levels of risk caused the immunological changes, suggesting that nestlings modulate their immune responses according to the perceived level of threat. Immune responses due to nest predation risk are partially mediated by the presence of parasites (i.e. *Leucocytozoon*) and the current health status of the individual as only nestlings not parasitized or in good body condition were able to maintain a high immune response.

5. This study highlights a previously unknown link between predation risk and the immune system, emphasizing the complex relationship among several selective pressures (predation, parasitism and environment) on developing organisms and accentuating the importance of studying predation from a physiological point of view.

Key-words: acquired immunity, alarm calls, H/L ratio, innate immunity, nest predation risk, parasites

Introduction

Predation is an antagonistic biological interaction that entails the killing of a prey by a predator, and represents therefore an important selective pressure in many natural systems, including birds (Caro 2005). Nestlings are generally more exposed to predation since nest predation represents the first cause of mortality in many birds, particularly in altricial species whose offspring are linked to a specific place, the nest, for the development period (Martin & Briskie 2009). Thus, nest predation pressure is able to model several avian life-history traits in nestlings (Martin 1995). Although many studies have analysed the role of nest predation on the anti-predatory behavioural responses, our knowledge on how nest predation may affect the physiological condition of prey is still poor (Ibáñez-Álamo *et al.* 2015). Predation risk is thought to modify prey's physiology mainly through the stimulation of a stress response (Sapolsky *et al.* 2000) and, therefore, is theoretically able to provoke important changes also in the immune system, another relevant physiological component. The immune system is a complex system of self-maintenance, usually divided in two components: (i) innate immunity, which represents the non-specific first line of defence, offering immediate protection; and (ii) acquired immunity, the pathogen-specific response, which allows for immunological memory (Roitt *et al.* 2001). The immune system promotes survival by limiting the negative impacts derived from pathogens, diseases or infections (Horrocks, Matson & Tieleman 2011a) and consequently, is strictly associated with the possible outcomes of an encounter with a predator, which can produce important injuries and wounds. This system, however, also incurs costs in term of production, maintenance and activation (Klasing 2004). Because of this, stress (e.g. predator threat) is generally considered immunosuppressive, as organisms reallocate resources towards activities that are vital for their immediate survival (Sapolsky *et al.* 2000). But some evidences showed that the effect of stress on immunity varies according to the characteristics of the stimulus (e.g. duration or intensity) as well as to the sensitivity of each immune component (Dhabhar 2009, Martin 2009), so a particular stressor may result in the reduction of some immune components, but the stimulation of others.

Despite this theoretical evidence and the critical fitness consequences resulting from nest predation, only a few studies have investigated the link between nest predation risk and immunity in nestlings (Ibáñez-Álamo *et al.* 2015). Moreover, knowledge about the short-term effects of nest predation risk on nestlings' immune system are practically absent. This is surprising since this circumstance, representing a potential predator attack, would reflect a commonly occurring prey-predator interactions in nature. To our knowledge, only three studies examine the effects of predation risk on immunity in nestlings.Tilgar *et al.* (2010) found an increase of heterophil to lymphocyte (H/L) ratio in nestlings of pied flycatcher *Ficedula hypoleuca* that were chronically exposed to the playback of conspecific distress calls, while no effect was found in response to an acute stress. In another study, Goedert *et al.* (2014) found changes in cell-mediated immune responses of campo flicker *Colaptes campestris* nestlings when they were captured by a potential predator (i.e. researcher). Finally, in a study conducted on ring-billed gull *Larus delawarensis*, a semi-precocial bird, an acute stress exposition reduced only the innate immunity (Chin *et al.* 2013). In light of these results, it seems that predation risk may play an important role in modulating nestlings' immunity, even though the direction of these immunological changes is not consistent.

In relation to this, it is also important to understand which cues associated to predators can produce these immune modifications. Nestlings can gather information about the presence of a potential predator both directly (i.e. sounds emitted by predators; Magrath, Pitcher & Dalziell 2007) and indirectly, through the signals given by other individuals that have already detected the predator, like parents (parent's alarm calls; Magrath, Pitcher & Dalziell 2007), or siblings and conspecific nestlings (distress calls; Tilgar *et al.* 2010). The ability of nestlings to recognize the potential predator by using different cues is decisive to evaluate the level of predation risk as they usually represent different steps in the predation sequence (i.e. distress calls are associated to extremely risky situations and are usually not emitted until the predator gets to the nest; (Hogstedt 1983, Goedert *et al.* 2014). Furthermore, it has been proposed that variability in the source of stress, as its intensity, novelty and duration, can differently affect immunity (Martin 2009), also supporting the relevance of studying immunological responses to different predation risk cues.

In this study we investigated the effects of acute nest predation risk on the immune responses of nestlings. We exposed nestlings of common blackbird *Turdus merula*; hereafter blackbird) to three progressive levels of nest predation risk (extreme –distress calls–, high –alarm calls– and moderate –predator calls –) for 1 hour and analysed their ability to adjust immune function according to the intensity of the risk. We measured eleven immunological parameters of both innate and acquired immunity following the suggestion to study and capture the complexity of the immune system using multiple measurements (Matson *et al.* 2006). Based on the general assumption that predator-induced stress produces an immunological suppression (Sapolsky *et al.* 2000), we predict that (1a) nestlings under an increased nest predation risk situation will reduce their immunity. We also predict that (1b) this reduction should be inversely related to the intensity of predation risk (see above), thus, the most marked decrease should occur in those nestlings exposed to the most extreme predation risk (distress calls). Alternatively, since recent studies on mammals have shown a recovery function of an increase in immune parameters following an acute stress event (Dhabhar 2002, Martin 2009), we can also predict that (2a) acute nest predation risk could stimulate the immune response of blackbird nestlings by increasing their immunological levels in order to promote their survival, by limiting the negative consequences of a predator attack. In this case we will expect (2b) a positive association between the immune function and intensity of predation risk, observing the most intense increase in nestlings under the most extreme predation risk treatment. Finally, given that the particular conditions in which the altricial nestlings develop (i.e. parasite loads or infection status; Navarro *et al.* 2004, De Coster *et al.* 2010) could generate additional sources of variation in immune response to nest predation risk (Møller, Allander & Dufva 1990), we predict that parasitism might affect the potential immunological responses of blackbird nestlings. Specifically, we expect that (3) those individuals infected by endoparasites will respond weakly to the risk of predation as the cost of immunosuppression in these individuals will be high (Forrester & Greiner 2009).

Materials and Methods

The experiment was conducted on a blackbird population during the breeding period of 2015 in the Valley of Lecrín 36° 56' N, 3° 33' W, a rural area situated at 580 m a.s.l. in the south east of Spain where the nest predation rate is intermediate (48.9%; Ibáñez-Álamo & Soler 2010). Nests were actively searched from the start of the breeding season. Once the nest was located we visited it every 2 days in order to know the exact hatching date. All the nests were checked by using a pole with a mirror placed at one extremity to minimize the disturbance produced by nest inspection.

Experimental design and data collection

We altered nest predation risk by manipulating the acoustic cues perceived by blackbird nestlings. (i) In the extreme predation risk group, nestlings were exposed to playbacks of blackbird nestling distress calls ("DC group") in order to simulate the direct attack of a predator to the nest. Distress calls are produced by nestlings in extremely threatening situations (e.g. when caught by a predator; Marler & Slabbekoorn 2004) and can induce anti-predatory behaviours and physiological changes in nestlings (e.g. Tilgar *et al.* 2010). (ii) The second group of nests experienced a high risk of predation but lower than the previous one. In this case, we simulated a direct threat to the nest by using playbacks of adult alarm calls ("AC group"); these calls usually warn the partner and the nestlings about the presence of a potential predator close to the nest and can trigger specific anti-predatory responses in nestlings of various species (e.g. Magrath, Pitcher & Dalziell 2007, Suzuki 2011). Finally, (iii) we used playbacks of calls from various blackbird nest predators ("PC group") to simulate a moderate threat to the nest. This situation simulated the presence of a potential nest predator in the surrounding of the nest. This group corresponded to the moderate predation risk group and is based on the ability of nestlings to independently assess the current risk of nest predation by recognizing direct cues of a predator's presence (e.g. Magrath, Pitcher & Dalziell 2007). We also created a fourth group, corresponding to the null level of predation risk (control group, CON) that involved exposure to the songs of other passerine species living in the study area (e.g. chaffinch), which do not represent any threat for blackbird nestlings.

Each playback consisted of 3 minutes of call activity (20 seconds of calls interspersed with 40 seconds of silence) followed by 5 minutes of silence. The playbacks were joined together in a single 1 h and 15 min long audio file, in which 10-20 calls of distinct individuals belonging to several species were reproduced. The order of playbacks within the audio file was changed randomly to avoid habituation of blackbirds to the sounds. The first 15 minutes of each audio file consisted of silence in order to calm the nestlings in the case they could perceive our presence while placing the speakers. We created 8 different audio files for each treatment group that were used in a sequential order to avoid the problem of pseudoreplication. Further information about the creation of the playbacks can be found in the supporting information (SI 1).

The speakers were hidden under a camouflaged cloth, connected to an MP3 player and broadcasted near the nest (6 m) at the mean of 70 dB. A similar methodology has been successfully used before in our model system to manipulate acoustic cues (Ibáñez-Álamo, Chastel & Soler 2011, Ibáñez-Álamo & Soler 2016). Each nest found was assigned to one of the treatments by following the order CON, PC, AC, DC. Doing this, we balanced evenly the experimental treatments throughout the season. We performed the experiment when nestlings were 10-11 days old, just before the nestlings leave the nest (Ibáñez-Álamo & Soler 2010). Nestlings of this age easily perceive the acoustic cues and therefore are able to "evaluate" the different degrees of risk according to each playback (Magrath, Pitcher & Dalziell 2007) in order to decide which anti-predator strategy to adopt. In fact, blackbird nestlings at this age are able to escape from an imminent predator attack (including a researcher checking the nest) by jumping and leaving the nest in order to hide in the nearby vegetation (pers. obs.).

Once we verified the presence of the nestlings in the nests we initiated the playback of each treatment. After broadcasting the playbacks, we stopped the speakers and immediately collected a blood sample (250-300 µl) from the brachial vein of the nestlings. All blood samples were collected between 10:00 and 14:00 h to standardize for the time of the day and stored at 4°C (maximum 5 hours after collection) until centrifugation (13000 rpm for 10 min). Plasma was stored at –25°C. A drop of blood was smeared on a marked glass slide and dried in open air. Common biometrical measures (i.e. body mass, tarsus, and wings) were also taken for each nestling as well as presence/absence of mites (subclass *Acari*).

Immunological assays

Innate humoral immunity

(i) Haemolysis/Haemagglutination titres (HL-HA)

Both HA and HL quantify levels of innate immunity. In particular, HA is indicative of the levels of circulating natural antibodies. These proteins facilitate the initial recognition of pathogens and promote the activation of adaptive immune responses (Carroll & Prodeus 1998). HL titers estimate the action of complement and other lytic enzymes (Carroll & Prodeus 1998). We performed HA and HL assays follwing Matson *et al.* (2005) with modifications described by Mauck *et al.* (2005) Scans of individual samples were randomized and scored by a single person (GR).

(ii) Haptoglobin (Hp)

Hp is an acute phase protein which is able to bind free hemoglobin released from erythrocytes in order to inhibit its oxidative activity. Under normal conditions, Hp is present in blood at low concentrations, while it can rapidly increase in response to acute infection, inflammation or trauma (Matson *et al.* 2012). We measured Hp concentration in plasma with a commercial kit (TP801; Tridelta Development Ltd., Maynooth, Ireland), which colorimetrically quantified the hemoglobin binding capacity of the plasma.

(iii) Ovotransferrin (OVT)

OVT acts as an acute phase protein by binding free iron, which is an essential nutrient for bacterial growth. High levels of OVT are usually considered as indicator of inflammation, infection, poor nutrition or disease(Horrocks, Matson & Tieleman. 2011a). We measured the OVT concentration following(Horrocks, Tieleman & Matson 2011b).

(iv) Nitric oxide (NOx)

Blood levels of NOx increase in response to the presence of inflammatory cytokines, microorganisms or endotoxins (Sild & Hõrak 2009). NOx is considered a measure of innate immunity as many cell types are capable to express it, especially macrophages, which release NOx by exocytose in order to destroy pathogens (Crippen *et al.* 2003). Quantification of plasmatic NOx was realized following Sild & Hõrak (2009).

Acquired humoral immunity

(v) Immunoglobulins (IgY)

Immunoglobulins are glycoproteins with antibody activity, produced by B lymphocytes. The antibodies neutralize pathogens, induce the activation of the complement system and promote cell migration to the sites of infection (Härtle *et al.* 2014). Total immunoglobulin concentrations (IgY) were measured from plasma samples using direct ELISA (following Martinez *et al.* 2003)). We adapted this method to common blackbird nestlings by calculating the optimal plasma dilution (1:9000). In this assay, when an IgY molecule binds to the detection antibody, a yellow-coloured compound is produced. Thus, the sample content of total immunoglobulins measured is directly proportional to the amount of coloured product measured with a spectrophotometer. Data obtained are expressed in optical density units. For each sample, mean absorbance value was calculated from three replicates and "corrected" by subtracting the mean value of "blank" absorbance to account for non-specific binding related to background activity.

Cellular component immunity

(vi) Leukocyte profile

In order to quantify white blood cells, we fixed blood smears in absolute methanol and stained them with Giemsa (GS500-500ml SIGMA-ALDRICH) diluted 1:10 in PBS (pH 7.2) for 45 min. Subsequently, smears were scanned with an optical microscope (1000x magnifications with oil immersion). We counted a minimum of 100 leukocytes on each slide. Each cell was classified as heterophil, lymphocyte, eosinophil, basophil or monocyte, following Campbell (2007). Leukocyte counts allowed us to calculate H/L ratio. Smears were also examined to evaluate the presence of hematozoan parasites (genera *Leucocytozoon*). To estimate the presence/absence of *Leucocytozoon* infection we inspected infected blood cells, which develop into gametocytes to complete its reproductive cycle (Forrester & Greiner 2009). Blood smears were examined by a single investigator (EC).

Statistical analysis

As a general procedure, the effect of predation risk treatment on the different immunological components was firstly tested analysing each immunological parameter separately. Subsequently, we investigated how all immunological parameters covaried among the four treatment groups, using discriminant analyses.

General linear mixed models (GLMM, *lme* function in the 'nlme' package; Pinheiro *et al.* 2016) were used to analyse each immunological parameter: HA, Hp, OVT, NOx, IgY, the number of leukocytes, lymphocytes, heterophils, eosinophils and finally, the H/L ratio. It was not possible to fit a model for HL and for the numbers of basophils and monocytes because data on these immunological parameters were insufficient. In each model we considered: treatment, the effect of breeding season (expressed by hatching date), the effect of body condition (calculated by the residuals of the regression between body mass and tarsus length; (Jakob, Marshall & Uetz 1996), and the presence of mites and *Leucocytozoon*. Moreover, we also considered the interactions between treatment and each of the other predictors in order to evaluate the possible mediated effect of these factors on nest predation risk and we included nest as a random factor in order to control for the non-independence of nestlings from the same nest. Before fitting the models, we checked for possible collinearity among predictors. We found a positive correlation between the presence of mites in the nest and hatching date (r Spearman = 0.6, $p < 0.001$) indicating that the proportion of nests infected by mites significantly increased throughout the season. Therefore, we decided to drop the presence of mites from our models (Quinn & Keough 2002) and kept the effect of breeding season (hatching date). In this way, we indirectly included the effect of mites and moreover we were able to indirectly control for the effect of other environmental factors not directly measured (such as temperature or food availability), but that may influence the immune response (Christe *et al.* 2001, Serra *et al.* 2012). During model simplification, the non-significant predictors were excluded, dropping firstly the non-significant interactions (Engqvist 2005). We did not remove treatment as it reflects the hypotheses to be tested. We used the Akaike Information Criterion score (AIC) to evaluate the resulting models. After checking the homogeneity of variance and the normal distribution of the residuals of our models (Zuur, Ieno & Elphick 2010), we used logarithmic transformations for those variables that violated these assumptions (i.e. NOx, the number of leukocytes, lymphocytes, heterophils, eosinophils and H/L ratio). When appropriate, we explored significant treatment effects using Fisher's post hoc tests (LSD).

Discriminant analysis was performed using function *lda* in the 'MASS' package (Venables & Ripley 2002). This analysis showed which immunological parameters contribute to classify nestlings into the four treatment groups. This method works by investigating the relationships among the groups' covariance to find a fixed number of linear functions that are used to discriminate between groups. Significance of a linear function indicates that the parameters with the high loadings on this function differ among the groups (Crawley 2007).

The analyses were performed using R version 3.1.1 for Windows (R Core Team 1999) and STATISTICA 10 (StatSoft Inc., Tulsa, Oklahoma, USA). Values provided in the manuscript are mean ± SE.

Results

Humoral innate immunity

We did not detect lysis activity (HL) in blackbird nestlings. HA was not affected by the treatment (Table 1), but we found a positive relationship between HA levels and hatching date (β = 0.08, Table 1). Moreover, those nestlings that carried *Leucocytozoon* had higher HA levels. Hp concentration did not vary with treatment or with any other predictor (Table 1). Our experimental increase of nest predation risk significantly affected OVT concentrations (Table 1). In particular the levels of OVT were significantly higher in the DC group compared to the AC group (Fig. 1a). Hatching date had a significant positive effect since nestlings that hatched later presented higher OVT concentration (β = 0.08; Table 1). NOx did not vary among groups or due to the other predictors (Table 1).

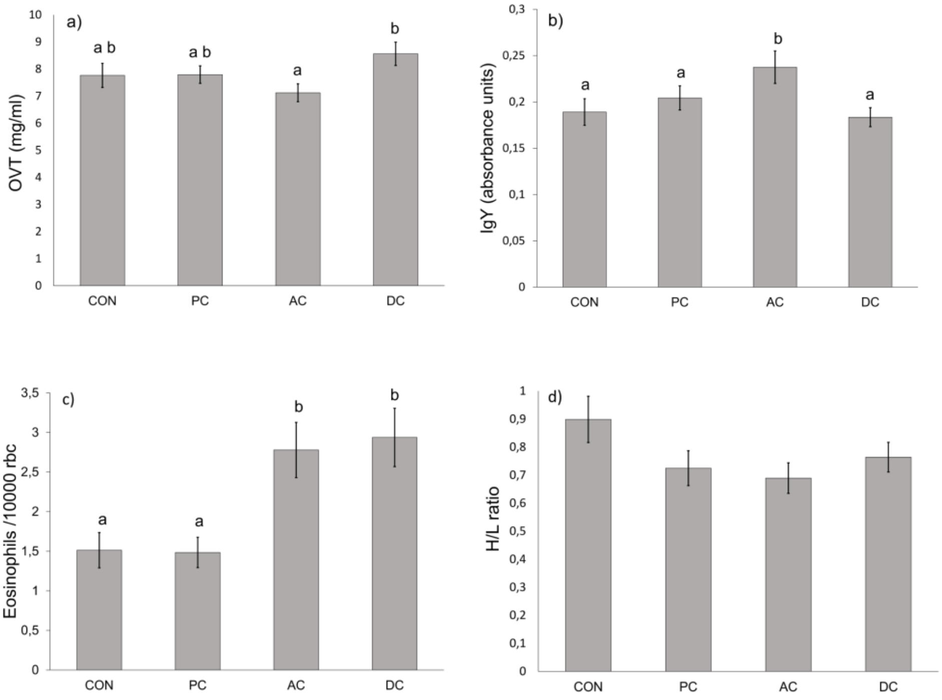

Figure 1: Effects of short-term increase of nest predation risk on the immunological parameters: (a) ovotransferrin, OVT, (b) immunoglobulin, IgY, (c) number of eosinophils, (d) H/L ratio. Means and standard errors are shown. Treatment groups that have distinct letter indicate significant differences for the post hoc test at the $p < 0.05$ level. Treatment had a significant effect on OVT, IgY and the number of eosinophils.

Table 1: Statistics of the fixed factor components of the simplify GLMM models for each of the immunological parameters. Significant predictors are marked with asterisk. β coefficient is showed for significant covariates in order to indicate the direction of the relationship between the covariate and the immunological parameter.

Humoral innate immunity	**β**	***df***	***F***	***p***	
HA					
Treatment		3, 56	0.83	0.48	
Hatching date	+0.08	1, 56	0.38	<0.001	*
Leucocytozoon		1, 69	4.85	0.03	*
HP					
Treatment		3, 59	0.90	0.44	
Hatching date		1, 59	1.05	0.31	
BMI		1, 85	1.90	0.17	
OVT					
Treatment		3, 48	3.14	0.03	*
Hatchind date	+0.08	1, 48	5.07	0.03	*
BMI		1, 49	1.92	0.17	
Leucocytozoon		1, 49	1.36	0.25	
Nox					
Treatment		3, 52	0.22	0.88	
BMI		1, 66	2.48	0.12	
Leucocytozoon		1, 66	1.51	0.23	
Humoral acquired immunity					
IgY					
Treatment		3, 58	2.85	0.04	*
Hatching date	+0.05	1, 58	6.56	0.01	*
BMI		1, 88	2.37	0.12	
Lymphocytes	+0.07	1, 88	5.36	0.01	*
Cellular component immunity					
Leukocytes					
Treatment		3, 58	1.27	0.28	
Hatching date	+0.001	1, 58	12.74	<0.001	*
BMI		1, 87	2.54	0.17	
Leucocytozoon		1, 87	1.95	0.14	
Heterophils					
Tratment		3, 58	0.02	0.99	
Hatching date	+0.78	1, 52	5.14	0.03	*
Lymphocytes					
Treatment		3, 58	1.36	0.25	
Hatching date	+0.46	1, 58	10.08	<0.001	*
BMI	+0.15	1, 84	6.21	0.01	*
Leucocytozoon		1, 84	7.67	0.006	*
Treatment*Leucocytozoon		3, 84	3.01	0.03	*
Eosinophils					
Treatment		3, 58	5.87	0.001	*
Hatching date		1, 58	0.62	0.43	

Leucocytozoon	1, 88	3.69	0.06	
H/L ratio				
Treatment	3, 58	1.21	0.31	
Hatching date	1, 58	0.25	0.62	
BMI	1, 85	0.89	0.35	
Leucocytozoon	1, 85	1.60	0.21	
Treatment*BMI	3, 85	3.08	0.03	*

Humoral acquired immunity

The nest predation treatment had a significant effect on the IgY concentrations (Table1). In particular, nestlings of the AC group had higher IgY levels (average of 26.3%) compared to the other experimental groups (Fig. 1b). Furthermore, IgY levels were positively correlated both with hatching date (β = 0.05) and with the number of lymphocytes (β = 0.07; Table 1).

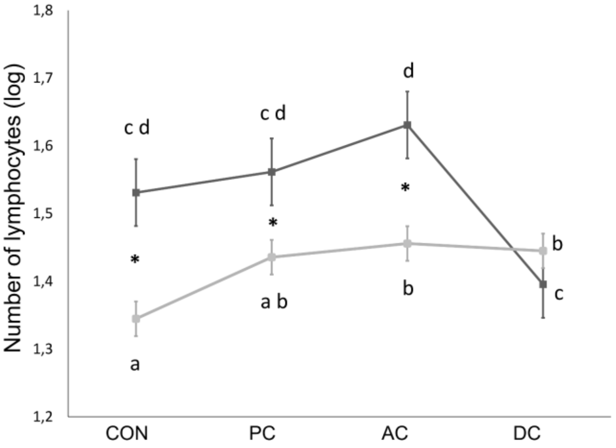

Figure 2: Effect of the interaction between treatment and Leucocytozoon presence on the number of lymphocytes. Light grey line indicates chicks without Leucocytozoon, while dark grey line indicates chicks with Leucocytozoon. Treatment groups that have distinct letter indicate significant differences among groups for the post hoc test at the $p < 0.05$ level. Nestlings without Leucocytozoon that belong to AC and DC groups had a higher number of lymphocytes compare to CON group, while in presence of Leucocytozoon, nestlings of AC group had a higher number of lymphocytes than DC group. Asterisks indicate the the significance of difference in the number of lymphocytes between healthy or infected nestlings within each treatment group. Nestlings with Leucocytozoon had a significant higher number of lymphocytes than healthy nestlings for the PC, AC and CON group, but not for the DC group.

Cellular component immunity

Our treatment did not affect the total number of white blood cells (leukocytes) in nestlings (Table 1). In addition, total number of leukocytes was highly correlated with hatching date, increasing in nestlings that hatched later in the breeding season (β = 0.001; Table 1). On the contrary, none of the other predictors affected this variable (Table 1). The number of heterophils was positively affected only by hatching date (β = 0.78; Table 1), increasing in nestlings that hatched later in the breeding season, while treatment had no effect (Table 1). Treatment did not directly affect the number of lymphocytes (Table 1), but its effect was mediated by the presence of *Leucocytozoon* in nestlings (Table 1). In particular, uninfected nestlings of AC and DC groups had higher numbers of lymphocytes than those of the CON group (LSD post hoc test *p* = 0.007 and *p* = 0.03 respectively; Fig 2), while in the presence of *Leucocytozoon*, nestlings of the AC group had more lymphocytes than those of the DC group (LSD post hoc test *p* = 0.003; Fig 2). Moreover, nestlings infected with *Leucocytozoon* had a significant higher number of lymphocytes than healthy nestlings for the PC, AC and CON group (LSD post hoc test *p* = 0.003, 0.02 and 0.05 respectively; Fig 2), but not for the DC group (LSD post hoc test *p* = 0.44; Fig 2). Moreover, the number of lymphocytes was positively correlated with both nestling hatching date (β = 0.46) as well as with body condition (β = 0.15; Table 1). The number of eosinophils was significantly affected by our treatment (Table 1). Nestlings of the AD and DC groups had significantly more eosinophils than those of the PC and CON groups (Fig 1c). We also found a marginally significant effect of infection by *Leucocytozoon* (Table 1) indicating that non-parasitized nestlings have more eosinophils than parasitized nestlings (Table 1). H/L ratio was not directly affected by our nest predation risk manipulation (Fig 1d), but changed in relation to the nestlings' body condition (Table 1). Nestlings in worse body condition had a higher H/L ratio compared to nestlings in good body condition, but

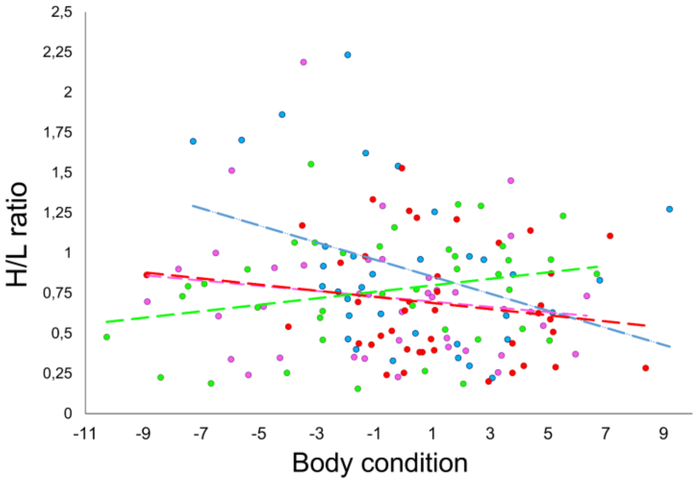

Figure 3: Patterns of the interaction between treatment and BMI on the H/L ratio. Blue, purple, red and green represent CON, PC, AC and DC groups respectively. Correlation was significant only in CON group (r Pearson = - 0.37, p = 0.03); H/L ratio decrease in chicks with better BMI (white solid line).

only in the control group (p = 0.03; Fig 3) suggesting that this relationship is lost under the risk of nest predation.

Table 2: Result of the discriminant analysis of the immunological parameters measured among the four treatment groups. The higher the eigenvalues, the larger proportion of variance explained. This indicates the function that better differentiates among groups. Bolded values of each function represent the highest loadings which contributes to the largest explanatory values compared within rows.

	Function 1	Function 2	Function 3
HA	-0.110	-0.174	**0.184**
HP	0.187	-0.192	**-0.314**
OVT	-0.111	**-0.721**	-0.231
Nox	0.270	-0.358	**0.372**
IgY	-0.276	**0.579**	0.138
Leukocytes	-0.065	-0.151	**0.202**
Heterophils	**0.321**	-0.090	0.067
Limphocytes	-0.324	-0.133	**0.416**
Eosinophils	**-0.414**	-0.058	-0.338
H/L ratio	**0.630**	0.005	-0.261
Eigenvalues	0.38	0.27	0.13
Prop. Variance explained (%)	48.39	35.03	16.58
Cumulative prop.	48.39	83.42	100
P-value	< 0.001	0.001	0.04

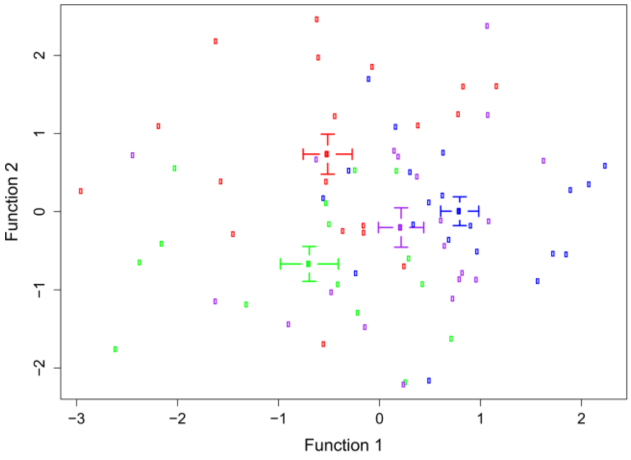

Figure 4: Distribution of nestlings according to the 1st and 2nd discriminant functions. Dots and whiskers refer to mean values ± standard error. Blue, purple, red and green represent CON, PC, AC and DC groups respectively.

Discriminant analysis

Discriminant analysis resulted in two highly significant discriminant functions which together explained 83% of the variance among treatment groups (Table 2). The first function indicated H/L ratio, eosinophils, and heterophils as the immunological parameters that contribute most to differences among groups (Table 2). AC and DC groups were more different than PC treatment with respect to the CON group (Fig. 4). The second function showed that nestlings in the DC and AC groups were the most different (Fig. 4) due to OVT and IgY parameters. The third discriminant function grouped the remaining of immune indices, explaining 17% of the total variation (Table 2).

Discussion

The experimental increase of nest predation risk directly affected OVT, IgY and the number of eosinophils while the effect on lymphocytes and H/L ratio were mediated by the presence of *Leucoctytozoon* and body condition respectively. We found an overall positive association between the immunological response and the intensity of nest predation risk, although not according to the level of detail expected, since nestlings of the most risky situations (DC and AC groups) showed variations in their immune parameters, whereas those of moderate predation risk (PC group) did not display any variation.

Immune responses to nest predation risk

Our results showed that OVT, IgY and the number of eosinophils increased in response to short-term increase of nest predation risk, indicating a reinforcement of the processes involved in immune defenses. This finding supports the idea that during acute stress conditions a stimulation of immune activity occurs (prediction 2a), whereas it contrasts with the assumption of the suppressive effect of stress on immunity (prediction 1a; Wingfield *et al.* 1997). Generally, the goal of the immune-suppression effect produced by stress is to redirect resources toward emergency biological functions (Sapolsky, Romero & Munck 2000). However, in certain situations, such as during a predator attack or a territorial conflict, which may lead to physical aggression and consequently provoke important injuries, the suppression of the immune function could not be adaptive (Dhabhar 2002). In these cases the rapid activation of the immune defenses may be critical for the survival of an individual. Our experiment supported this hypothesis as blackbird nestlings, by perceiving an imminent attack, displayed stimulated immune responses probably to increase survival following the attack of a nest predator. In particular, the observed increase in OVT levels for the extreme level of nest predation risk (DC group) would be suitable to promptly contrast the spread of pathogens and to limit the negative consequences of an imminent injury. OVT is a multifunctional protein whose concentration increases in the bloodstream in response to inflammation and is involved in bactericidal, antiviral, and immunomodulatory activity (Giansanti *et al.* 2012). Interestingly, although in absence of an immunological stimulus (e.g. injuries or contact with new antigens), nestlings exposed to adult alarm calls produced more IgY. This finding suggests that predator stress-induced immunomodulation can affect the antibody-mediated immunity, thus possibly contributing to a general state of preparedness of the immune system to cope with an incoming infections (Dhabhar & McEwen 1997). Part of the nestling leukocyte profile was stimulated too. Eosinophils increased in nestling of DC and AC groups and lymphocytes increased in AC group, as occurred with IgY levels, whereas the number of heterophils did not significantly change, suggesting that different types of leukocytes could respond at different times after the treatment started (Buehler *et al.* 2008). This mechanism seems to be associated with the two stages of leukocyte response to stress observed in mammals: in the initial phase, leukocytes increase in blood circulation and subsequently, they exit the bloodstream and migrate towards the sites of interest, such as wounds, infection sites or lymphoid tissues, where they could perform their specific functions, (Dhabhar & McEwen 1999). In the context of nest predation, the redistribution of leukocytes is evolutionarily explained as an important component of the fight or flight reaction during predatory attacks (Dhabhar & McEwen 1997). According to these premises, it is likely that the immune suppression benefits (prediction 1a) are favorable only over a long period (chronic stress), but not during short-term stress situations, such as a predatory attack (Dhabhar & McEwen 1999).

The significant increase in the number of eosinophils that we observed seem to indicate that these cells could have an important role in the initial stage of immunomodulation, at least in response to nest predation risk (Jacobsen *et al.* 2007). This is an helpful results, since the information about avian eosinophils are limited; their function is generally associated with parasite exposure, antibody-mediated response and the regulation of the inflammatory response (Campbell 2007, Davis, Maney & Maerz 2008).

Responses according to the intensity of nest predation risk

Predictions 2b, which correlated the increase in immune response to the intensity of nest predation risk was supported in general, although not to the level of detail expected initially. Based on the discriminant analysis nestlings' responses are gathered into two different groups: low risk (CON and PC groups) *vs* high risk situations (AC and DC groups; Fig. 4). While high and extreme predation risk induced an increase in several immune components, the use of predator cues did not provoke significant changes. This finding is of special relevance as it is a common manipulation used for nest predation experiments (e.g. Mougeot & Bretagnolle 2000, Ibáñez-Álamo *et al.* 2011, Caetano *et al.* 2014). Our results suggest that low levels of predation risk are not sufficient to induce immune response, which is activated only when the threat to be preyed upon becomes severe (AC and DC group). Alternatively, conspecific calls (alarm or distress calls) are a better indicator of nest predation risk than predator calls. Parental alarm calls contain detailed information for nestlings about the nature of predators (Platzen & Magrath 2005), predator distance or behavior (Suzuki 2011), thus parent-offspring communication would represent the main informative process through which nestlings can monitor the current nest predation risk. Despite the explanation for these results, our findings indicate that using even simple gradients of (nest) predation intensity can help to better understand predator-prey interactions.

Another important result highlighted by the second function of the discriminant analysis concerns the differences in the OVT and IgY concentrations shown by the AC and DC groups (Fig. 4). Increased OVT concentration seems to be efficient in response to an imminent predator attack (DC group), whereas IgY increased when nestlings experienced a level of predation risk just lower than the previous one (AC group). This fact could be due to the temporal component of a predatory event. In a natural situation, alarm calls usually correspond to an earlier stage in the predatory sequence (i.e. the predator is located in the surrounding of the nest) in comparison with nestling distress calls, which start when the predator is already at the nest (Caro 2005). In this context, distress calls could trigger different (quicker) immune responses while the activation of the acquired component (IgY) would need more time (Lee 2006). Another non-exclusive explanation might be that nestlings do not need to elevate IgY levels in response to distress calls because they are already increased as a consequence of an earlier stage of a predator attack (i.e. due to parental alarm calls). In the latest stage of the predatory process, when the predator is already at the nest, IgY would still be active and the defensive responses might be more oriented towards other type of strategies (i.e. escape from the nest).

Interaction with parasites and environmental variables

According to our third prediction, the parasitism status mediated the ability of nestlings to cope with a predatory situation, at least for some components of the immune system (Table 1). Nestlings parasitized with *Leucocytozoon* were not able to maintain their elevated lymphocyte levels in the extreme predation risk situation (Fig. 2). Considering that healthy nestlings have those lymphocyte levels, they could still be useful to cope future costs associated to potential injuries also for parasitized chicks, but probably the will not be enough to fight against the *Leucocytozoon* infection.

Another very interesting result indicated that the effect of nest predation risk on the H/L ratio was influenced by nestlings' body condition, thus, suggesting that variations of H/L ratio were strongly associated to the healthy status of nestlings (Masello *et al.* 2009). Here, nestlings exposed to non-predation risk (CON group) showed a negative correlation between body condition and H/L ratio, whereas those exposed to a predator threat lost this relationship, indicating that nest predation may alter the normal trade off of other physiological traits (Fig. 3). This might have important implications in those studies measuring H/L ratio if parents perceived researchers as potential predators giving alarm calls, and thus making more difficult to find biologically meaningful results.

Finally, we detected a strong effect of breeding season on the immune parameters (Table 1) as the nestlings hatching later in the season showed a larger investment in most of the immune parameters. Higher levels of immunity may indicate two situations: (i) higher quality nestlings (Roulin *et al.* 2003), or (ii) an activation of the immune system which usually occurred under poor conditions(De Coster *et al.* 2010, Lindström *et al.* 2004). The deterioration of environmental conditions that occurs at the end of the breeding period in the study areas, when temperatures are higher (Spanish Meteorological Agency) and the number of ectoparasites increases (i.e. mites), suggests that nestlings would have to invest more in immune defense, supporting thus, the later explanation.

Conclusions

Summarizing, we conclude that (i) a short-term increase in nest predation risk induced changes in the immune system of nestlings by increasing specially the acquired component. (ii) This effect is complex and multifactorial and depends on the immune variable measure and the type of manipulation of nest predation risk. (iii) The different levels of nest predation risk caused different immunological changes, suggesting that nestlings are able to modulate their immune system according to the threat posed by a predator but always mediated by conspecific (alarm or distress) calls. (iv) Relevant natural factors, as parasites and seasonal variability can affect and impede the normal physiological response to nest predation, while at the same time, (iv) this selective pressure can imbalance some trade-offs between immune indexes (H/L ratio) and body condition. Finally, (v) this study highlights the relevance of studying the physiological consequences of predator-prey interactions, not only to better understand the proximate mechanisms behind them but also to reveal new trade-offs among several selective pressures.

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Authors' Contributions

- Conceived the ideas and designed methodology: GR, MS, JDI
- Field work and data collection: GR, FRR, EC
- Immunological assays: GR, EC, MV, MGS, BIT
- Analyzed the data: GR, EC
- Writing of the first draft: GR, JDI

All authors contributed critically to the manuscript and gave final approval for publication.

Data accessibility

The data sources are uploaded as supporting information (SI 2 Humoral Immunity and SI 3 Cellular immunity).

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CHAPTER 5

IMMUNE RESPONSE TO NEST PREDATION RISK DURING NESTLING PERIOD DEPENDS ON THE DEVELOPMENTAL CONDITIONS OF NESTLINGS

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Ready to submit

Abstract

Among the physiological anti-predator strategies, those that involve the immune system have been generally overlooked, despite the importance that immunity could have in enhancing preys' probabilities of survival to a predator's encounter, for example by limiting the negative consequence after that encounter. Nestlings are excellent models for the study of these anti-predator strategies because they suffer an extremely high predation pressure while experiencing at the same time the majority of their development, which maximises potential trade-offs between the immune system and other important functions. In this study and using common blackbirds (*Turdus merula*), we investigated whether an elevated nest predation risk during the whole nestling period affects nestlings' immune system and explored the possible interactions with intrinsic (i.e. body condition and growth rate) and extrinsic (i.e. breeding season) factors. Nestlings under increase predation risk modified some components of their immune system (i.e. immunoglobulins and lymphocites) but not others (i.e. haptoglobin or ovotransferrin), indicating that the immunological response can be complex and multifaceted. Predation risk induced a suppression of IgY levels and an increase in lymphocytes in nestlings with poor body condition, suggesting a mediator role of parents through their investment in nestlings' condition. We also found support for the fact that nest predation risk can affect the trade-off between growth and immune response, since experimental but not control nestlings showed a negative correlation between structural growth and the number of heterophils. Finally, our results indicate that immunological responses to predation risk do not depend on the breeding season, even though we found higher general immunological levels in nestlings towards the end of the season. This study highlights the important role of immunity as an anti-predator response in nestlings and shows the relevance of including a physiological component to the study of predation risk.

Introduction

Predation is one of the most important selective pressures in nature (Caro 2005). Beyond the direct impact on fitness caused by the killing of preys (Preisser, Bolnick & Benard 2005), the consequences related to the prey's perception of predation risk can be decisive (Lima 1998, Cresswell 2008, Zanette *et al.* 2011). The effects of predation risk induce preys to respond with a wide variety of anti-predator defenses including behavioural and physiological changes (Caro 2005, Creel *et al.* 2005, Lima & Dill 1990, Díaz *et al.* 2013, Hawlena & Schmitz 2010). However, the scientific community has been traditionally focused on behavioral rather than physiological responses despite the benefits of the latter to fully comprehend the costs associated to predation risk (Clinchy *et al.* 2004, Zanette *et al.* 2011, Clinchy *et al.* 2013). The single exception maybe the hormonal responses to predation, which are studied within the more general framework of stress-induced factors (Sapolsky *et al.* 2000, Hawlena & Schmitz 2010). But other physiological components, like the immune system, have been greatly overlooked (Hawlena & Schmitz 2010). This is surprising given that the immune system, which protects the organism from pathogens, diseases and infection (Roitt *et al.* 2001), can also play an important role enhancing preys' probabilities of survival to a predator's attack and its consequences (Dhabhar & McEwen 1997, Dhabhar 2002). This is based in the fact that the risk of predation could induce three different anti-predator responses: (i) to avoid the encounter with the predator, (ii) to escape the potential predator if the encounter happens, and (iii) to overcome the consequences of that encounter (i.e. injuries). In this context, the immune system seems to be involved in the second and third set of anti-predator defenses. Regarding the second, there is evidence, for example, indicating that a high cell-mediated immune response is associated with higher probabilities of escaping a predator in campo flicker (*Colaptes campestris*) nestlings as they can produce more efficient distress calls that attract a greater number of adults to mob the predator (Goedert *et al.* 2014). Another study with pied flycatchers (*Ficedula hypoleuca*) also relates a significant elevation in immunoglobulins with an increased risk of predation by sparrowhawks (*Accipiter nissus*) although these changes are probably related to both, the second or third type of anti-predator responses (Thomson *et al.* 2010).

Despite this potential link, the activation of the immune system can be costly in terms of energy, nutrients or the alteration of other important physiological functions like oxidative stress (Hasselquist & Nilsson 2012), which in addition to its complexity (Janeway *et al.* 1997) suggest that the immunological response to predation risk should be carefully studied controlling for other potential confounding factors (i.e. condition of the individual; Ibáñez-Álamo *et al.* 2015, Roncalli et al. submitted). Alternatively, predation risk could produce an immunosuppressive effect mediated by the endocrinological changes associated to that situation (Duffy *et al.* 2000, Butler *et al.* 2009). For instance, it is known that stressful situations, like that provoked by a predator, can increase stress hormone levels which simultaneously causes a decrease of certain components of the immune system (Boonstra *et al.* 1998, Clinchy *et al.* 2004).

Birds are particularly interesting to test these hypotheses given the peculiarities of their reproductive cycle that always involves a relative long period in which they are attached to a specific location, the nest (Bosque & Bosque 1995). In fact, nest predation represents the first cause of nestling mortality for many bird species (Martin & Briskie, 2009). Furthermore, the nestling period is particularly critical for birds as it is where the majority of their development occurs (Lack 1968, Ricklefs 1983), and is known to determine future survival prospects (Lobato *et al.* 2005, Noguera, Kim & Velando 2011). Despite this suitability, only two studies have investigated the effect of nest predation risk on nestlings' immune system, but all of them finding support for the immunoenhancement effect. One of them using pied flycatchers found an elevation of H/L ratio when exposed to conspecific distress calls for some days (Tilgar *et al.* 2010). In the second we demonstrated that common blackbird (*Turdus merula*) nestlings activate several but not all immune parameters measured as a response to an experimental and punctual increased in predation risk (Roncalli et al. submitted) suggesting that this interaction is complex and multifaceted and clearly indicating the need to analyze more than just a single immunological variable in this context (Matson *et al.* 2006). Interestingly, pied flycatchers breeding in high nest predation risk sites lay eggs with more immunoglobulins (Morosinotto et al. 2013) which highlight even more the potential importance of an activated immune system in an environment threatened by predators.

The main aim of our study was to investigate whether an experimentally increased risk of nest predation during the whole nestling period could induce an immune response and in which immunological parameters. The long-term perspective of our manipulation is particularly relevant as it has been demonstrated that these manipulation rather than short-term changes are optimal to study the “ecology of fear” (Brown *et al.* 1999, Cresswell *et al.* 2010, Clinchy *et al.* 2013). Furthermore, the few studies on the topic have manipulated predation risk only during some hours or days, but never since the hatching until the fledgling period (Tilgar et al. 2010, Roncalli et al. submitted, reviewed in Ibáñez-Álamo et al. 2015), even though correlational studies with adults or eggs suggest important effects (Thomson et al. 2010, Morosinotto et al. 2013) and the fact that short- and long-term risks can produce different anti-predator responses (Lima 2009). According to previous studies the most plausible prediction would be that chicks exposed to an increased predation risk during the nestling stage will activate (at least) part of their immune system (prediction 1a). More difficult is to make specific predictions regarding each immune parameter due to the lack of previous information for many of them although there seems to be an agreement to predict an elevation of immunoglobulin levels (Roncalli et al. submitted, Thomson et al. 2010, Morosinotto et al. 2013). Alternatively, and given the general immunosuppression effect on the immune system during long periods of stress (Dhabhar 2002, Saino *et al.* 2003, Stier *et al.* 2009) we predict also that (1b) a down-regulation of immune parameters in nestlings exposed to predation risk. In order to test these predictions we experimentally modified the risk of nest predation of common blackbird nestlings, from which we already have detailed information on their immunological responses to a short-term manipulation in predation risk (Roncalli et al. submitted), from the moment of hatching until they left the nest. We measured 12 immunological variables to obtain a complete overview of the potential changes associated to our experiment and controlled for different intrinsic (i.e. body condition) and extrinsic (i.e. breeding period) factors that are known to affect the immune system of nestlings (Navarro *et al.* 2003, Dubiec & Cichon 2005, Roncalli et al. submitted).

Material and Methods

Study model

We conducted our study during the spring of 2014 in a common blackbird population located in the Valley of Lecrín (36° 56' N, 3° 33' W; 580 m a.s.l.), an agricultural area in south eastern Spain. In this population blackbird females incubate for 13–14 days and nestlings stay in the nest for 11–13 days (Ibáñez-Álamo & Soler 2010). Predators occurring in the study area consist of avian species (e.g. Eurasian sparrowhawks), mammals (e.g. genets *Viverra genetta*) and snakes, with an overall nest predation rate of 48.9% (Ibáñez-Álamo & Soler 2010).

We actively searched the nests from the start of the breeding period (early March). Once a nest was located we visited it every 2 days using a pole with a mirror in order to know the content and the exact hatching date.

Experimental design

To create a chronic increase in predation risk we reproduced predator acoustic cues (experimental group) or passerine sounds (control group) during the entire duration of the nestling period (Fig. 1) following previously made similar experimental manipulations (Zanette *et al.* 2011, Coslovsky & Richner 2012, Hua *et al.* 2014). To compose playbacks we carefully selected calls from a virtual platform on the web (www.xeno-canto.org). Playbacks for the experimental group included calls emitted by predators that live or were observed in the study areas. To compose playbacks for the control group we selected songs and call vocalizations of several passerine species present in the area. We avoid using alarm call vocalizations of these species as they may indirectly indicate the presence of a predator (Haff & Magrath 2012, Haff & Magrath 2013). Each playback consisted of 6 minutes and 40 seconds of call activity (40 seconds of calls interspersed with 1 minutes of silence for 4 times) followed by 7 minutes of silence. We joined 9 different playbacks to compose a single 1 h and 45 min long audio file, which was then broadcasted repeatedly. Eight different audio files were created for each group.

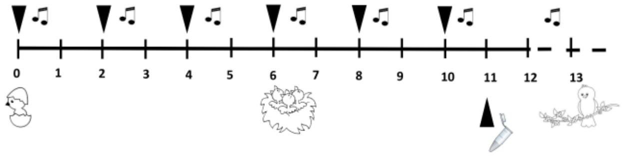

Figure 1: Experimental design. The speaker played every other day during the whole nestling period. We collected biometric measurements on every visit before connecting the speakers. At day 11 we collected blood samples from each nestling but continue with the nest predation risk situation until all chicks left the nest.

We broadcasted sounds in plots, which consisted in circles of 100 m of radius including 1-3 blackbird nests. In order to avoid potential confounding effects due to maternal effects (Morosinotto *et al.* 2013), we started our manipulation the day of hatching and randomly assigned each plot to the experimental or control group. We left a buffer zone (100 m) between plots to grant acoustic isolation regarding our manipulation. Speakers operated during the day (from 8:00 a.m. to 20:00 p.m.) at 70 dB. We tried to avoid the habituation of blackbirds to the speakers by: (i) carefully selecting calls of different bird species and from different individuals for each species (i.e. approximately 15 distinct calls were reproduced in each audio file); (ii), randomising the order of playbacks within the audio file; (iii) changing the location of speakers within the plot; and (iv) operating the speakers every other day (Fig. 1).

Blood sampling and storage

When the second hatched nestling was 11 days old, we collected a blood sample (250-300 µl) from the brachial vein of all chicks of that nest. We always collected the blood sample in a day in which the speakers did not play to ensure that the effect of the increase of nest predation risk referred to the accumulate threat of being preued upon during the entire nestling period and not to the short-term increase in nest predation risk (Roncalli et al. submitted). All blood samples were collected between 10:00 and 14:00 h and kept refrigerated (4ºC, maximum 5 hours) until centrifugation (13000 rpm for 10 min). Plasma was separated and stored at –25°C. We also collected a drop of blood that was smeared on a marked glass slide and dried in open air.

Immunological assays

We performed several immunological assays in order to quantify 12 parameters belonging to both the humoral and cellular components of the immune system as well as to innate and acquired immunity (Janeway *et al.* 1997). The objective of this multiple measurements was to capture the complexity of the immune system (Matson *et al.* 2006), since we previously found that not all components of the immune system respond similarly to a punctual increase of nest predation risk in blackbird (Roncalli et al. submitted).

Innate humoral immunity

(i) Haemolysis/Haemagglutination titres (HL-HA)

Haemagglutination (HA) and haemolysis (HL) assays were used to quantify the levels of non-specific natural antibodies and titers of complement-like lytic enzymes respectively. These molecules are part of the innate immunity facilitating the initial recognition of pathogens and promoting the activation of adaptive immunity (Carroll & Prodeus 1998). We followed the procedure described by Matson, Ricklefs & Klasing (2005) for these analyses. Scan of individual samples was randomized among all plates and scored by a single person (GR; see Matson et al. 2005 for more details).

(ii) Haptoglobin (HP)

Haptoglobin (HP) is an acute phase protein found in several species, including birds, which is able to inhibit the oxidative activity of erythrocytes, by binding the free hemoglobin released (Galicia & Ceuppens 2011). In response to acute infection or inflammation, HP results in a high blood concentration (Matson *et al.* 2012). A commercial colorimetric assay kit (TP801; Tridelta Development Ltd., Maynooth, Ireland) was used to quantify its concentration following Matson et al. (2006).

(iii) Ovotransferrin (OVT)

OVT is another acute phase protein, which can bind free iron, an essential nutrient for bacterial growth. Ovotransferrin is a protein with antibacterial, antiviral and antifungal activities (Giansanti *et al.* 2012) and therefore, it is usually considered as an indicator of inflammation and infection, poor nutritional state or diseases (Horrocks *et al.* 2011b). OVT concentration was quantify following Horrocks et al. (2011).

Acquired humoral immunity

(v) Immunoglobulins (IgY)

Immunoglobulins are important serum proteins produced by B lymphocytes that are used by the immune system to identify and counteract pathogens and to promote leukocytes migration to the sites of infection (Härtle *et al.* 2014). Total immunoglobulin concentrations (IgY) were assessed using a sensitive enzyme-linked immune absorbent assay (ELISA) method. Antichicken antibodies were used following the procedure developed by (Martinez *et al.* 2003). We adapted this method for common blackbird nestlings following (Roncalli *et al* submitted).

Cellular component immunity

(vi) Leucocyte profile

In order to quantify white blood cells, blood smears were fixed in absolute methanol immediately the day of collection and stained for 45 min with Giemsa (GS500-500ml SIGMA-ALDRICH Giemsa stain). The smears were scanned with an optical microscope (1000x magnifications with oil immersion) in order to count a minimum of 100 leucocytes in each slide. Each cell was classified as heterophils, lymphocytes, eosinophils, basophils or monocytes following the description of Campbell (2007) in order to estimate the relative proportion of each cell type and to calculate H/L ratio.

Developmental factors and breeding season

To control the possible effect of developmental condition on immunity during nestling period, we calculated the growth rates and body condition of each nestling. Growth negatively affect the immune system (Soler *et al.* 2003, Hawlena & Schmitz 2010, Van der Most *et al.* 2011), whereas body condition is observed to be positively related to immunity (Navarro *et al.* 2003, Roncalli et al. submitted).

To obtain growth rates, we marked each hatched nestling with a different colour by means of non-toxic markers (FaberCastel Multimark) in order to recognize it in the following visits. Once every two days, just before connecting the speakers, the same researcher (GR) collected body mass (±0.1 g) and tarsus length (0.01 mm) of each nestling. Because growth is typically S-shaped, we modulated growth rate of body mass and tarsus length using the following logistic function (Starck & Ricklefs 1998):

$$w_t = \frac{A}{1 + e^{(\mathrm{k}(I-t))}}$$

w_t represents the biometric parameter (body mass or tarsus length) at time *t*. *A* is the asymptotic growth parameter of nestlings while *I* is the inflection point of the growth curve (in days), and *t* corresponds to the nestling age (in days). The growth rate constant (*k*) obtained from the formula, which is considered a standardized parameter for comparing growth rates (Starck & Ricklefs 1998), was used to estimate growth rates for the body mass (*km*) and tarsus length (*kt*) to included them in our statistical models. Body condition was calculated as the residuals of the regression between body mass and the tarsus length (Jakob *et al.* 1996) measured the day of blood sampling. In the same day we also obtained the length of both wings (0.1 cm) to calculate the alar symmetry as it was observed that nest predation may affect the locomotor traits involved in flight performance (Lima 2009) (Coslovsky & Richner 2011). Symmetry was calculated as the ratio between the length of the right and the left wing. Value of 1 unit indicates a perfect symmetry.

The possible effects of environmental factors on the immune system (Dubiec & Cichon 2005, Roncalli et al. submitted) were estimated by considering the breeding season, which provide an indirect measure of the seasonal variation of the environment (Dubiec & Cichon 2005). Breeding season was estimated from the hatching date, that is the day on which the first nestling of each brood hatched (day 1= 1 March). Further, we also controlled for another extrinsic factor, brood size, since its negative relationships with immunity was observed in several species (Saino, Calza & Møller 1997) (Ilmonen *et al.* 2003) (Bourgeon, Guindre-Parker & Williams 2011).

Statistical analysis

To examine the effect of nest predation risk and its possible interaction with developmental and environmental conditions on each immunological parameter, we fitted two set of models, one referring to developmental (intrinsic) factors and the other referring to the environmental (extrinsic) factors. Each set of models were run independently for each immunological parameter measured (HA, HP, OVT, IgY levels, number of leukocytes, heterophils, lymphocytes, eosinophils, basophils and H/L ratio). It was not possible to fit any model for the lysis activity (HL) or monocytes because the values of these immunological parameters were very rare. We used general linear mixed models (GLMM, *lme* function in the ‘nlme’ package; Pinheiro et al. 2016) considering the immunological parameters as dependent variables. For the first set of models, we included nest predation treatment, body condition, the growth rate of the body mass (*km*) and tarsus length (*kt*) and finally the alar symmetry. In the second set of the models, we considered nest predation treatment, hatching date and brood size. In addition, we also consider the interaction between treatment and each of the other factors in the model to check for the possible mediator effect of each factor on nest predation risk. For both sets of models we fitted a nested random structure in which nest identity was nested within plot identity in order to control for the non-independence of nestlings from the same nest, and nests from the same plot. Successively, following a backward selection procedure, we excluded the factors that showed the highest (non-significant) *p* values in order to obtain simpler alternative models (Engqvist 2005). We did not remove treatment when it was not significant as it reflects the hypotheses to be tested. Because we fitted the same models based on two set of simultaneous hypotheses (developmental and environmental models), we applied Bonferroni correction (Cabin & Mitchell 2000), which, in our statistical models, fixed the significant threshold of the p values at 0.025. We controlled for the homogeneity of variance and the normal distribution of the residuals of our models (Zuur *et al.* 2010). We used the logarithmic transformation for those variable that violate these conditions (i.e. IgY, the number of eosinophils and H/L ratio). Data were analyzed using R version 3.3.2 (R Core Team, 2016).

Results

Nest predation risk and developmental conditions

Our nest predation risk manipulation significantly affected the acquired immunity (IgY and lymphocytes) of blackbird nestlings and some aspects of their innate component (i.e. heterophils). Interestingly, both effects were mediated by body condition or growth rate respectively (Table 1). In particular nestlings in poor body condition from the experimental group showed lower IgY levels than those of the control group (Fig. 2A). A similar mediator effect was found for lymphocytes as we found a positive significant correlation between body condition and lymphocytes for control nestlings (*r* Pearson = 0.40, $p = 0.01$) but not for those suffering an elevated nest predation risk (*r* Pearson = -0.02, $p = 0.88$; Fig. 2B). This effect was marginally significant according to our Bonferroni corrections ($p = 0.03$ for the interaction, slightly larger than 0.025; Table 1). With respect to the innate immunity, we found no effect of our treatment but a marginally negative significant effect of structural growth showing that nestlings investing more in growth had lower levels of HP and OVT ($p = 0.04$; Table 1). We found similar associations for different cellular parameters. Higher growth in body mass was related to significantly lower numbers of eosinophils and basophils ($p < 0.002$) and marginally for leukocytes ($p = 0.04$). Additionally, our results showed a trend ($p = 0.04$) for the interaction between our treatment and structural growth rate, indicating no association between tarsus growth and the number of heterophils of control nestlings (*r* Pearson = - 0.20, $p = 0.28$), but a significant negative

Table 1: GLMM table of development predictors on immune system. Statistics of the final GLMM models obtained after the backward selection procedure for each of the immunological parameters. Significant predictors are marked with point, significant predictors after Bonferroni correction are marked with asterisk.

Humoral innate immunity	**Coef. (SE)**	**df**	**F**	**P-values**
HA				
Treatment		1, 34	0.07	0.79
Body condition		1, 32	1.24	0.27
km		1, 24	0.04	0.84
kt		1, 23	0.02	0.89
Alar symmetry		1, 31	1.24	0.35
Treatment X Body condition		1, 21	0.84	0.36
Treatment X *km*		1, 24	0.01	0.91
Treatment X *kt*		1, 23	0.07	0.30
Treatment X Alar symmetry		1, 19	0.01	0.91
HP				
Treatment		1, 35	0.03	0.87
Body condition		1, 31	0.19	0.66
km		1, 30	0.03	0.87
kt	-0.26 (0.12)	**1, 33**	**4.43**	**0.04 .**
Alar symmetry		1, 32	0.54	0.47
Treatment X Body condition		1, 28	1.12	0.29
Treatment X *km*		1, 27	0.47	0.49
Treatment X *kt*		1, 29	1.58	0.22
Treatment X Alar symmetry		1, 26	0.53	0.47
OVT				
Treatment		1, 26	< 0.01	0.97
Body condition		1, 17	0.20	0.66
km		1, 16	0.03	0.86
kt	-14.71 (6.88)	**1, 19**	**4.57**	**0.04 .**
Alar symmetry		1, 18	0.83	0.37
Treatment X Body condition		1, 12	1.12	0.95
Treatment X *km*		1, 14	0.34	0.57
Treatment X *kt*		1, 15	1.94	0.18
Treatment X Alar symmetry		1, 13	0.17	0.69
Humoral acquired immunity				
IgY				
Treatment		1, 35	0.22	0.64
Body condition		1, 43	< 0.01	0.95
km		1, 33	< 0.01	0.95
kt		1, 31	0.04	0.84
Alar symmetry		1, 42	0.40	0.53
Treatment X Body condition	+0.06 (0.02)	**1, 43**	**7.37**	**0.009 ***
Treatment X *km*		1, 30	0.38	0.54
Treatment X *kt*		1, 28	< 0.01	0.98
Treatment X Alar symmetry		1, 29	0.18	0.74
Cellular component immunity				
Leukocytes				
Treatment		1, 29	3.22	0.08
Body condition		1, 28	3.04	0.09

km	-96.54 (46.75)	**1, 29**	**4.26**	**0.04 .**
kt		1, 25	0.03	0.56
Alar symmetry		1, 27	0.50	0.49
Treatment X Body condition		1, 24	3.08	0.09
Treatment X *km*		1, 23	1.48	0.24
Treatment X *kt*		1, 22	0.35	0.56
Treatment X Alar symmetry		1, 21	0.03	0.86
Heterophils				
Treatment		1, 26	0.57	0.46
Body condition		1, 25	0.80	0.37
km		1, 26	2.68	0.11
kt		1, 27	0.13	0.72
Alar symmetry		1, 24	0.02	0.88
Treatment X Body condition		1, 23	1.11	0.30
Treatment X *km*		1, 21	4.43	0.74
Treatment X *kt*	-110.72 (52.61)	**1, 27**	**0.12**	**0.04 .**
Treatment X Alar symmetry		1, 22	0.92	0.35
Lymphocytes				
Treatment		1, 26	2.41	0.13
Body condition		1, 26	0.94	0.34
km		1, 25	0.75	0.39
kt		1, 26	0.41	0.53
Alar symmetry		1, 24	0.07	0.79
Treatment X Body condition	-1.69 (0.75)	**1, 26**	**5.06**	**0.03 .**
Treatment X *km*		1, 23	1.34	0.26
Treatment X *kt*		1, 22	0.08	0.77
Treatment X Alar symmetry		1, 21	< 0.01	0.99
Eosinophils				
Treatment		1, 27	2.78	0.11
Body condition	+0.08 (0.03)	**1, 28**	**5.3**	**0.029 .**
km	-4.52 (1.54)	**1, 28**	**8.61**	**0.007 ***
kt		1, 26	0.06	0.81
Alar symmetry		1, 25	< 0.01	0.93
Treatment X Body condition		1, 23	0.47	0.50
Treatment X *km*		1, 24	1.04	0.32
Treatment X *kt*		1, 22	0.36	0.56
Treatment X Alar symmetry		1, 21	0.05	0.83
Basophils				
Treatment		1, 27	1.02	0.32
Body condition		1, 27	0.36	0.55
km	-13.65 (5.36)	**1, 29**	**6.49**	**0.016 ***
kt		1, 25	0.38	0.54
Alar symmetry		1, 28	1.07	0.31
Treatment X Body condition		1, 23	0.71	0.41
Treatment X *km*		1, 22	0.18	0.67
Treatment X *kt*		1, 21	0.03	0.87
Treatment X Alar symmetry		1, 24	2.9	0.10
H/L ratio				
Treatment		1, 25	0.56	0.45
Body condition		1, 25	0.25	0.62
km		1, 22	0.62	0.43
kt		1, 24	0.50	0.48

Alar symmetry	1, 24	1.42	0.24
Treatment X Body condition	1, 21	0.66	0.42
Treatment X *km*	1, 20	0.16	0.70
Treatment X *kt*	1, 24	3.91	0.06
Treatment X Alar symmetry	1, 19	1.56	0.74

relationship for those of the experimental group (*r* Pearson = - 0.59, *p* = 0.001; Fig. 2C). Alar symmetry did not affect any immunological parameter (Table 1).

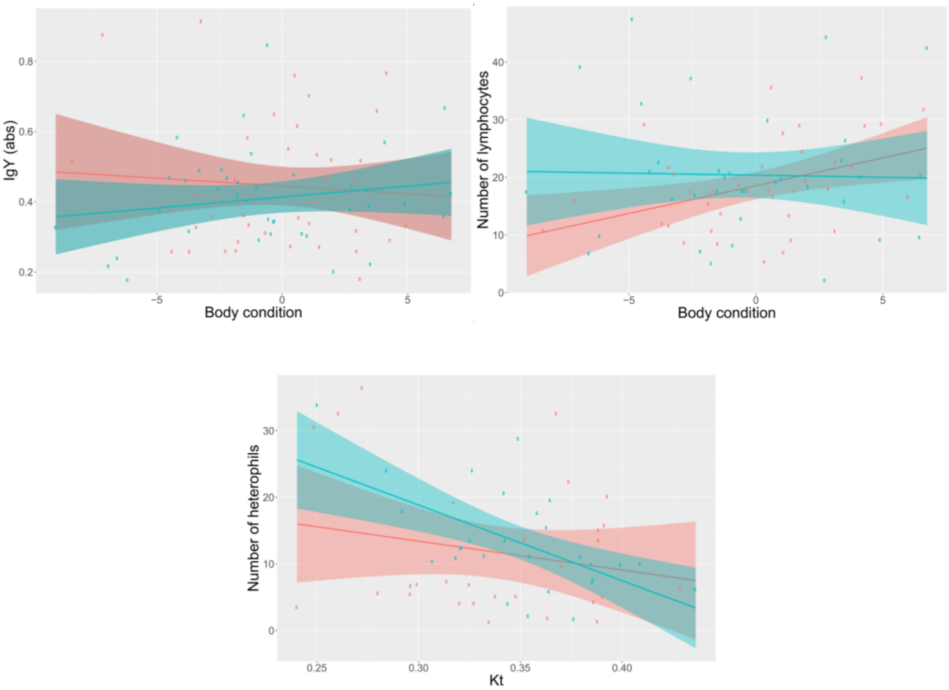

Figure 2: A) Effects of the interaction between the treatment and the body condition on the IgY levels. B) Effects of the interaction between the treatment and the body condition on the number of lymphocytes. C) Effects of the interaction between the treatment and the kt on the number of heterophils. Blue squares indicate nestlings of treatment group, whereas red squares indicate nestlings of control group.

Nest predation risk and environmental conditions

We found a strong significant positive effect of hatching date for most white blood cells, as well as for IgY (Table 2), which highlighted the important influence of seasonal variation of the environment on the cellular and acquire components of the immune system. On the contrary, we did not find any significant effects of brood size (Table 2). None of this two extrinsic factors interacted with our nest predation risk treatment, which similarly as in the other set of models did not showed an effect by itself.

Table 2: GLMM table of environment predictors on immune system. Statistics of the final GLMM models obtained after the backward selection procedure for each of the immunological parameters. Significant predictors are marked with point, significant predictors after Bonferroni correction are marked with asterisk.

Humoral innate immunity	**Coef. (SE)**	**df**	**F**	**P-value**
HA				
Treatment		1, 34	0.07	0.79
Hatching date		1, 33	0.03	0.86
Brood size		1, 32	2.31	0.14
Treatment X Hatching date		1, 31	0.03	0.95
Treatment X Brood size		1, 32	0.84	0.36
HP				
Treatment		1, 35	0.02	0.87
Hatching date		1, 33	0.11	0.75
Brood size		1, 34	1.17	0.29
Treatment X Hatching date		1, 31	0.003	0.95
Treatment X Brood size		1, 32	0.22	0.64
OVT				
Treatment		1, 26	0.002	0.97
Hatching date		1, 24	< 0.001	0.99
Brood size		1, 25	0.22	0.64
Treatment X Hatching date		1, 23	0.18	0.67
Treatment X Brood size		1, 22	0.001	0.97
Humoral acquired immunity				
IgY				
Treatment		1, 35	0.25	0.61
Hatching date	+0.02 (0.004)	**1, 34**	**10.04**	**0.003 ***
Brood size		1, 44	1.16	0.28
Treatment X Hatching date		1, 33	0.63	0.45
Treatment X Brood size		1, 32	0.58	0.43
Cellular component immunity				
Leukocytes				
Treatment		1, 29	3.22	0.08
Hatching date	+0.73 (0.24)	**1, 33**	**9.51**	**0.004 ***
Brood size		1, 32	0.08	0.78
Treatment X Hatching date		1, 3	0.21	0.65
Treatment X Brood size		1, 31	0.37	0.55
Heterophils				
Treatment		1, 26	0.25	0.61
Hatching date	+0.30 (0.10)	**1, 33**	**8.26**	**0.007 ***
Brood size		1, 32	0.60	0.44
Treatment X Hatching date		1, 3	< 0.001	0.99
Treatment X Brood size		1, 31	1.46	0.24
Lymphocytes				
Treatment		1, 34	0.61	0.44
Hatching date		1, 33	3.51	0.07
Brood size		1, 32	0.05	0.82

Treatment X Hatching date		1, 3	0.11	0.82
Treatment X Brood size		1, 31	0.29	0.74
Eosinophils				
Treatment		1, 34	2.06	0.16
Hatching date	+0.02 (0.009)	**1, 33**	**6.4**	**0.02 ***
Brood size		1, 32	0.005	0.94
Treatment X Hatching date		1, 31	1.96	0.17
Treatment X Brood size		1, 3	0.05	0.82
Basophils				
Treatment		1, 34	0.99	0.33
Hatching date	+0.64 (0.64)	**1, 33**	**5.08**	**0.03 .**
Brood size		1, 32	1.68	0.20
Treatment X Hatching date		1, 31	1.41	0.24
Treatment X Brood size		1, 3	1.11	0.28
H/L ratio				
Treatment		1, 33	1.01	0.32
Hatching date		1, 32	2.65	0.11
Brood size		1, 31	0.13	0.72
Treatment X Hatching date		1, 3	0.55	0.46
Treatment X Brood size		1, 29	0.01	0.91

Discussion

Our long-term increase of nest predation risk significantly affected the immune system of blackbird nestlings. Predation risk induced changes in acquired immunity (IgY levels and the number of lymphocytes) and in some innate components (i.e. number of heterophils). Nevertheless, the direction of these immunological changes differed in each parameter and depended on the interaction with the developmental conditions of chicks. The effect of nest predation on IgY and lymphocytes depended on the body condition of nestlings, while the variation in heterophils was affected by their structural growth.

Nest predation risk and developmental conditions

Mediated effect of body condition

We found that the effect of our predation risk manipulation on IgY levels, which correspond to the humoral acquired component, depended on the body condition of chicks. In fact, the slopes of the relationship between IgY levels and body condition are contrasting for experimental and control nestlings (Table 1; Fig. 2A). This result does not fit with our prediction 1a or 1b, as the immunoenhancement or immunosuppressive effect will be highly dependent on the present condition of the chick. If the nestling is in poor body condition our results suggest an immunosuppressive effect given that the experimental chicks showed lower IgY levels compared to control ones. A potential mediator effect of stress hormones could be behind this result as it has been described that chronic exposure to stress can down-regulate the immune system (Martin 2009). However, the evidence for the link between glucocorticoids and nest predation is not clear and seems to depend on different factors like the age (Tilgar *et al.* 2010) and type of cue perceived by nestlings (Ibáñez-Álamo et al. 2015). For instance, blackbird nestlings decrease, rather than increase, corticosterone to a short-term elevation in predation risk (Ibáñez-Álamo *et al.* 2011). Despite the modification in IgY levels is direct or mediated by hormones, our results suggest that nestlings in poor body condition had a limited availability of resources to use for responding immunologically to the risk of predation. In such a weakened situation, these resources could be critical for other functions (i.e. sibling competition; Bourgeon *et al.* 2011) and thus, the costs associated to divert them from these functions would be greater than the benefits provided by the immune response towards a potential predator. By contrast, nestling in good body condition would have enough resources to invest in immune function in response to the possibility of being preyed upon. Immunoglobulins are particularly important to fight infections and pathogens (Lochmiller & Deerenberg 2000) and thus could prepare the organism to cope with the negative consequences following the encounter with the predator (group 3 of anti-predator responses) as this will likely produce injuries facilitating the action of pathogens and harmful microorganisms (Dhabhar & McEwen 1997). IgY levels did not vary in the only two studies in which this immune parameter was measured in a predation risk context (Chin et al., 2013, Roncalli *et al.* submitted). Nevertheless, both studies simulated a short-term (i.e. hours) increase in predation risk, so the differences with respect to our findings could depend on the duration of the exposure to the risk.

In contrast to the results for IgY levels, nestlings in poor condition increased part of their cellular immune response (lymphocytes) as a response to the risk of being depredated (Fig. 2B). This result supports the immunoenhancement effect (prediction 1a) even though according to our Bonferroni correction it is only marginally significant. Generally, chronic stress conditions tend to reduce the number of leukocytes (Dhabhar 2002), but contrasting results were found in the few previous studies that measured white blood cells in adult birds in relation to a chronic elevation of predation risk (Boonstra *et al.* 1998, Navarro *et al.* 2004, Clinchy *et al.* 2004). In a recent study using a short-term manipulation of the predation risk we found no significant changes in blackbirds' leukocyte profile (Roncalli et al. submitted), confirming the importance of the duration of risk in order to explore physiological responses to predation. Our findings also highlight the importance of nest predation to alter the trade-off between immunology and body condition (Fig. 2B). As it has been demonstrated in other studies (e.g. Navarro et al. 2003), control nestlings showed a significantly positive relationship between lymphocytes and body condition, but this association is lost under the risk of predation showing, for the first time, that long-term changes in predation risk can affect this trade-off, probably due to a redistribution of resources and a new balance of cost-benefits under the new situation.

Body condition is directly related to parental care and food provisioning (Karell *et al.* 2009, Brommer *et al.* 2011) and, some studies also found a positive association between immune defenses of chicks and parental effort or food supply (Saino *et al.* 1997, Hoi-Leitner *et al.* 2001, De Neve *et al.* 2007). This suggest that the role of parents to indirectly regulate the immune system of their offspring for preparing them to a threatening environment would occur therefore, not only by transferring immune factors *via* maternal effect during egg-laying (Morosinotto *et al.* 2013), but also through the parental behaviours carry out during the nestling period. This idea will need further experimental support but provides an interesting direction for future researces on the topic.

Mediated effect of growth

Despite the general importance of growth for the immune response of nestlings (Table 1) that has been describe in other studies (De Neve *et al.* 2007, Romano *et al.* 2011, Van der Most *et al.* 2011), we found that it is also important in an anti-predator context. The exposure to nest predation risk provoked a marginally significant change in the association between the number of heterophils and structural growth rate (Table 2, Fig. 2C). This means that those nestlings with slower growth rate for their tarsus had a higher number of heterophils in the experimental groups with respect to control nestlings, partially supporting for the immunoenhancement effect of nest predation risk (prediction 1a). As for body condition, this result also supports the idea that predation might alter established trade-offs in growing organisms. Growth is costly in energy and nutrient and therefore nestlings undergo trade-offs with other competing activities, such as immunity (Soler *et al.* 2003, Hawlena & Schmitz 2010, Van der Most *et al.* 2011). Our results indicated that nestlings with lower growth rate in tarsus length might employ the resources saved in growth to invest them in innate immunity response under nest predation risk, at least regarding their cellular component. Heterophils represents the predominant white blood cells in several bird species, carrying out important functions, such as the participation in inflammatory responses and the control of antimicrobial activity (Harmon 1998). Its participation in overcoming the consequences of a predator attack could be determinant.

Nest predation risk and environmental conditions

Hatching date is an important estimator of the seasonal variability of the environment where birds breed (Dubiec & Cichon 2005). Predation risk may change over the breeding season as predators need to cover the food demand of their offspring (Newton 1986). Seasonal variations in predation pressure are able to influence the behavioural mechanisms in anti-predatory responses (Roncalli et al. submitted). On the other hand, the effect of breeding season on immunity has been found in the number of white blood cells, both stimulating (Roncalli et al. submitted) and suppressing them (Sorci, Soler & Møller 1997, Dubiec & Cichon 2005). Our results are in accordance with those obtained in a previous study that found that blackbird nestlings hatching later in the season had higher number of white blood cells and IgY levels (Roncalli et al. submitted). Similarly to that study, this relationship was however unrelated to nest predation risk and no interaction between our treatment and extrinsic factors was found (Table 3). These results in addition to the interaction found for intrinsic factors, suggest that the latters are more important to modulate physiological anti-predator responses of nestlings, at least those involving the immune system.

Conclusions

The long-term increase in nest predation risk provoked the modifications of some components of blackbird nestlings' immune system, affecting principally to the acquired immunity (IgY and lymphocytes) and the innate cellular component (heterophils).

The different directions of the responses of each immunological component reflect the complexity of the relationship between the immune system and nest predation risk. These findings underline the importance of measuring several immunological parameters when studying nest predation (or other selective pressures) in order to fully understand their effect. In addition, we found that the developmental conditions of nestlings seem to play an important role in modulating the effect of nest predation risk on the immune system. But at the same time they suggest that nest predation risk seems to be able to alter the normal trade-off between development and the immune system which might have subtle, previously unknown consequences in developing organisms such as nestlings. Finally, our results also highlight the important role that parents can play to indirectly promote physiological anti-predator responses in altricial nestlings.

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GENERAL DISCUSSION

The study of the mechanisms by which selective pressures drive evolution is decisive to understand the adaptations that enhance the survival or reproduction of organisms (Endler 1986). Predation and brood parasitism are two biological interactions that exert important selective pressures (Møller *et al.* 1993, Abrams 2000). Birds are characterized by the fact that their reproductive cycle develops for a relative long period in a specific location, the nest (Bosque & Bosque 1995) and consequently, the selective force of both predation (i.e. nest predation) and brood parasitism is particularly intense during this phase. The findings of this thesis confirm that brood parasitism and nest predation strongly affect different traits of the two studied species and, more importantly, they provide the evidence that these two biological interactions may influence each other and interact simultaneously in relation to the nest environment.

The **first chapter** of the thesis allowed us to collect novel and detailed information on the breeding biology of Bonelli's warblers. No information on the breeding biology of this species was available for its core distribution area, the Iberian Peninsula, where more than 60% of its pairs breed. Obtaining information on the breeding parameters of a species is the first step to understand its biology and its conservation status (Green 2004), but also offered us the needed information in which to base subsequent studies (i.e. Chapter 2). Importantly, we found an extremely low breeding success in our study area, which draws the attention of the potential risk that Bonelli's warbler populations might face in Sierra Nevada and other Mediterranean mountains, and could explain the reduction of this species in this region during the last decades (Urios *et al.* 1991). Also in **Chapter 1**, and in agreement with several studies indicating that altitude represents an important abiotic factor capable of conditioning several biological and ecological traits in bird (Badyaev 1997, Lu *et al.* 2009, Hille & Cooper 2015), we found that this factor affected several breeding parameters of Bonelli's warblers, like clutch size or nestlings' growth rates. All information collected during this part allowed us to focus on the following one.

Regarding brood parasitism, we firstly explored a methodological question by analysing the possibility that the material used when manufacturing model eggs could affect the egg-rejection behaviour (**Chapter 2**). Many egg-rejection experiments have been performed through the use of artificial egg models (Davies & Brooke 1989, Soler & Møller 1990, Marchetti 1992, Lotem, Nakamura & Zahavi 1995, Marchetti 2000) and only recently some researchers have started to use natural eggs (Antonov *et al.* 2008, Soler *et al.* 2015, Ruiz-Raya *et al.* 2015 and 2016). But even though several studies have called attention about the reliability of using hard egg models (Moksnes *et al.* 1991a, Martín-Vivaldi *et al.* 2002, Prather *et al.* 2007), no previous study analysed the effect of soft egg models. We decided to test the hypothesis that soft egg models would be easier to eject using the Bonelli's warbler as model species because it is a small host and thus the costs associated to egg-rejection are particular severe; small hosts are puncture-ejectors, but sometimes they cannot eject parasitic eggs and, thus, are forced to desert the nest or to accept the egg (Antonov *et al.* 2009). We found that plasticine eggs were always ejected by Bonelli's warblers, while only 40% of the females that had been parasitized with natural eggs (of the same size) rejected them by deserting the nest. These results clearly demonstrated that this soft material overestimated ejection rate probably because the plasticine egg was easier to handle and eject. This finding suggests caution for the conclusions obtained from egg-rejection experiments using soft material and recommend the use of natural eggs whenever possible for this kind of studies.

In addition to the effect of material, also the size of the model egg used in the manipulation was determinant in conditioning the behaviour of Bonelli's warbler females and the rejection rates. The intensity by which parasites shape adaptive defences in hosts has been found to depend on several factors, including the possibility to incur in rejection costs (Antonov *et al.* 2008 and 2009), parasitism risk (Lindholm & Thomas 2000) and the internal status of motivation (Soler *et al.* 2012). The high rejection costs are maybe the most important factor that conditions the response to parasitism in small hosts (Antonov *et al.* 2009). In **Chapter 2** we found that, despite females of Bonelli's warblers clearly recognized the model eggs, ultimately, only 50% of them was able to eject the large model egg, while both the medium-sized and small eggs were always easily ejected. Physical constrains in managing large eggs seem to prevent their ejection, favouring nest desertion. Low rates of ejection support the result found for blackbirds, a medium-sized grasp-ejector host, which also reduced the ejection when a large egg was introduced in the nest (Soler *et al.* 2015). However, differently from blackbirds in which nest desertion cannot be considered an egg-rejection mechanism because its size allows to easily grasp the parasitic egg (Soler *et al.* 2015), deserting the nests would be the main response in Bonelli's warbler, as occurs for other *Phylloscopus* specie (Moksnes *et al.* 1991a). Importantly, the presence of large model eggs in the clutch reduced the time of incubation by 15% (at least in the first two hours after the introduction of the egg model), possibly originating concerns about egg viability. To our knowledge no other studies have quantified the effect of parasitic eggs on incubation time.

Despite the egg-recognition and rejection defences of Bonelli's warblers, none of the 71 nests monitored during the laying period was parasitized by common cuckoos. This result is in agreement with the extremely low parasitism rate found in southern Italy (1 of 45 nests) by Campobello and Sealy (2009) and suggest that Bonelli's warbler would be rarely parasitized nowadays. In Sierra Nevada, common cuckoos would prefer to parasitize other abundant passerines, such as European stonechats or European robins (Martínez *et al.* 2010, Martín-Vivaldi *et al.* 2013). More studies in other locations are however needed to confirm whether this warbler species is regularly used as host or not by common cuckoos.

Nevertheless, in addition to the characteristic of the parasitic egg, also external factors to the brood parasite-host system may be crucial. In **Chapter 3** we explored the effects of one of such external factors, predation risk. Predation, in particular nest predation, is an important driving force capable of producing adaptive responses in birds throughout their breeding cycle (Lima 2009), affecting for example the nest construction, clutch size, incubation activity or parental feeding behaviour (reviewed in Martin & Briskie 2009). To our knowledge, this is the first study that explores the effect of predation on brood parasitism, specifically on host's rejection behaviour, since most of the investigations done so far focused on the effect of parasite on predation (Hannon *et al.* 2009, Ibáñez-Álamo *et al.* 2012). Predation risk would modulate host responses to brood parasitism principally by affecting the decision of ejecting the parasitic eggs, because this activity could attract predators. Interestingly, this effect was found in those blackbird females exposed to an adult predation risk, but not in those exposed to an offspring predation risk, demonstrating that different kind of predators or a different predation context may affect the selective pressure on hosts. Predation risk would induce females to evaluate the costs and benefits related to ejection. Females in a risky situation for themselves (adult risk) ejected significantly fewer model eggs compared with females of the control group, probably in order to avoid the detection and the attack of a predator. Since the blackbird is a multi-brooded species (up to three broods per season in Spain; Aparicio 2008) and its maximum longevity is on average 10-13 years (Wasser & Sherman 2010), the likelihood to suffer important injuries, including death, while ejecting the egg could entail the loss of all future reproductive events. On the contrary, the costs associated to the acceptance of the putative parasitic egg would be lower as females would lose "only" a single reproductive event. This result matched with the fact that we found the lowest ejection rate at the end of the breeding period, which correspond to the maximum activity of sparrowhawks (Newton 1986), the predator that we used in our adult predation risk manipulation and that is considered one of the main predator of blackbirds (Gotmark & Post 1996, Nielsen & Møller 2006, Ibáñez-Álamo & Soler 2012). Therefore, the increase of predation pressure during the final phase of the reproductive season forced females to decide to keep the egg.

Another interesting result of this experiment is the absence of responses of blackbird females to offspring predation risk. This contrasts with the findings of another study which the use of magpie acoustic cues affected the incubation behaviour of blackbird females (Ibáñez-Álamo and Soler 2012). Plasticity in anti-predator strategies has been observed in several species suggesting that they change depending on the intensity of the predation pressure (Åbjörnsson *et al.* 2004, Vervust, Grbac & Van Damme 2007, Shaffery & Relyea 2016). Daily predation rate in the study area used by Ibáñez-Álamo and Soler (2012) is twice as high as that in the Valley of Lecrín (Ibáñez-Álamo & Soler 2010). Probably this is the reason behind our contrasting results. Nevertheless, these results point out that the effect of predation on brood parasitism should present a great degree of plasticity depending on the different predators and the intensity of the risk perceived by females during incubation.

The role of nest predation in driving the adaptive responses in birds throughout their breeding cycle is even more decisive for young than for adult individuals. Nest predation exerts an important selective pressure on nestlings since it is the primary cause of mortality in altricial species (Martin 1995). In fact, nest predation would be more costly for nestlings than for parents: in the first case predation would mean the complete loss of nestlings' fitness, whereas parents would lose "only" the fitness related to a single reproductive event. In **Chapters 4 and 5** we provided novel results indicating that the exposure to nest predation risk was able to induce changes in blackbird nestlings' immune system. In fact, studies analysing anti-predator strategies have focused mainly in morphological (Lima & Dill 1990, Swaddle & Lockwood 1998) and behavioural defences (Martín & López 2005, Wirsing & Ripple 2010, Creel *et al.* 2014). Only recently, researchers have started to consider also the physiological component, mainly the hormonal response, but its mechanisms are still poorly known, specifically for young individuals (Ibáñez-Álamo *et al.* 2015).

Both short-term (**Chapter 4**) and long-term (**Chapter 5**) increases in nest predation risk provoked changes in the nestling's immune system, indicating the importance of immunity in relation to the anti-predator defences. The few studies investigating the effect of predation on immunity used only one or few immunological parameters (Tilgar *et al.* 2010, Thomson *et al.* 2010, Chin *et al.* 2013, Goedert *et al.* 2014), whereas in our study we collected 12 parameters belonging to both the innate and acquired component. This procedure allowed us to obtain a complete overview of the immune response and actually, we found that not all immunological components respond to nest predation risk in the same way (**Chapters 4 and 5**). Furthermore, the direction of some responses is different from others (**Chapter 5**). This confirms the complexity of the interaction between predation risk and the immune system, whose components may act with different intensity (Roitt *et al.* 2001). Moreover, it should be stressed that immunological responses to nest predation could have been mediated by variation in hormones (i.e. corticosterone), which are known to have an important impact on immunity, mainly by suppressing certain components (Boonstra et al. 1998, Clinchy et al. 2004). Hormones usually increase in stressful situations (Clinchy et al. 2004, Hawlena & Schmitz 2010), but the evidence for the link between corticosterone and nest-predation induced stress is not clear (Tilgar et al. 2010; Ibáñez-Álamo et al. 2011, rewieved in Ibáñez-Álamo et al. 2015), hence it would be of interest for future studies to explore both the hormonal and immunological variations, as well as their interaction, in response to predation risk.

The acute short-term exposure to nest predation risk induced a general activation of the immune system (**Chapter 4**), supporting previous studies conducted in mammals (Dhabhar 2009, Martin 2009) in which the immediate response to an acute stress situation was a general activation of the immune system, while the immunosuppression occurred only after some time (i.e. hours). The immediate activation of immunity in the context of a predator attack could be decisive for the reinforcement of nestlings' conditions in order to promote the survival of the organisms. In fact, the injuries provoked by a predator encounter could facilitate inflammation, possible infections and diseases, which may ultimately result in a significant fitness reduction (even death). Curiously, the increase in immunity significantly occurred only under an extreme and high nest predation risk, whereas a moderate risk (predator calls) did not affect nestlings' response. This contrast with what we found in **Chapter 5**, where the same moderate risk, but covering the whole nestling period, was sufficient to provoke an immune response. It seems clear that the duration of the exposure to predator acoustic calls would be determinant in the activation of the immune response (Martin 2009). Growing under a constant nest predation risk would provoke more subtle but continuous changes (Zanette *et al.* 2011, Clinchy *et al.* 2013). The exposure to long-term nest predation risk induced an immunosuppression in the humoral acquired immunity (i.e. immunoglobulins) and confirms therefore the general assumption that immunosuppression probably occurs when stress situations become chronic (Martin 2009). On the contrary, the cellular component showed an increase, which is in agreement with the findings of Chapter 4 already underlining the complexity of the relationship between the immune system and nest predation risk.

An interesting and relevant result found in both **Chapters 4 and 5** is the influence of the condition of nestlings, such as the health status or body condition, on the immunological response to nest predation. This would indicate the constrain of nestlings exposed to nest predation in adjusting their immunological responses (increase or reduction) depending on the consequences that such immunological variations may provoke on other biological functions. Immune system participates in the protection of parasitic infestation (Navarro *et al.* 2004, Wojczulanis-Jakubas *et al.* 2012), and this function is particularly important in nestlings (De Coster *et al.* 2010). In addition, the nestling stage involves an important energetic and nutritional investment associated to their development (Ricklefs 1983) which can interact with immunity resources (Soler *et al.* 2003). In accordance to this premises, we found that the effects of nestlings' condition on immune response were stronger during the long-term manipulation (**Chapter 5**) since the reduction of immunoglobulins and the increase of lymphocyte and heterophils were mediated by body condition and growth rate. In contrast, for the short-term manipulation (**Chapter 4**), the levels of ovotransferrin, immunoglobulins and eosinophils directly increased. These differences seem to suggest that the immunological response against nest predation risk during the whole nestling period would be particularly costly for nestlings because, during this critical phase of their life, they must optimize the trade-offs between allocating resources to anti-predator defences or to their growth and development (Hawlena & Schmitz, 2010, Van der Most *et al.* 2011). On the contrary, costs associated to a short-term stress situation are generally transitory and can be compensated after the end of the event which provoked stress (Eggers, Griesser & Ekman 2008).

In both **Chapters 4 and 5**, we found a strong effect of breeding season on immunological parameters, and this effect was unrelated with nest predation risk. Breeding season reflect the seasonal variation of environmental conditions (Dubiec & Cichon 2005). Higher levels of immunity usually suggest an activation of the immune system which usually occurred under poor environmental conditions (De Coster *et al.* 2010, Lindström *et al.* 2004). Our study area is characterized by an increase in temperature (Spanish Meteorological Agency) and in the number of ectoparasites (per. obs) throughout the season, which probably would be the responsible of the deterioration of the environmental conditions and therefore impose an important investment in immunity for nestlings. Further studies on this topic would be helpful to understand which environmental factors provoke the observed increase.

Finally, it is important to point out the interesting results in relation to the acoustic cues used to increase nest predation risk in **Chapter 4**. Our nest predation risk manipulation involved the use of predator calls, parental alarm calls and distress calls in order to create a moderate, high and extreme level of nest predation risk respectively. Among the three experimental manipulations, only that using predator calls cues did not provoke significant immunological changes, thus contrasting with the results found in the long-term manipulation where the same predator cues induced immunological responses in nestlings (**Chapter 5**). Studies involving the use of parental alarm calls and the distress calls also in the long-term manipulation would be helpful to investigate the existence of a progression in the intensity of the immunological responses according to the acoustic cues. The strong variation of the immune system in response to adult and distress calls suggests that the direct communicative channels between parents and offspring would be more efficient in assessing the predation threat (Magrath *et al.* 2007, Suzuki 2011, Suzuki 2014). Parental alarm calls contain detailed information about the nature of predators (Platzen & Magrath 2005), predator distance or behaviour (Suzuki 2011), which can be processed by nestlings in order to decide the best defence to adopt. Thus, our results are of special interest for those studies in which researchers need to manipulate nest predation risk by acoustic cues, but at the same time indicate that the selection of acoustic cues for this kind of studies is of utmost importance.

To sum up, the findings of this thesis suggest that brood parasitism and nest predation are important forces affecting behavioural and physiological traits in birds and, more importantly, that they may condition each other while doing so. Given the few studies investigating the relationships between these two biological interactions, this work could be a stimulus to steer future research in order to better comprehend the mechanisms by which they interact. An interesting future research line could be exploring, for example, the plasticity of hosts’ responses under different levels of predation pressure and in different phases of brood parasitism (i.e. pre-laying or chick stage). Especially interesting would be to investigate this topic in those hosts that are currently being parasitized and that simultaneously suffer high predation rate (i.e. reed warbler (*Acrocephalus scirpaceus*) or European robins). In addition, we demonstrated that the immune system could play an important role in avian anti-predator responses, probably by reinforcing the immunity of the nestlings in order to prevent possible negative effects of the predator’s attack. Future studies investigating the role of immune responses in different context of predation pressure might improve the understanding of how nestlings cope with the immediate consequence of a predator’s attack on the one hand, and with the constant threat of living under predation risk (the ecology of fear) on the other.

CONCLUSIONS/CONCLUSIONES

CONCLUSIONS

1. We provide for the first time detailed information about Bonelli's warbler breeding biology in the core area of its distribution. The low breeding success of 25 % addresses concerns about the potential risk that Bonelli's warbler might face in the near future, considering its reduction in Sierra Nevada during the recent decades.

2. The size of the parasitic egg affected rejection responses in Bonelli's warblers. Large model eggs hampered ejection, while favouring desertion, probably as a consequence of a physical constraint in manipulating the parasitic egg. Moreover, large model egg reduced the incubation time in about 15%, possibly to the detriment of eggs viability.

3. The plasticine model eggs do not provide reliable estimates of ejection and desertion rates, since this soft material facilitates the ejection and so overestimates ejection rate. Conclusions of egg-rejection experiments using egg models made of soft material should be considered with caution and the use of real eggs would be preferred whenever possible.

4. Predation risk affected the host's defences to brood parasitism. Blackbird females changed their egg-rejection behaviour depending on the type of predator. Adult predation risk modified part of the egg-rejection process, since females of this group touched more frequently the eggs during the incubation. Despite the egg-recognition, some of the females exposed to an adult predator decided not to eject the foreign egg, suggesting that the parasitic egg is not a stimulus strong enough to induce female blackbirds to assume the very high (deadly) potential cost of ejecting it in the presence of an adult predator.

5. Offspring predation risk did not affect egg-rejection process, probably because the loss of a single brood is not so costly, for a blackbird, as the death of the adult individual, which corresponds to the loss of all future reproductive events. Females thus would optimise the investment between the current brood and the self-maintenance in the framework of life-history theory.

6. Both the short- and long-term increase of nest predation risk induced changes in the immune response of blackbird nestlings, indicating that anti-predator strategies in offspring could have an important immunological component that requires further investigation.

7. A short-term increase of nest predation risk induced an increase in both humoral and cellular immunity (ovotransferrin, immunoglobulins levels, lymphocytes and

eosinophils), suggesting that the perception of a potential predator could lead blackbird nestlings to stimulate their immunity to prepare the organism to cope with the deleterious consequences of a predator attack, such as inflammation and infections.

8. The increase of nest predation risk during the whole breeding period determined both a suppression of immunoglobulins and an enhancement of lymphocytes and heterophils. Growing under a constant nest predation risk would provoke therefore more subtle but chronic changes, which may become physiologically costly and negatively affect the nestling's future fitness.

9. The condition of nestlings seem to be relevant in mediating the immunological changes associated to nest predation risk. In particular, health status (i.e. endoparasites), body condition and structural growth conditioned the response of several components of immunity to nest predation.

10. The level of threat presented by different acoustic cues influence the immunological response to nest predators in blackbird nestlings. Alarm and distress calls are more effective than direct sounds of potential predators which highlight the importance of testing different types of predation risk when studying physiological anti-predator responses.

CONCLUSIONES

1. Hemos aportado por primera vez información detallada sobre la biología reproductiva del mosquitero papialbo en el centro de su área de distribución. Su escaso éxito reproductivo (25%) explica el descenso del número de individuos detectado durante las últimas décadas en Sierra Nevada y apoya la preocupación existente sobre el futuro del mosquitero papialbo en esta zona

2. El tamaño del huevo parásito afectó a la respuesta de expulsión en el mosquitero papialbo. Los modelos de mayor tamaño dificultaron la expulsión, favoreciendo el abandono del nido, probablemente como consecuencia de un impedimento físico en la manipulación del huevo parásito. Además, el modelo de mayor tamaño redujo el tiempo de incubación en un 15%, lo que podría tener un efecto en la viabilidad de los huevos.

3. Los huevos hechos con plastilina no proporcionaron la tasa real de expulsión y de abandono, ya que este material blando favoreció la expulsión del huevo y por tanto sobreestimó la tasa de expulsión. Por lo tanto, las conclusiones obtenidas en experimentos de expulsión de huevos que utilizan modelos hechos de material más blando deberían de ser consideradas con precaución. Siempre que sea posible deberían de utilizarse huevos reales.

4. El riesgo de depredación afectó las defensas de los hospedadores frente al parasitismo de cría. Las hembras del mirlo común cambiaron su comportamiento de expulsión de huevos dependiendo del tipo de depredador. El riesgo provocado por depredadores de adultos modificó parte del proceso de expulsión del huevo parásito, ya que las hembras de este grupo tocaron los huevos con su pico con más frecuencia durante la incubación. A pesar de reconocer el huevo parásito, algunas de las hembras expuestas al riesgo de depredación de adultos decidieron no expulsarlo, lo que sugiere que ese huevo no sería un estímulo lo suficientemente fuerte como para inducir a las hembras a asumir el alto coste potencial (depredación) asociado a su expulsión en presencia de un depredador de adultos.

5. El riesgo de depredación del nido no afectó al proceso de expulsión de huevos, probablemente porque la pérdida de un solo evento reproductor no es tan costoso para un mirlo como su propia muerte que le supondría la pérdida de todas las reproducciones futuras. Por lo tanto, las hembras de mirlo optimizarían la inversión entre su reproduccíon presente y el mantenimiento de su condición en el marco teórico de la teoría de estrategias vitales.

6. El aumento del riesgo de depredación del nido, tanto a corto como a largo plazo produjo una variación en el sistema inmune de los pollos de mirlo, lo que indica

una gran importancia del sistema inmune como componente de las respuestas fisiológicas anti-depredadoras.

7. El aumento a corto plazo del riesgo de depredación de nidos produjo un aumento tanto en el componente humoral como en el celular del sistema inmune (ovotransferrina, inmunoglobulina, linfocitos y eosinófilos), lo que sugiere que la percepción de un depredador potencial podría conducir a los pollos de mirlo a estimular su sistema inmune para preparar su organismo para hacer frente a las consecuencias deletéreas, como procesos inflamatorios e infecciones, de un ataque por parte de un depredador.

8. El incremento del riesgo de depredación durante el periodo de estancia de los pollos en el nido determinó tanto la supresión de las inmunoglobulinas como el incremento del número de linfocitos y heterófilos. Desarrollarse en condiciones de riesgo de depredación permanente provocaría por lo tanto cambios más sutiles, pero constantes, que podrían ser fisiológicamente costosos y afectar negativamente a la futura eficacia biológica de los pollos.

9. La condición física de los pollos parece jugar un papel relevante en la mediación de los cambios inmunológicos asociados al riesgo de depredación de nidos. Concretamente, el estado de salud (i.e. endoparásitos), la condición corporal y la tasa de crecimiento condicionan las respuestas de numerosos componentes del sistema inmune frente a la depredación de nidos.

10. Los niveles altos y extremos de riesgo de depredación del nido son los que produjeron cambios significativos en el sistema inmune, lo que indica que los pollos de mirlo serían capaces de modular su sistema inmune en respuesta al riesgo de depredación según el nivel de amenaza que perciben.

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SUPPLEMENTAL INFORMATION

SI 1

Short-term increase in predation risk affects the immune system of nestlings

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Calls used to compose playbacks of AC, PC and CON groups were selected from a virtual platform on the web (www.xeno-canto.org). PC playbacks included calls emitted by different predators that were observed in the study area, such as Eurasian sparrowhawks *Accipiter nisus*, Booted eagles *Hieraeetus pennatus* or Common kestrels *Falco tinnunculus*. Playbacks for CON group included songs and calls of several passerine species present in the area, such as European serin *Serinus serinus*, European goldfinch *Carduelis carduelis*, Sardinian warbler *Sylvia melanocephala* or common chaffinch *Fringilla coelebs*. We excluded alarm call vocalizations of these species, which may indirectly indicate the potential presence of a predator in the surroundings of the nest. For AC playbacks we selected 15 blackbird alarm calls from both males and females, by including the several kind of alarm calls emitted by this species. For example, females emit a typical rhythmical whistle especially when predator is at the proximity of the nest, while a strong and impetuous call is used when predator gets closer. Regarding the playbacks of DF group, we could not use the virtual platform as only few distress calls of blackbird nestlings were available on the web. Thus, we used distress calls that were previously recorded from nestlings of our population. To do this, we chose 10 nestlings from 6 broods and we recorded their distress calls (Sony ICD-PX333 Digital Voice Recorder), while we was handling them. This operation was done inside the car to ensure that the other nestlings in the area could not listen to. By this, we avoided undesirable background sounds (i.e. alarm calls) and we standardized the recording parameters. All recorded sounds were successively converted to digital audio files by using Audacity software (version 2.1.0).

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SI 2: Humoral immunity

ID	NEST	CHICK	TREAT	H. DATE	L.ZOON	BODY COND	HA	HP	OVT	NOX	IGY
TM15111AP1	TM15111A	P1	pred	81	1	1,5049	10,75	0,1625	7,5187	0,006507	0,2564
TM15111AP2	TM15111A	P2	pred	81	0	5,9555	11	0,1382	9,8577	0,017167	0,1262
TM15111P1	TM15111	P1	pred	46	0	3,3959	3,75	0,1468	8,2135	0,035140	0,1624
TM15111P2	TM15111	P2	pred	46	0	1,5572	NA	0,1982	NA	NA	0,0907
TM15111P3	TM15111	P3	pred	46	0	-0,719	4	0,0871	8,7822	0,010185	0,0793
TM15113P1	TM15113	P1	pred	55	0	-4,8356	3	0,1224	3,6111	0,003560	0,2324
TM15113P2	TM15113	P2	pred	55	0	-0,1851	6	0,1460	5,6089	0,000903	0,2084
TM15113P3	TM15113	P3	pred	55	0	-4,2664	5,5	0,1578	NA	0,002355	0,2039
TM15119P1	TM15119	P1	control	41	0	1,0806	4	0,1795	NA	0,002757	0,1010
TM15123P1	TM15123	P1	control	46	0	3,6029	NA	NA	NA	NA	0,1697
TM15123P2	TM15123	P2	control	46	0	3,0758	NA	NA	6,7840	NA	0,1210
TM15123P3	TM15123	P3	control	46	0	-0,1819	NA	NA	4,7562	NA	0,1197
TM15126P1	TM15126	P1	control	46	0	-0,3628	NA	NA	NA	0,004361	0,1290
TM15126P2	TM15126	P2	control	46	NA	1,3046	7	NA	NA	0,002703	0,3824
TM15130P1	TM15130	P1	pred	49	0	1,7983	6	0,1409	NA	0,003450	0,1208
TM15130P2	TM15130	P2	pred	49	0	0,852	4,25	0,1218	3,7319	0,007505	0,1534
TM15130P3	TM15130	P3	pred	49	0	3,7182	2	0,1371	7,2058	0,005329	0,2326
TM15137P1	TM15137	P1	adult	48	0	0,2063	4,5	NA	NA	NA	0,1280
TM15137P2	TM15137	P2	adult	48	0	1,1791	7	0,1935	7,9004	0,003650	0,1547
TM15143P1	TM15143	P1	adult	59	0	0,6575	4,75	0,1723	NA	0,003560	0,1392
TM15143P2	TM15143	P2	adult	59	0	-2,1746	3,5	0,1604	7,7272	0,003622	0,1986
TM15161P1	TM15161	P1	pred	65	0	3,2744	6	0,1987	NA	0,001755	0,2581
TM15161P2	TM15161	P2	pred	65	0	-5,3579	6	0,2229	NA	NA	0,1434
TM15167P1	TM15167	P1	control	58	1	5,1588	6	0,1547	8,1951	0,004108	0,1483
TM15167P2	TM15167	P2	control	58	1	-2,2557	4,75	0,1455	5,4840	0,004771	0,1243

TM15170P1	TM15170	P1	control	58	0	-1,7238	4,5	0,1751	8,1469	0,005604	0,0632
TM15170P2	TM15170	P2	control	58	0	3,5589	5	0,1753	10,2386	0,009253	0,0460
TM15170P3	TM15170	P3	control	58	0	6,792	4,5	0,1007	NA	0,004143	0,0898
TM15179P1	TM15179	P1	control	39	1	-1,9286	6	0,2229	6,7680	0,014677	0,3151
TM15179P2	TM15179	P2	control	39	0	-2,7016	7,5	0,1012	5,0503	0,017271	0,1989
TM15182P1	TM15182	P1	control	39	0	3,7948	2,5	0,2183	NA	0,005308	0,1236
TM15182P2	TM15182	P2	control	39	0	2,2876	2,75	0,1889	7,5274	0,012342	0,2646
TM15182P3	TM15182	P3	control	39	0	-2,7988	5,5	0,1570	8,5133	0,003698	0,1490
TM15186P1	TM15186	P1	juvenile	57	0	3,4352	2	0,2008	NA	0,007817	0,0779
TM15186P2	TM15186	P2	juvenile	57	0	3,6658	4,75	0,2405	NA	0,005764	0,0738
TM15187P1	TM15187	P1	juvenile	47	1	1,445	3,5	0,1528	6,2084	NA	0,1908
TM15191P1	TM15191	P1	pred	62	0	2,1594	10,5	0,2460	8,0770	NA	0,2627
TM15191P2	TM15191	P2	pred	62	0	-0,0458	11	0,2492	9,7754	0,014370	0,2966
TM15194P1	TM15194	P1	adult	62	0	7,1509	NA	NA	NA	NA	0,2357
TM15194P2	TM15194	P2	adult	62	0	4,6716	NA	NA	NA	NA	0,1763
TM15194P3	TM15194	P3	adult	62	0	4,3957	NA	NA	NA	NA	0,1098
TM15197P1	TM15197	P1	control	71	0	9,2083	2	0,2034	7,2366	0,005444	0,1611
TM15197P2	TM15197	P2	control	71	0	1,897	2,5	0,2277	9,4346	NA	0,1958
TM15213P1	TM15213	P1	juvenile	67	0	-6,8834	11	0,1703	NA	0,024777	0,2020
TM15213P2	TM15213	P2	juvenile	67	0	-7,6413	4	0,1310	NA	0,003657	0,1027
TM15222P1	TM15222	P1	juvenile	58	0	4,1216	3,25	0,1351	NA	0,004856	0,2752
TM15222P2	TM15222	P2	juvenile	58	0	2,5832	3	0,1651	7,5683	0,003553	0,1709
TM15254P1	TM15254	P1	pred	64	1	4,8371	10,5	0,2461	NA	0,003286	0,2959
TM15254P2	TM15254	P2	pred	64	0	-1,7	2	0,2984	NA	0,005715	0,2382
TM15254P3	TM15254	P3	pred	64	1	-1,3356	11	0,2642	5,1444	0,005673	0,3036
TM15254P4	TM15254	P4	pred	64	1	-8,8545	11	0,1439	7,3493	0,006532	0,1546
TM15260P1	TM15260	P1	pred	70	NA	-0,438	11	0,1653	7,1983	0,003555	0,1369
TM15260P2	TM15260	P2	pred	70	NA	3,3655	7	0,2153	5,4500	0,002265	0,1579
TM15262P1	TM15262	P1	adult	58	0	8,3829	5,5	0,2044	NA	0,006730	0,1437
TM15266P1	TM15266	P1	juvenile	70	NA	-0,9118	11	0,1975	NA	NA	0,1032

TM15266P2	TM15266	P2	juvenile	70	NA	2,1948	3	0,2148	9,0937	0,009810	0,1295
TM15284P1	TM15284	P1	juvenile	67	0	1,8926	5	0,1898	11,1565	0,005953	0,1020
TM15284P2	TM15284	P2	juvenile	67	1	5,0572	4	0,1695	4,9251	NA	0,1014
TM15284P3	TM15284	P3	juvenile	67	1	1,7893	7,5	0,2007	8,4061	0,016413	0,0867
TM15285P1	TM15285	P1	control	72	0	-1,9244	5	0,2442	7,4188	0,005919	0,1420
TM15285P2	TM15285	P2	control	72	1	-1,4394	11	0,1943	NA	0,010766	0,1912
TM15285P3	TM15285	P3	control	72	1	-4,1935	11	0,1527	6,9486	0,003175	0,1748
TM15288P1	TM15288	P1	control	72	0	2,7811	8	0,2132	8,4178	0,008052	0,2459
TM15288P2	TM15288	P2	control	72	0	-1,8746	5	0,2220	NA	0,010241	0,2359
TM15288P3	TM15288	P3	control	72	0	-0,778	6,75	0,1373	1,5871	0,005988	0,2454
TM15300P1	TM15300	P1	control	83	0	0,5887	11	0,0927	7,3894	0,007027	0,3429
TM15308P1	TM15308	P1	pred	85	0	-5,9599	NA	0,1328	NA	0,007274	0,3184
TM15317P1	TM15317	P1	adult	85	0	-3,9767	2	0,1128	6,9099	0,003656	0,3472
TM15317P2	TM15317	P2	adult	85	0	-1,3189	11	0,1858	9,0819	0,004731	0,3694
TM15317P3	TM15317	P3	adult	85	0	4,757	NA	0,1446	6,6489	NA	0,2611
TM15320P1	TM15320	P1	adult	91	0	0,4689	NA	0,1519	7,4658	NA	0,3564
TM15324P1	TM15324	P1	juvenile	72	0	-3,7629	NA	0,2301	10,7158	0,008069	0,2627
TM15324P2	TM15324	P2	juvenile	72	0	-2,0862	11	0,2114	7,7596	0,003648	0,2666
TM15324P3	TM15324	P3	juvenile	72	0	-4,027	2	0,1953	8,2768	0,006865	0,2001
TM15329P1	TM15329	P1	adult	68	1	0,0187	8,5	0,1763	6,7678	NA	0,2655
TM15335P1	TM15335	P1	adult	78	0	5,0849	5	0,1409	NA	0,015085	0,1971
TM15335P2	TM15335	P2	adult	78	1	2,9457	9,25	0,1065	NA	0,017703	0,1452
TM15335P3	TM15335	P3	adult	78	1	3,3051	11	0,1057	NA	0,025558	0,2354
TM15340P1	TM15340	P1	adult	97	0	0,1306	11	0,1679	NA	NA	0,2470
TM15340P2	TM15340	P2	adult	97	0	-0,3983	10	0,1881	NA	NA	0,4548
TM15340P3	TM15340	P3	adult	97	0	1,1695	8,5	0,1369	4,9986	0,005257	0,4447
TM1534P1	TM1534	P1	adult	36	NA	0,5602	6,5	0,1651	6,6547	0,002939	0,0956
TM1534P2	TM1534	P2	adult	36	0		6,5	0,1504	4,8361	NA	0,1247
TM1534P3	TM1534	P3	adult	36	0	0,9554	3	0,1576	7,1410	0,003184	0,1889
TM15362P1	TM15362	P1	juvenile	84	1	1,8557	NA	NA	12,1362	0,005121	0,2384

TM15362P2	TM15362	P2	juvenile	84	0	-7,4474	NA	0,1777	NA	0,006124	0,1444
TM15362P3	TM15362	P3	juvenile	84	1	-2,878	11	0,2051	12,1497	0,007471	0,3006
TM15367P1	TM15367	P1	pred	94	0	-6,4885	11	0,1293	NA	0,015399	0,1467
TM15367P2	TM15367	P2	pred	94	0	-6,3951	11	0,1618	9,2656	0,009802	0,2714
TM15367P3	TM15367	P3	pred	94	0	-5,9374	2,25	0,1807	6,8428	0,012347	0,2982
TM15367P4	TM15367	P4	pred	94	0	-4,4561	NA	0,1627	8,4193	0,004324	0,1186
TM15371P1	TM15371	P1	pred	98	0	6,347	11	0,0630	9,0752	0,004722	0,2190
TM15371P2	TM15371	P2	pred	98	0	-0,7261	9	0,1299	8,1724	0,007087	0,2880
TM15371P3	TM15371	P3	pred	98	0	1,1591	NA	0,1078	8,2994	0,004064	0,3384
TM15409P1	TM15409	P1	adult	93	0	1,0641	11	0,2075	4,6318	0,003808	0,3687
TM15409P2	TM15409	P2	adult	93	0	-8,8775	11	0,1115	4,3745	0,008540	0,4085
TM15409P3	TM15409	P3	adult	93	0	-1,5409	7	0,1045	4,3686	0,004892	0,3966
TM15409P4	TM15409	P4	adult	93	1	5,1811	9	0,1382	8,8707	0,004460	0,4369
TM15415P1	TM15415	P1	pred	101	0	4,4018	6,75	0,1471	6,7265	0,004571	0,1299
TM15415P2	TM15415	P2	pred	101	0	-7,78	NA	0,1485	8,9412	0,003493	0,1979
TM15415P3	TM15415	P3	pred	101	0	-0,1492	NA	0,1500	7,3190	0,004000	0,1662
TM15417P1	TM15417	P1	pred	98	1	0,8799	NA	0,1141	7,7845	0,003349	0,1126
TM15417P2	TM15417	P2	pred	98	0	-1,5234	5	0,1419	7,9890	NA	0,2033
TM15417P3	TM15417	P3	pred	98	0	3,4798	3	0,2117	11,4027	0,004724	0,1098
TM15439P1	TM15439	P1	juvenile	96	0	-2,7896	NA	NA	NA	NA	0,1465
TM15439P2	TM15439	P2	juvenile	96	0	-3,1948	7,75	NA	NA	NA	0,2197
TM15439P3	TM15439	P3	juvenile	96	0	-0,3105	11	NA	NA	NA	0,1832
TM15443P1	TM15443	P1	control	83	0	-2,768	11	0,1444	8,0711	0,002821	0,3220
TM15443P2	TM15443	P2	control	83	0	-7,2725	11	0,1881	NA	0,003732	0,3488
TM15443P3	TM15443	P3	control	83	0	0,4184	9,5	0,1908	10,4153	0,006976	0,2472
TM15444P1	TM15444	P1	juvenile	85	1	5,103	8	0,1157	5,1620	0,003986	0,1841
TM15444P2	TM15444	P2	juvenile	85	0	-0,8169	11	NA	8,1122	0,002863	0,2578
TM15446P1	TM15446	P1	juvenile	85	0	-10,261	NA	0,1198	NA	0,009707	0,1578
TM15446P2	TM15446	P2	juvenile	85	0	-3,0562	11	NA	10,2985	0,007377	0,1649
TM15446P3	TM15446	P3	juvenile	85	0	-2,7935	11	0,1151	8,0946	0,007411	0,2027

TM15449P1	TM15449	P1	adult	99	1	-0,578	5,75	0,1111	7,2132	0,004680	0,2175
TM15449P2	TM15449	P2	adult	99	0	-1,1055	3,5	0,1212	6,8041	0,002699	0,1093
TM15450P1	TM15450	P1	adult	80	0	5,2661	NA	0,1633	NA	0,003023	0,1384
TM15450P2	TM15450	P2	adult	80	0	4,1624	NA	0,1477	NA	0,003605	0,2014
TM15450P3	TM15450	P3	adult	80	0	3,7728	11	0,1449	7,1709	0,003192	0,2901
TM15451P1	TM15451	P1	control	90	1	-1,9159	NA	NA	NA	NA	0,1390
TM15451P2	TM15451	P2	control	90	0	-1,6293	11	NA	NA	NA	0,2093
TM15451P3	TM15451	P3	control	90	0	1,8642	NA	NA	NA	NA	0,2336
TM15453P1	TM15453	P1	adult	100	0	0,051	NA	0,1597	10,2742	0,006625	0,1545
TM15453P2	TM15453	P2	adult	100	0	-0,051	4	0,1799	9,9709	0,005896	0,0951
TM15456P1	TM15456	P1	juvenile	102	0	-8,396	11	0,1484	9,2515	0,003341	0,1675
TM15462P1	TM15462	P1	juvenile	100	0	-0,7292	10	0,1416	8,5815	0,006495	0,1982
TM15462P2	TM15462	P2	juvenile	100	0	-6,6545	4	0,1938	13,1518	0,003702	0,2378
TM15463P1	TM15463	P1	juvenile	101	0	3,2911	NA	0,1416	10,0556	0,005475	0,1750
TM15463P2	TM15463	P2	juvenile	101	0	3,6405	11	0,1222	9,3573	0,003469	0,3295
TM1552P1	TM1552	P1	control	35	0	-1,3039	2	0,1244	11,0047	0,003609	0,1341
TM1552P2	TM1552	P2	control	35	0	-5,592	2,75	0,0869	6,1551	0,006101	0,0761
TM1552P3	TM1552	P3	control	35	NA	-2,277	4	0,1229	4,8161	0,002189	0,1125
TM1563P1	TM1563	P1	adult	40	0	1,0928	5,5	0,1947	6,3483	0,002898	0,3209
TM1563P2	TM1563	P2	adult	40	0	-3,4986	5	0,1215	4,9093	0,005699	0,1730
TM1563P3	TM1563	P3	adult	40	0	-1,5679	4	0,1405	5,2158	0,003245	0,2607
TM1567AP1	TM1567A	P1	control	69	0	2,2863	3,5	0,1983	8,1272	0,002050	0,2077
TM1567AP2	TM1567A	P2	control	69	0	-1,0646	11	0,3317	10,4160	0,003758	0,2946
TM1568P1	TM1568	P1	adult	55	0	3,7705	3,25	0,2237	8,3537	NA	0,4720
TM1568P2	TM1568	P2	adult	55	0	-0,7771	5	0,2002	8,0970	0,015778	0,1836
TM1568P3	TM1568	P3	adult	55	0	0,2026	9,75	0,1035	NA	0,007270	0,3409
TM1573P1	TM1573	P1	juvenile	49	0	-5,3899	6,5	0,1137	NA	0,003816	0,2720
TM1573P2	TM1573	P2	juvenile	49	0	2,6847	2,5	0,2697	NA	0,015273	0,2426
TM1579AP1	TM1579A	P1	juvenile	53	0	-1,5749	6	0,0851	NA	0,002609	0,1947
TM1579AP2	TM1579A	P2	juvenile	53	1	0,7475	3	0,1479	7,0699	0,009435	0,2442

TM1579P1	TM1579	P1	pred	45	0	-1,1958	3,5	0,1419	8,1651	0,002576	0,1901
TM1579P2	TM1579	P2	pred	45	0	0,9944	5	0,1483	NA	NA	0,0493
TM1579P3	TM1579	P3	pred	45	0	-3,4381	7	0,1737	9,4725	0,003955	0,1363
TM1581AP1	TM1581A	P1	juvenile	40	0	-5,0409	NA	NA	NA	NA	0,1152
TM1581AP2	TM1581A	P2	juvenile	40	0	0,45	NA	NA	NA	NA	0,2870
TM1581AP3	TM1581A	P3	juvenile	40	0	2,0721	NA	NA	NA	NA	0,1357
TM1583AP1	TM1583A	P1	adult	39	0	5,1076	4,5	0,1256	11,3622	NA	0,1746
TM1583AP2	TM1583A	P2	adult	39	0	1,8457	4	0,1767	NA	0,003990	0,0898
TM1583AP3	TM1583A	P3	adult	39	0	-1,0542	3,75	0,2097	7,2010	NA	0,1206
TM1591P1	TM1591	P1	juvenile	48	1	0,317	5	0,1364	6,8479	0,002435	0,1781
TM1591P2	TM1591	P2	juvenile	48	0	5,5249	2,75	0,1125	NA	0,004629	0,1426
TM1591P3	TM1591	P3	juvenile	48	0	1,5732	3	0,1282	NA	0,004956	0,1331
TM1591P4	TM1591	P4	juvenile	48	1	6,6926	4,75	0,1777	8,6132	0,008402	0,1053
TM15FRANP1	TM15FRAN	P1	pred	49	0	3,7168	10,5	0,2099	6,7429	0,011586	0,3430
TM15FRANP2	TM15FRAN	P2	pred	49	0	-1,2098	11,25	0,2190	NA	NA	0,2040

Short-term increase in predation risk affects the immune system of nestlings

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SI 3: Cellular immunity

ID	NEST	CHICK	TREAT	H. DATE	L.ZOON	BODY COND	LEU	HET	LYM	EOS	H/L
TM15111AP1	TM15111A	P1	pred	81	1	1,5049	69,0131	22,0842	46,9289	0,0000	0,4706
TM15111AP2	TM15111A	P2	pred	81	0	5,9555	29,8401	8,0568	21,7832	0,0000	0,3699
TM15111P1	TM15111	P1	pred	46	0	3,3959	40,3097	9,9777	27,5383	2,3946	0,3623
TM15111P2	TM15111	P2	pred	46	0	1,5572	23,8601	6,8509	16,5367	0,4772	0,4143
TM15111P3	TM15111	P3	pred	46	0	-0,719	51,5043	27,0270	20,9077	3,6053	1,2927
TM15113P1	TM15113	P1	pred	55	0	-4,8356	34,7826	13,9130	20,8696	0,0000	0,6667
TM15113P2	TM15113	P2	pred	55	0	-0,1851	59,0319	10,6257	46,6352	1,7710	0,2278
TM15113P3	TM15113	P3	pred	55	0	-4,2664	47,9226	11,9806	34,5042	1,4377	0,3472
TM15119P1	TM15119	P1	control	41	0	1,0806	37,2273	20,1027	16,0077	1,1168	1,2558
TM15123P1	TM15123	P1	control	46	0	3,6029	30,1350	9,0405	19,5878	1,5068	0,4615
TM15123P2	TM15123	P2	control	46	0	3,0758	53,4232	9,3361	42,0124	2,1369	0,2222
TM15123P3	TM15123	P3	control	46	0	-0,1819	33,8352	19,2861	12,5190	2,0301	1,5405
TM15126P1	TM15126	P1	control	46	0	-0,3628	18,3503	4,5422	13,8081	0,0000	0,3289
TM15126P2	TM15126	P2	control	46	NA	1,3046	NA	NA	NA	NA	NA
TM15130P1	TM15130	P1	pred	49	0	1,7983	31,5736	12,6295	16,7340	2,2102	0,7547
TM15130P2	TM15130	P2	pred	49	0	0,852	38,3142	14,9425	19,9234	3,4483	0,7500
TM15130P3	TM15130	P3	pred	49	0	3,7182	40,6995	20,9542	18,9394	0,8140	1,1064
TM15137P1	TM15137	P1	adult	48	0	0,2063	37,1416	14,4852	20,7993	1,8571	0,6964
TM15137P2	TM15137	P2	adult	48	0	1,1791	42,6694	17,4945	20,4813	4,2669	0,8542
TM15143P1	TM15143	P1	adult	59	0	0,6575	42,3096	10,7848	28,2064	3,3848	0,3824
TM15143P2	TM15143	P2	adult	59	0	-2,1746	26,7762	12,1951	12,9905	1,6066	0,9388
TM15161P1	TM15161	P1	pred	65	0	3,2744	51,3822	10,2764	40,0781	1,0276	0,2564
TM15161P2	TM15161	P2	pred	65	0	-5,3579	23,9564	4,5066	18,7381	0,7187	0,2405
TM15167P1	TM15167	P1	control	58	1	5,1588	65,3976	25,2525	40,1450	0,0000	0,6290

TM15167P2	TM15167	P2	control	58	1	-2,2557	44,2087	18,1256	23,8727	2,2104	0,7593
TM15170P1	TM15170	P1	control	58	0	-1,7238	33,8685	16,5955	16,9342	0,3387	0,9800
TM15170P2	TM15170	P2	control	58	0	3,5589	43,3182	15,5945	25,5577	2,1659	0,6102
TM15170P3	TM15170	P3	control	58	0	6,792	56,2988	24,5262	29,5429	2,2520	0,8302
TM15179P1	TM15179	P1	control	39	1	-1,9286	51,0204	20,0080	28,0112	2,5510	0,7143
TM15179P2	TM15179	P2	control	39	0	-2,7016	31,1748	15,7417	15,1244	0,3117	1,0408
TM15182P1	TM15182	P1	control	39	0	3,7948	NA	NA	NA	NA	0,8654
TM15182P2	TM15182	P2	control	39	0	2,2876	26,1144	5,7452	19,3247	0,5223	0,2973
TM15182P3	TM15182	P3	control	39	0	-2,7988	28,8193	11,9842	15,1230	0,8646	0,7925
TM15186P1	TM15186	P1	juvenile	57	0	3,4352	44,5916	22,0751	21,1921	0,8918	1,0417
TM15186P2	TM15186	P2	juvenile	57	0	3,6658	38,2614	14,1567	18,3655	5,7392	0,7708
TM15187P1	TM15187	P1	juvenile	47	1	1,445	56,2549	18,9373	36,2036	1,1251	0,5231
TM15191P1	TM15191	P1	pred	62	0	2,1594	52,2585	13,9701	35,7014	2,6129	0,3913
TM15191P2	TM15191	P2	pred	62	0	-0,0458	76,7127	28,8622	45,5719	2,3014	0,6333
TM15194P1	TM15194	P1	adult	62	0	7,1509	49,8405	25,9171	23,4250	0,0000	1,1064
TM15194P2	TM15194	P2	adult	62	0	4,6716	60,7263	21,0438	33,6700	6,0726	0,6250
TM15194P3	TM15194	P3	adult	62	0	4,3957	52,8989	25,9204	22,7465	4,2319	1,1395
TM15197P1	TM15197	P1	control	71	0	9,2083	74,5167	41,3162	32,4627	0,7452	1,2727
TM15197P2	TM15197	P2	control	71	0	1,897	40,3226	10,2783	29,6490	0,4032	0,3467
TM15213P1	TM15213	P1	juvenile	67	0	-6,8834	34,3407	14,4231	17,8571	2,0604	0,8077
TM15213P2	TM15213	P2	juvenile	67	0	-7,6413	22,6943	8,6238	11,8010	1,5886	0,7308
TM15222P1	TM15222	P1	juvenile	58	0	4,1216	38,2219	11,0844	21,0221	3,4400	0,5273
TM15222P2	TM15222	P2	juvenile	58	0	2,5832	41,4654	12,0773	26,1675	3,3172	0,4615
TM15254P1	TM15254	P1	pred	64	1	4,8371	53,7291	18,6190	34,0462	1,0746	0,5469
TM15254P2	TM15254	P2	pred	64	0	-1,7	41,6008	10,8162	30,7846	0,0000	0,3514
TM15254P3	TM15254	P3	pred	64	1	-1,3356	54,8198	13,5693	39,6222	0,5482	0,3425
TM15254P4	TM15254	P4	pred	64	1	-8,8545	54,4722	21,2441	30,5044	2,7236	0,6964
TM15260P1	TM15260	P1	pred	70	NA	-0,438	NA	NA	NA	NA	NA
TM15260P2	TM15260	P2	pred	70	NA	3,3655	NA	NA	NA	NA	NA
TM15262P1	TM15262	P1	adult	58	0	8,3829	61,9534	12,7551	44,9466	1,8586	0,2838

TM15266P1	TM15266	P1	juvenile	70	NA	-0,9118	NA	NA	NA	NA	NA
TM15266P2	TM15266	P2	juvenile	70	NA	2,1948	NA	NA	NA	NA	NA
TM15284P1	TM15284	P1	juvenile	67	0	1,8926	72,0297	39,9372	30,6661	1,4406	1,3023
TM15284P2	TM15284	P2	juvenile	67	1	5,0572	24,0292	7,2088	15,8593	0,7209	0,4545
TM15284P3	TM15284	P3	juvenile	67	1	1,7893	79,6004	36,6786	37,4590	5,5720	0,9792
TM15285P1	TM15285	P1	control	72	0	-1,9244	139,3534	93,3668	41,8060	2,7871	2,2333
TM15285P2	TM15285	P2	control	72	1	-1,4394	60,6385	26,1578	33,2917	0,6064	0,7857
TM15285P3	TM15285	P3	control	72	1	-4,1935	100,0389	65,0738	34,9650	0,0000	1,8611
TM15288P1	TM15288	P1	control	72	0	2,7811	63,7755	29,3367	30,6122	3,1888	0,9583
TM15288P2	TM15288	P2	control	72	0	-1,8746	39,9209	14,2292	23,3202	1,9960	0,6102
TM15288P3	TM15288	P3	control	72	0	-0,778	53,5103	19,2637	31,0360	3,2106	0,6207
TM15300P1	TM15300	P1	control	83	0	0,5887	84,7742	40,2887	41,9674	2,5432	0,9600
TM15308P1	TM15308	P1	pred	85	0	-5,9599	34,0623	8,1749	24,1842	0,6812	0,3380
TM15317P1	TM15317	P1	adult	85	0	-3,9767	36,0647	11,6680	21,5681	2,8852	0,5410
TM15317P2	TM15317	P2	adult	85	0	-1,3189	74,1345	34,8432	35,5846	3,7067	0,9792
TM15317P3	TM15317	P3	adult	85	0	4,757	54,7885	20,2718	30,1337	4,3831	0,6727
TM15320P1	TM15320	P1	adult	91	0	0,4689	89,4614	44,7307	36,6792	7,1569	1,2195
TM15324P1	TM15324	P1	juvenile	72	0	-3,7629	75,9878	37,2340	34,9544	3,0395	1,0652
TM15324P2	TM15324	P2	juvenile	72	0	-2,0862	75,8590	34,2109	34,2109	6,8273	1,0000
TM15324P3	TM15324	P3	juvenile	72	0	-4,027	109,1822	21,8364	86,2540	1,0918	0,2532
TM15329P1	TM15329	P1	adult	68	1	0,0187	70,1194	13,8850	54,8459	1,4024	0,2532
TM15335P1	TM15335	P1	adult	78	0	5,0849	39,3701	14,5669	24,8032	0,0000	0,5873
TM15335P2	TM15335	P2	adult	78	1	2,9457	75,4438	12,5740	62,8698	0,0000	0,2000
TM15335P3	TM15335	P3	adult	78	1	3,3051	63,4024	32,0151	30,1318	1,2555	1,0625
TM15340P1	TM15340	P1	adult	97	0	0,1306	44,9568	11,5730	28,9326	4,4957	0,4000
TM15340P2	TM15340	P2	adult	97	0	-0,3983	81,7460	27,7778	53,9683	0,0000	0,5147
TM15340P3	TM15340	P3	adult	97	0	1,1695	86,8056	35,5903	46,8750	4,3403	0,7593
TM1534P1	TM1534	P1	adult	36	NA	0,5602	32,3782	8,4183	22,0172	0,3238	0,3824
TM1534P2	TM1534	P2	adult	36	0		28,0647	12,6291	14,0323	1,4032	0,9000
TM1534P3	TM1534	P3	adult	36	0	0,9554	41,9428	10,9051	23,4880	1,6777	0,4643

TM15362P1	TM15362	P1	juvenile	84	1	1,8557	63,6213	28,6296	31,8107	1,9086	0,9000
TM15362P2	TM15362	P2	juvenile	84	0	-7,4474	62,2510	26,1454	32,9930	3,1126	0,7925
TM15362P3	TM15362	P3	juvenile	84	1	-2,878	64,7410	23,0329	38,5956	1,2948	0,5968
TM15367P1	TM15367	P1	pred	94	0	-6,4885	49,8654	23,9354	23,9354	1,9946	1,0000
TM15367P2	TM15367	P2	pred	94	0	-6,3951	62,3519	23,0702	38,0347	0,0000	0,6066
TM15367P3	TM15367	P3	pred	94	0	-5,9374	145,4493	84,9654	56,1636	4,3635	1,5128
TM15367P4	TM15367	P4	pred	94	0	-4,4561	38,5307	17,9810	19,8158	0,0000	0,9074
TM15371P1	TM15371	P1	pred	98	0	6,347	76,8411	31,1929	42,6050	3,0736	0,7321
TM15371P2	TM15371	P2	pred	98	0	-0,7261	88,0712	42,7276	44,4716	0,0000	0,9608
TM15371P3	TM15371	P3	pred	98	0	1,1591	44,5633	19,1622	24,9554	0,4456	0,7679
TM15409P1	TM15409	P1	adult	93	0	1,0641	56,4653	15,8103	40,0903	0,0000	0,3944
TM15409P2	TM15409	P2	adult	93	0	-8,8775	96,5251	42,4710	49,2278	3,8610	0,8627
TM15409P3	TM15409	P3	adult	93	0	-1,5409	43,2119	13,0055	29,7869	0,4321	0,4366
TM15409P4	TM15409	P4	adult	93	1	5,1811	70,0574	22,4184	44,1362	2,8023	0,5079
TM15415P1	TM15415	P1	pred	101	0	4,4018	39,7583	15,1081	23,8550	0,7952	0,6333
TM15415P2	TM15415	P2	pred	101	0	-7,78	38,7077	17,2460	19,1622	2,3225	0,9000
TM15415P3	TM15415	P3	pred	101	0	-0,1492	61,9404	19,0114	41,7024	1,2388	0,4559
TM15417P1	TM15417	P1	pred	98	1	0,8799	63,9304	28,1294	33,2438	2,5572	0,8462
TM15417P2	TM15417	P2	pred	98	0	-1,5234	38,2966	14,5527	19,5313	1,9148	0,7451
TM15417P3	TM15417	P3	pred	98	0	3,4798	31,6614	12,2257	18,8088	0,6332	0,6500
TM15439P1	TM15439	P1	juvenile	96	0	-2,7896	44,5991	12,4877	27,2054	3,5679	0,4590
TM15439P2	TM15439	P2	juvenile	96	0	-3,1948	55,7255	32,2334	20,7605	2,7863	1,5526
TM15439P3	TM15439	P3	juvenile	96	0	-0,3105	79,6253	39,8126	34,3482	3,1850	1,1591
TM15443P1	TM15443	P1	control	83	0	-2,768	28,5949	12,7403	13,8728	1,7157	0,9184
TM15443P2	TM15443	P2	control	83	0	-7,2725	41,6146	25,3849	14,9813	0,8323	1,6944
TM15443P3	TM15443	P3	control	83	0	0,4184	34,9186	10,4756	20,9512	0,6984	0,5000
TM15444P1	TM15444	P1	juvenile	85	1	5,103	51,2821	24,1026	25,1282	2,0513	0,9592
TM15444P2	TM15444	P2	juvenile	85	0	-0,8169	104,6762	49,8924	47,9358	7,3273	1,0408
TM15446P1	TM15446	P1	juvenile	85	0	-10,261	32,5806	10,0000	20,9677	1,3032	0,4769
TM15446P2	TM15446	P2	juvenile	85	0	-3,0562	29,8063	14,9031	14,0089	0,8942	1,0638

TM15446P3	TM15446	P3	juvenile	85	0	-2,7935	47,7281	17,6594	27,6823	1,9091	0,6379
TM15449P1	TM15449	P1	adult	99	1	-0,578	39,7419	7,4762	31,0852	1,1923	0,2405
TM15449P2	TM15449	P2	adult	99	0	-1,1055	50,2364	14,7754	34,4760	1,0047	0,4286
TM15450P1	TM15450	P1	adult	80	0	5,2661	61,7737	13,4557	46,4832	1,8532	0,2895
TM15450P2	TM15450	P2	adult	80	0	4,1624	54,4474	11,8598	39,8922	2,7224	0,2973
TM15450P3	TM15450	P3	adult	80	0	3,7728	45,1008	8,8433	34,9310	1,3530	0,2532
TM15451P1	TM15451	P1	control	90	1	-1,9159	71,8861	22,7758	49,1103	0,0000	0,4638
TM15451P2	TM15451	P2	control	90	0	-1,6293	64,1270	17,7778	44,4444	1,9238	0,4000
TM15451P3	TM15451	P3	control	90	0	1,8642	36,1781	10,4917	24,2394	1,0853	0,4328
TM15453P1	TM15453	P1	adult	100	0	0,051	58,2751	19,8135	30,8858	7,5758	0,6415
TM15453P2	TM15453	P2	adult	100	0	-0,051	84,1667	45,8333	30,0000	8,4167	1,5278
TM15456P1	TM15456	P1	juvenile	102	0	-8,396	53,5156	9,5374	42,3886	1,6055	0,2250
TM15462P1	TM15462	P1	juvenile	100	0	-0,7292	44,7467	18,3462	24,6107	0,4475	0,7455
TM15462P2	TM15462	P2	juvenile	100	0	-6,6545	33,5593	5,3163	28,2430	0,0000	0,1882
TM15463P1	TM15463	P1	juvenile	101	0	3,2911	72,1120	32,8431	37,8409	0,7211	0,8679
TM15463P2	TM15463	P2	juvenile	101	0	3,6405	81,5794	34,2633	35,8949	11,4211	0,9545
TM1552P1	TM1552	P1	control	35	0	-1,3039	32,6030	19,5618	12,0631	0,3260	1,6216
TM1552P2	TM1552	P2	control	35	0	-5,592	20,0957	12,6603	7,4354	0,0000	1,7027
TM1552P3	TM1552	P3	control	35	NA	-2,277	NA	NA	NA	NA	NA
TM1563P1	TM1563	P1	adult	40	0	1,0928	49,5511	18,6430	28,9457	1,9820	0,6441
TM1563P2	TM1563	P2	adult	40	0	-3,4986	19,4655	9,3434	7,9808	1,9465	1,1707
TM1563P3	TM1563	P3	adult	40	0	-1,5679	65,0533	26,6719	38,3815	0,0000	0,6949
TM1567AP1	TM1567A	P1	control	69	0	2,2863	69,0268	31,4379	32,1214	5,5221	0,9787
TM1567AP2	TM1567A	P2	control	69	0	-1,0646	62,0318	26,9195	31,0159	4,3422	0,8679
TM1568P1	TM1568	P1	adult	55	0	3,7705	39,1321	10,8485	24,7966	3,1306	0,4375
TM1568P2	TM1568	P2	adult	55	0	-0,7771	41,7711	12,9490	26,7335	2,0886	0,4844
TM1568P3	TM1568	P3	adult	55	0	0,2026	46,3392	24,5598	19,4625	2,3170	1,2619
TM1573P1	TM1573	P1	juvenile	49	0	-5,3899	73,5835	32,3767	36,0559	5,1508	0,8980
TM1573P2	TM1573	P2	juvenile	49	0	2,6847	78,2913	40,2858	31,1645	4,6975	1,2927
TM1579AP1	TM1579A	P1	juvenile	53	0	-1,5749	19,9553	2,5942	16,7625	0,5987	0,1548

TM1579AP2	TM1579A	P2	juvenile	53	1	0,7475	17,5365	3,1566	11,9248	2,4551	0,2647
TM1579P1	TM1579	P1	pred	45	0	-1,1958	40,2382	16,0953	21,7286	2,4143	0,7407
TM1579P2	TM1579	P2	pred	45	0	0,9944	32,2404	11,9289	16,4426	3,5464	0,7255
TM1579P3	TM1579	P3	pred	45	0	-3,4381	63,5700	30,2115	32,7291	0,6357	0,9231
TM1581AP1	TM1581A	P1	juvenile	40	0	-5,0409	55,2577	20,2429	30,6379	4,4206	0,6607
TM1581AP2	TM1581A	P2	juvenile	40	0	0,45	46,4296	19,0361	24,6077	2,7858	0,7736
TM1581AP3	TM1581A	P3	juvenile	40	0	2,0721	39,1988	5,8798	31,7510	1,5680	0,1852
TM1583AP1	TM1583A	P1	adult	39	0	5,1076	53,8068	22,0608	25,2892	6,4568	0,8723
TM1583AP2	TM1583A	P2	adult	39	0	1,8457	97,1817	50,5345	41,7881	4,8591	1,2093
TM1583AP3	TM1583A	P3	adult	39	0	-1,0542	71,3287	36,3636	27,2727	6,4196	1,3333
TM1591P1	TM1591	P1	juvenile	48	1	0,317	58,7648	22,9183	34,0836	1,1753	0,6724
TM1591P2	TM1591	P2	juvenile	48	0	5,5249	71,4694	34,3053	27,8731	7,8616	1,2308
TM1591P3	TM1591	P3	juvenile	48	0	1,5732	75,1547	35,3669	34,6301	3,7577	1,0213
TM1591P4	TM1591	P4	juvenile	48	1	6,6926	29,5097	11,6870	13,4401	2,6559	0,8696
TM15FRANP1	TM15FRAN	P1	pred	49	0	3,7168	55,7377	31,6940	21,8579	1,6721	1,4500
TM15FRANP2	TM15FRAN	P2	pred	49	0	-1,2098	49,2203	22,4172	23,3918	2,4610	0,9583

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ATLANTICO