

0 202087

UNIVERSIDAD DE ALCALA



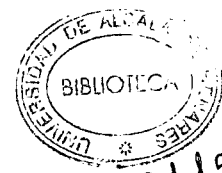
5904201157

57-04

U11

MAR

2011



D

C-4201157

Los abajo firmantes como codirectores de Tesis y Director del Departamento de Biología Animal de la Universidad de Alcalá, hacemos constar que la Tesis titulada *“Ecología reproductiva de Cernicalo vulgar: ecofisiología, asincronía de eclosión e interacciones de los pollos en el nido según el sexo”* y realizada por Jesús Martínez Padilla, licenciado en Ciencias Ambientales, reúne los requisitos necesarios para su defensa y aprobación, y por tanto para optar al grado de Doctor.

UNIVERSIDAD DE ALCALÁ	
REGISTRO GENERAL	
SECCIÓN I	
29 OCT. 2003	
ENTRADA	SALIDA
Nº 5395	AÑO

El Director del Departamento



Vº Bº de los Directores

Javier Viñuela Madera

Juan Moreno Klemming

Guillermo Blanco Hervás

Octubre de 2003

---

DEPARTAMENTO DE BIOLOGÍA ANIMAL

El Dr. **JAIME POTTI SÁNCHEZ**, Profesor Titular del Departamento de Biología Animal de la Universidad de Alcalá y Tutor del Doctorando,

RATIFICA:

Que la Tesis Doctoral titulada “*Ecología reproductiva del Cernícalo vulgar: ecofisiología, asincronía de eclosión e interacciones de los pollos en el nido según el sexo*”, presentada por D. **Jesús Martínez Padilla** y dirigida por los doctores **Juan Moreno Klemming**, **Javier Viñuela Madera** y **Guillermo Blanco Hervás**, reúne los requisitos necesarios para su defensa y aprobación.



Fdo: Jaime Potti Sánchez

Alcalá de Henares, octubre de 2003

Ilustración de portada: © Jesús García González

Ilustración interior: © Lorenzo Pérez Rodríguez



*A quien alimenta mi vocación,  
a pesar de soportarla.*

## CONTENIDOS

---

<b>Agradecimientos</b>	<b>1</b>
<b>Introducción general</b>	
<b>Antecedentes y líneas de estudio</b>	<b>3</b>
<b>Objetivos</b>	<b>9</b>
<b>Métodos generales</b>	<b>10</b>
<b>Resultados y discusión</b>	<b>14</b>
<b>Conclusiones y perspectivas</b>	<b>26</b>
<b>Capítulo I</b>	<b>41</b>
First evidence of sex differences in the length of avian embryonic period: consequences for sibling competition in sexually dimorphic birds.	
<b>Capítulo II</b>	<b>51</b>
Mass provisioning to different-sex eggs within the laying sequence: consequences for adjustment of reproductive effort in a sexually dimorphic bird.	
<b>Capítulo III</b>	<b>67</b>
Prelaying maternal condition mediates the association between egg mass and nestling immunity in the Eurasian kestrel	
<b>Capítulo IV</b>	<b>81</b>
Intestinal parasitism is not related with reproductive parameters and nestling immune response in wild Eurasian kestrels <i>Falco tinnunculus</i> .	
<b>Capítulo V</b>	<b>93</b>
Brood hierarchies, sex and parasites determine stress in Eurasian kestrel ( <i>Falco tinnunculus</i> ) nestlings: a study of heat-shock proteins.	
<b>Capítulo VI</b>	<b>111</b>
Influence of hatching asynchrony, sex and brood reduction on immunity of Eurasian kestrel nestlings.	

## *Agradecimientos...o agrade-cimientos*

Es realmente agradable dedicar este momento tan minúsculo, después de tanto trabajo, a agradecer. No más. Supongo que como muchos en esta misma situación, se han visto tentados a hacer algo diferente, algo más que enumerar a gentes. Más ironía, más humor, menos ciencia, menos objetividad, más sujeto..., en definitiva algo más de sí mismos. Más huella personal, si cabe. Pensé en hacer lo mismo, y eso haré: pensar en los cercanos. Sin embargo, parece curioso que para escribir estas líneas, normalmente uno mire hacia atrás cuando la propia palabra de este apartado también puede hacer mirar hacia delante: “Agrade”, del verbo agradar (de la que no sé qué tiempo conjugación y persona) y “cimientos” (3ª acepción de su significado según la RAE: principio y raíz de algo). Un agradecimiento a lo que podría ser por lo que fuisteis. Así que enumeraré a gente, pero que no como personas que estuvieron, sino en gentes que están y estarán. Más hoy, más mañana y menos ayer.

Ya me gustaría que este País hubiera apoyado mínimamente la investigación en general y la mía en particular, pero agradezco (aunque no necesito) su indiferencia. Me hizo pensar más. Mi primer agradecimiento al mínimo interés por la ciencia de este país (con minúscula). Además, me hizo conocer sus fisuras y a los individuos que la conforman, que merecen mucho más la pena. Individuos como sujetos no sujetos. Dentro de estos a Araceli, la más eficiente y paciente secretaria que conozco. Nunca superaría tanto papel junto y para tantas cosas. También al Departamento de Biología Animal de la Universidad de Alcalá, que favoreció la entrada en el departamento, tras mi tropezón en otro lugar. Dentro del Departamento, Jaime Potti siempre estuvo cerca facilitándome todo lo que estuvo en su mano. También a la familia Finat, que nos permitió trabajar en su espacio, que cunda el ejemplo. Juan y Pilar nunca negaron y siempre facilitaron.

Carlos, Nico y Maite quizá sean los andamios de esta Tesis. No por discusiones sobre rapaces, pájaros o incluso la ecología (aunque las hubo, y mucho). Si no por la filosofía de vivir, la comprensión, la entereza y el ánimo. Un año en Alcalá quizá fuera el empujón y vosotros sois los hostigadores. Los de la carrera están en otro plano. Por supuesto a Txuso, que me basé en su tesis sobre las relaciones ecológicas entre dos especies simpátricas: la perdiz roja y la griega. Fueron mi guía en estos días. La Vir y Alfonso, también a vosotros por que al fin y al cabo os habéis tragado algunas “primitivas” ideas sobre el papel que cumplimos en nuestra vida desde el punto de vista de la evolución, y por que siempre estáis con los brazos abiertos. Agnès y Angel, especialmente a la primera por las discusiones sobre lo masculino y lo femenino, que me sugirieron ideas... y por que aprendo catalán. También a los IRECIanos, especialmente Lorenzo y paquillo, por las charlitas, cafés y compañía los fines de semana del final de la escritura de la Tesis.

Por lo que sea me gustan las rapaces y una vez conoces a alguien que sabe lo que tú quieres saber y hace lo que tú quieres hacer. Y entonces cambia todo y de algún modo, para bien o para mal, todo se alinea. A partir de que conocí a Aurelio, lo vi más claro. Quieres estar con rapaces y saber todo y más de rapaces. No hay mucho más. De toda mi generación, cuánto es debido a las imágenes del Hombre y la Tierra?. No lo sé, pero al menos una parte de esta Tesis es para y por Aurelio.



De los directores de Tesis no puedo más que quitarme el sombrero. Sin ellos esta Tesis, no sería como es, ni siquiera sería. Javier Viñuela, no dudó un instante (creo que nunca) de apoyarme con todos los medios que tuvo en su mano. No sólo desde la ciencia, sino también desde las pelás, el ánimo y el apoyo constante. No sé cómo agradecerte, quizá pueda algún día. No es poesía. Juan Moreno abrió sus puertas a un pipiolo que no tenía ni idea (aseguro que no sabía lo que era un paper en esa época), pagó los costes del primer año de campo y ha estado cuando lo he requerido. Confió en alguien que únicamente tenía ganas de aprender, machacarse en el campo y no miró expedientes. Espero que cunda el ejemplo, por mi parte, tengo muy bien aprendida esa lección. De Guillermo, aprendo de él la practicidad y el pragmatismo. Además, me introdujo en esto de los modelos mixtos, que se lo agradezco, pero me gustaría que no hubieran tenido que existir. También me apoyó con pelillas, que nos sacaron de un pozo más que profundo por aquellos entonces.

Si hay algo que agradezco a esto de la ciencia, es haber conocido a buenas personas con mentes redondas, de las que las ideas salen y entran en todas las direcciones...aunque también calvas: Juanto. Paradójicamente es con quien más tiempo he pasado en el campo, aunque nunca hemos podido trabajar juntos de modo intensivo. Estadística, papers, discusiones sobre temas variopintos, etc. y siempre la cordialidad. Aprendí/o sobre esto de la ciencia en su más amplio contexto. Además, te debo una (cerveza). También a las Anas, las partes contratantes de Javi y Juanto, por su cercanía y calor... y por manifestar la otra cara de la parte contratante de la primera parte.

Esto también es de y con quienes me criaron. Cazorla, Ainsa, Sotres, Potes, Llanes, Ribadesella, Mieres, Cangas de Onís, Fuente Dé, la senda de los Cazadores, la cola de caballo, Ordesa, Añisclo, San Juan de la Peña, Morillo de Tou, Broto, Linás de Broto, Cotefablo, Alpe D'Huez, Peguerinos, Sacedón, etc. Tanto bicho y tanto monte dan lugar a esto, esa es la causa. Quienes me educaron desde la dificultad. De quienes aprendí la capacidad de superación. De romper cadenas sociales, esta va por mi hermano. Quienes JAMÁS me pidieron cuentas. Este es uno de vuestros muchos resultados y logros. Espero llegar a dar vuestra misma lección. A mis padres y hermano. Por supuesto, a Dana, que fue con quien me inicié en esto de los pájaros, aunque ella buscara lagartos en los largos paseos por Peguerinos.

Todos ellos han estado más o menos en el camino de la Tesis, pero Claudia estuvo SIEMPRE, desde antes (Alcalá) hasta después (Scotland?), desde el campo hasta la desesperación, es un andamio y mi buzón de tiempo. Con constancia y sin pausa. Alejando las enfermedades y a los enfermos para no enfermar. Jamás dudó nada y Todo (con mayúscula) fue poco para apoyarme, para no bajar. Independientemente de cualquier cosa, siempre una ternura, mucho cariño y lo mismo de ti. Eso es, siempre tú conmigo. Esta tesis también la has sudado y sufrido tú, por lo que debes considerarte autora y todo aquel loco que ose leer este trabajo deben verlo igual. Este es nuestro trabajo. Hasta hace poco, no sabía por qué hay gente que me llama poeta, hasta que un día estando contigo, disfrutándote, me di cuenta. Ellos te vieron a ti: la poesía.

# INTRODUCCIÓN GENERAL

## 1. ANTECEDENTES Y LÍNEAS DE ESTUDIO

---

### 1.1. El dimorfismo sexual en la competencia entre hermanos

Fisher (1930) formuló matemáticamente por primera vez la idea de que en especies diploides habría una tendencia a producir descendencia con una razón de sexos (“sex ratio”) equilibrada, aunque no obstante esta idea fue originalmente señalada por Darwin (1871). Sin embargo, trabajos más recientes han indicado que, debido al dimorfismo sexual y a diferencias ecológicas o etológicas entre ambos sexos, la inversión necesaria para criar un macho o una hembra, y/o las expectativas de valor reproductivo futuro de ambos sexos, pueden variar en función de condicionantes ecológicos (Ej.: Charnov, 1982). La teoría de las estrategias vitales (Roff 2002) sugiere que los padres pueden favorecer a determinados miembros de la descendencia sobre otros con el fin de incrementar la eficacia biológica global (Clutton-Brock, 1991). Dado que la crianza de distintos sexos puede diferir en costes de inversión o en expectativas futuras de eficacia biológica (o fitness), se pueden dar casos de inversión diferencial sesgada por el sexo de la descendencia, o la producción de camadas con razón de sexos sesgada, todo ello afectado por las circunstancias ecológicas concretas a que se ven expuestos los individuos en cada intento reproductivo. Por tanto, la inversión parental requerida para criar los pollos de distinto sexo puede ser diferente (Slagsvold et al. 1986, Bennett et al. 1995, Riedstra et al. 1998), y la razón de sexos final de la inversión parental podría

estar sesgada hacia el sexo menos costoso de criar (Kolman 1960). Por ejemplo, en algunas especies puede existir un sesgo en la mortalidad hacia machos, debido a su mayor tamaño, y por tanto mayor demanda de recursos durante la cría (Clutton-Brock et al. 1985; Clutton-Brock 1986; Sheldon et al. 1998). Es decir, cuando ambos sexos no son similares en costes para los padres, la relación de sexos en una camada podría sesgarse hacia el sexo que menor coste unitario requiera, al menos en padres con limitación de recursos (por ejemplo baja condición física). Sin embargo, Lack (1954) propuso que el mayor tamaño de un sexo podría favorecer su supervivencia en la nidada debido a su mayor capacidad competitiva, cuando existe competencia directa entre hermanos por los recursos tróficos aportados por los padres (especies nidícolas, en el caso de las aves). Por ejemplo, en las aves rapaces donde el dimorfismo sexual está invertido, por causas evolutivas inciertas (Massemin et al. 2000), parece existir un sesgo en la mortalidad en nido hacia el sexo menor, los machos (Arroyo, 2002; Korpimäki et al. 2000; Dijkstra, 1990), posiblemente por su menor capacidad competitiva respecto a las hembras de mayor tamaño, aunque pueden encontrarse evidencias contrarias (Newton 1979; Olsen & Cockburn 1991).

Por tanto, existe cierta controversia en los patrones de inversión con respecto al sexo, y hay una amplia bibliografía reciente sobre el tema. Curiosamente, esta bibliografía podría estar sesgada hacia casos en los que se observan razones de sexos sesgadas en la descendencia, o patrones de inversión o mortalidad diferencial según los

sexos que producen razones de sexos sesgadas. Sin embargo, y volviendo a la idea original de Fisher, es posible que haya adaptaciones para conseguir razones de sexos equilibradas cuando los condicionantes ecológicos hacen más fácil o rentable la inversión en un sexo determinado, y esta posibilidad ha sido mucho menos explorada en la literatura reciente. Además, mientras que el estudio de la proporción de sexos ha recibido gran atención en las últimas décadas, mucho menos conocimiento científico se ha generado en relación a los efectos en la eficacia biológica de los pollos derivados de la competencia entre hermanos mediada por el sexo.

En rapaces se ha demostrado la ventaja competitiva del pollo mayor en cuanto a supervivencia o crecimiento tanto en cautividad (Anderson, 1993) como en poblaciones naturales (Fargallo et al, 2003). También se ha comprobado la existencia de esta ventaja en otros componentes de la eficacia biológica, como la respuesta inmunitaria (ver punto 1.3.) de volantones, donde las hembras tendrían una mayor respuesta bajo restricciones alimentarias debido a su mayor capacidad competitiva (Fargallo, et al. 2002). Por otro lado, dicha diferencia podría deberse a diferencias sexuales en niveles circulantes de andrógenos ya en el nido. En estos casos un mayor tamaño podría tener una ventaja adaptativa sobre el sexo menor, a pesar de tener unos requerimientos mayores para el crecimiento. Este mayor requerimiento energético del sexo mayor, también detectado en pollos de Cernícalo vulgar (Pen, 2000), podría sin embargo representar costes mayores bajo condiciones desfavorables en la cría (Drummond 1991; Velando, 2003; Velando, 2002). Por tanto,

existe controversia en relación a los resultados publicados, por lo que estudios que evalúen de manera novedosa la influencia del sexo de los pollos en el nido, especialmente en especies dimórficas, son necesarios para aportar resultados a este campo científico. Para ello, se usaron como indicadores de estrés ambiental los niveles de proteínas de estrés térmico o 'heat shock proteins' (HSPs en adelante) que fueron descubiertas a inicios de la década de los 60 (Ritossa, 1962) y que son empleadas por los organismos para mantener los procesos homeostáticos celulares tanto en condiciones naturales como de estrés (Leppä & Sistonen, 1997; Welch, 1992; Schlesinger, 1990; Lindquist, 1986) y que son altamente estables evolutivamente (Ingolia, et al. 1980; Karch, et al. 1981; Bienz, 1984).

El problema se complica además si tenemos en cuenta la posible existencia de un conflicto paterno-filial sobre la reducción de nidada (Trivers 1974; Mock & Parker, 1997; Viñuela 1999), en el contexto del compromiso cantidad-calidad de la descendencia. Los padres pueden intentar conseguir la supervivencia de toda la nidada, aún a costa de sacrificar algo de la calidad de los pollos dominantes, dado que el grado de parentesco con todos los pollos es en principio igual (con la excepción de la posible aparición de fertilizaciones extrapareja que podría afectar a la inversión de los machos). En cambio, los pollos dominantes, por su menor parentesco genético con sus hermanos que consigo mismo, estarían más dispuestos a "eliminar" la competencia de los hermanos de menor tamaño. Este conflicto paterno-filial puede dar lugar a "estrategias" parentales que permitan a los padres controlar la competencia entre hermanos (Viñuela

1999). Existen evidencias más recientes que sugieren incluso la existencia de mecanismos fisiológicos para facilitar este control parental de la competencia entre hermanos, como por ejemplo, la inversión hormonal diferencial en los huevos como modo de mejorar la capacidad competitiva de alguno de los pollos tras la eclosión (Ricklefs, 1993; Sockman & Schwabl, 2000; Eising et al., 2001) o bien invirtiendo diferencialmente en miembros de la pollada para minimizar los efectos de la competencia entre hermanos a través de la jerarquía de tamaños (Wiebe et al., 1998b; Viñuela, 2000). Dado que la competencia mediada por el sexo de los pollos en el nido puede ser causa de mortalidad, como hemos visto anteriormente, sería esperable que la inversión maternal pudiera modular y minimizar la competencia que fuera a tener su descendencia debida al dimorfismo sexual.

Sin embargo, al contrario que en Petrie et al. (2001), son escasas las evidencias que sugieren inversiones diferenciales en los huevos en función del sexo para maximizar la supervivencia. Únicamente, 4 estudios (Mead et al., 1987; Andersson et al., 1997; Cordero et al., 2001, 2001; Cunningham & Russell, 2001) sugieren diferencias sexuales en la inversión en huevos, y además los patrones detectados son contradictorios entre especies, ya que las diferencias observadas pueden ser sesgos hacia el macho o la hembra, o sólo hay dimorfismo en el primer huevo puesto (Emlen, 1997). Este hecho, unido a las implicaciones ecológicas que puede tener el tamaño del huevo (Williams, 1994), sugieren un control de los recursos para la descendencia mediado por el sexo de los embriones por parte de la madre para mejorar la eficacia biológica global de la pareja. Sin embargo, el efecto de este tipo

de control parental diferencial entre huevos macho o hembra no ha sido estudiado, ni (1) en relación a la puesta, ni (2) a la eclosión, ni (3) las implicaciones que puede tener esta inversión durante su etapa en el nido.

## **1.2. Asincronía de eclosión.**

El inicio de la incubación en aves antes de la finalización de la puesta es común. Este hecho deriva en que el desarrollo embrionario de los primeros huevos puestos se inicia antes que el de los últimos huevos puestos. Este hecho trasladado a la eclosión, implica que existe una jerarquía temporal de nacimientos (Foto 1) y consecuentemente una jerarquía de tamaños (Lack, 1954; Mock & Parker, 1997), que variará en función del intervalo de puesta. Hasta el momento se han elaborado múltiples hipótesis que pretenden explicar el significado adaptativo o no que pudiera tener este patrón y en qué fase del desarrollo actuarían las fuerzas selectivas que condicionan a la hembra a adoptar un patrón u otro en el inicio de la incubación (Tabla 1. Revisiones en Magrath, 1990; Stoleson & Beissinger, 1995). Sin embargo, existe un notable desacuerdo en la comunidad científica a la hora de establecer un consenso al respecto derivado de los resultados científicos contradictorios según una u otra hipótesis, aunque sí se admite que: (1) el patrón de eclosión debe maximizar el éxito reproductivo (Dawson & Bortolotti, 2002), y (2) que existen numerosos factores involucrados y que estos pueden variar entre especies y ambientes (Stoleson & Beissinger, 1995; Stenning, 1996; Viñuela & Carrascal 1999; Viñuela 2000).

La más discutida de las hipótesis ha sido la planteada por David Lack (1954): la

Significado evolutivo	Fuerza selectiva	Hipótesis	Periodo Crítico	Autores
No adaptativo	Fisiología	Hormonas	Puesta	Mead & Morton, 1985
No adaptativo	Fisiología	Limitación energética	Puesta	Greig-Smith, 1985
Adaptativo	Condiciones ambientales	Viabilidad de los huevos	Puesta	Arnold et al. 1987
Adaptativo	Condiciones ambientales	Oportunidad limitada de cría	Puesta	Beissinger & Waltman, 1991
Adaptativo	Predación	Protección de los huevos	Puesta	Oring, 1982
Adaptativo	Sistema social	Parasitismo de nidada	Puesta	Jackson, 1993
Adaptativo	Eficiencia parental	Reducción del pico de demanda	Pollos	Mock & Schwagmeyer, 1990
Adaptativo	Eficiencia parental	Diversidad en la dieta	Pollos	Magrath, 1990
Adaptativo	Eficiencia parental	Despensa	Pollos	Alexander, 1974
Adaptativo	Eficiencia parental	Revalidad entre hermanos	Pollos	Hahn, 1981
Adaptativo	Dimorfismo sexual	Manipulación de la razón de sexos	Pollos	Slagsvold, 1990
Adaptativo	Condiciones ambientales	Reducción de nidada	Pollos	Lack, 1954
Adaptativo	Condiciones ambientales	'Hurry-up'	Pollos	Hussel, 1972
Adaptativo	Sucesos estocásticos	Hipótesis del seguro	Pollos	Stinson, 1979
Adaptativo	Parasitismo	Pollo sabroso	Pollos	Christie, et al., 1998
Adaptativo	Condiciones ambientales	Diversidad volantones	Pollos	Laaksonen, 2003
Adaptativo	Condiciones ambientales	Cantidad de comida	Pollos	Mock & Ploger, 1987
Adaptativo	Condiciones ambientales	Garantía de la calidad del pollo	Pollos	Slagsvold, 1986
Adaptativo	Sistema de apareamiento	Fallo de la nidificación	Puesta/nidada	Clark & Wilson, 1981
Adaptativo	Sistema de apareamiento	Conflicto sexual	Puesta/nidada	Slagsvold & Lifjeld, 1989
Adaptativo	Predación	Predación sobre adultos	Puesta/nidada	Magrath, 1988

**Tabla 1.** Hipótesis explicativas de la asincronía de eclosión. Esta tabla, aunque actualizada hasta la fecha de publicación de esta tesis, está realizada a partir de la sugerida por Stoleson & Beisinger (1995).

hipótesis de la reducción de nidada. Esta hipótesis sugiere que bajo períodos de escasez de alimento, una eclosión asincrónica podría facilitar la eliminación del último pollo eclosionado de la nidada, facilitando la supervivencia del resto de los pollos. Esta hipótesis se basa en el hecho de que las aves no serían capaces de predecir la abundancia de alimento en el periodo de cría de los pollos cuando van a realizar la puesta. En esas circunstancias realizarían una puesta "optimista" (ajustada al número de pollos



**Foto 1.** Diferencias en el tiempo de eclosión

que podrían criar en condiciones óptimas), que sería reducida si las condiciones de disponibilidad de recursos son inferiores a las óptimas, y la jerarquía de tamaños impuesta por la asincronía de eclosión sería un mecanismo para facilitar dicha reducción de nidada. Además, esta eliminación de los pollos más tardíos en eclosionar en condiciones de escasez de alimento podría contribuir a mejorar la calidad de los pollos supervivientes (Wiebe & Bortolotti 1994). De este modo, bajo condiciones de restricción alimentaria, las puestas de eclosión asincrónica obtendrían un mayor éxito reproductivo que las puestas de eclosión sincrónica. En este sentido, diferentes trabajos apoyan o refutan la idea de Lack, señalando algunos que la hipótesis no responde a la cuestión de por qué existen puestas asincrónicas en períodos de abundancia de alimento (Wiebe et al. 1998a; Wiehn et al. 2000). No obstante, la mayor parte de los estudios que han estudiado la asincronía de eclosión en relación a la reducción de nidada, se han centrado en



variables clásicas como crecimiento, peso del volantón, o supervivencia, mientras que variables relacionadas con otros componentes de la eficacia biológica de los volantones, como puede ser su calidad fisiológica, han sido relegadas a un segundo plano a pesar de que su estudio puede sugerir nuevas evidencias que contradigan o verifiquen la hipótesis de Lack (aunque ver Chirste, et al. 1998). La especie de estudio de esta tesis (Cernícalo vulgar, *Falco tinnunculus*) es particularmente interesante para el estudio de estos problemas, por tener una asincronía de eclosión relativamente pequeña en comparación con otras rapaces. Además, no existe agresión entre hermanos, ni hay registros de fratricidios, lo cual puede simplificar el estudio del significado de la asincronía de eclosión. Estudios anteriores con esta especie en latitudes norteadas sugieren que el grado de asincronía de eclosión estaría ajustado a la predecibilidad del alimento, resultado que apoya, al menos parcialmente, la hipótesis de Lack (Wiebe & Bortolotti, 1994).

### 1.3. Sistema inmunitario.

Las interacciones parásito-hospedador, quizá sean una de las relaciones más extendidas en la naturaleza. Sin embargo, hasta los años 70, este tipo de relaciones fueron campo casi exclusivo de parasitólogos, que centraron sus estudios en la descripción de sus ciclos vitales, patología y su conocimiento como agentes causantes de enfermedades (Zuk & Stoehr, 2002). Sin embargo, en los últimos 20 años, la ecología ha empezado a tratar las relaciones parásito-hospedador desde un punto de vista evolutivo debido al alto impacto que puede tener los parásitos sobre las estrategias vitales de los hospedadores. Este interés se ha mostrado tanto desde la

perspectiva de la evolución de la virulencia como desde el efecto que tienen los parásitos en los hospedadores y cómo pueden influir estos en sus estrategias vitales (Zuk & Stoehr, 2002), siendo este último enfoque en el que nos centraremos. En este sentido, una creciente bibliografía en temas relacionados con la ecología evolutiva asumen un efecto sobre diferentes componentes en la vida de los hospedadores como, por ejemplo: en la selección sexual (Hamilton & Zuk, 1982; Folstad & Karter, 1992), el éxito reproductor (Merino & Potti, 1995), el comportamiento (Barnard & Behnke, 1999) o la fisiología (Veiga, 1998).

Puesto que los parásitos tienen un efecto notable sobre algunos componentes de la eficacia biológica de los hospedadores, estos han tenido que desarrollar mecanismos de defensa para minimizar los costes asociados. Es decir, los hospedadores han tenido que desarrollar modos de resistencia frente a parásitos que prevengan la colonización de los mismos (infección), o bien minimicen los efectos fisiológicos debilitadores (enfermedad) debidos a la infección parasitaria (Apanius, 1998). Por un lado, el hospedador puede desarrollar estrategias comportamentales que reduzcan el riesgo de parasitación (Moore, 2002), cumpliéndose el requerimiento de que los parásitos disminuyan la eficacia biológica del hospedador y que el comportamiento adoptado reduzca o elimine al parásito (Hart, 1990; Hart, 1994). Sin embargo, el mecanismo directo que combate la invasión de patógenos, es el sistema inmunitario. Dentro de éste, se diferencian dos sistemas principales de defensa para evitar la entrada de patógenos en Vertebrados: la inmunidad innata o inespecífica y la inmunidad adquirida o específica (Schmid-Hempel & Ebert 2003). La inmunidad innata puede

frenar la entrada de patógenos o combatirlos a través de procesos de detección y fagocitosis llevados a cabo por glóbulos blancos formados en la médula ósea. Por este mecanismo, los granulocitos (neutrófilos, eosinófilos y basófilos) y los monocitos, una vez llegados al tejido infectado, detectan a los antígenos conservados de numerosos patógenos y destruyen a los organismos portadores.

Por otro lado, la *inmunidad adquirida*, es el modo de resistencia más específico frente a patógenos y en el que se han centrado los estudios recientes en ecología evolutiva de vertebrados. Los mecanismos relacionados con la inmunidad adquirida no son activos hasta que los hospedadores son invadidos por un patógeno. Es por ello que la inmunidad adquirida se sustenta en tres atributos clave: inducibilidad, especificidad y memoria (Apanius, 1998). La inmunidad adquirida, a su vez, se agrupa en dos componentes principales: inmunidad mediada por células y la inmunidad humoral. La inmunidad humoral es un método de defensa basado en la inducción y proliferación de clones de linfocitos B que detectan a un número indefinido y potencialmente vastísimo de antígenos y a su vez producen anticuerpos circulantes (inmunoglobulinas), capaces a su vez de identificarlos y presentarlos a células destructoras. La restricción y proliferación clonal sustenta a la memoria del sistema, al permitir la detección más rápida de antígenos en infecciones subsiguientes. La inmunidad celular está basada en la acción de los linfocitos T que regulan la acción de otras células del sistema inmunitario una vez les han sido presentados los antígenos. En esta serie de acciones reguladoras y destructoras inducidas por linfocitos T, no intervienen los anticuerpos. Los linfocitos T

y B de aves se forman en el timo y en la bolsa de Fabricio antes de su involución en la vida adulta. Estos órganos proporcionan un ambiente apropiado para la proliferación de líneas de linfocitos.

Sin embargo, ¿qué interés puede tener en estudios de ecología la medición de la calidad del sistema inmunitario? Los parásitos sustraen recursos de los hospedadores, por lo que estos evitan mediante el sistema inmune esta pérdida de recursos, que puede ser empleada en otras funciones como, por ejemplo, reproducirse o crecer. En este sentido, el estudio de las estrategias vitales se sustenta en el principio de asignación enunciado por Levins (1968), según el cual los recursos destinados a una función no pueden ser dedicados a otra. Es decir, un individuo determinado tiene que asignar de una manera competitiva sus recursos a crecer, sobrevivir y reproducirse (Reznick, 1992), tres componentes costosos que agrupan a su vez a multitud de estimadores de la eficacia biológica. Parte de este coste de supervivencia viene determinado por la calidad del sistema inmunitario, del que se sabe que implica un alto coste energético para su funcionamiento (Lochmiller & Deerenberg, 2000). Por ello, debido a las condiciones de limitación energética de los individuos en la naturaleza, estos deben distribuir sus recursos entre el sistema inmunitario y otros componentes. Recientemente, estudios en ecología evolutiva se han centrado en las implicaciones que puede tener para los individuos la calidad del sistema inmunitario, medida como respuesta inmunitaria celular (linfocitos T). Diferentes resultados sugieren la importancia evolutiva del sistema inmunitario y sus implicaciones (ver por ejemplo, Moreno et al., 1999; Tella et al. 2000, Sorci et al., 1997; Hōrak et al.

1999, González et al. 1999; Velando et al., 2001; Johnsen et al. 2000, Fair et al., 1999; Whitaker & Fair, 2002; Saino et al., 1998; Christe et al., 1998; Tella, et al., 2002), demostrando que es una herramienta válida como estimador de la calidad del individuo. En este sentido, el estudio de la respuesta inmunitaria puede aportar una nueva visión sobre problemas “clásicos” en ecología reproductiva de aves, como la reducción de nidada y la asincronía de eclosión (ver punto anterior).

Paralelamente, existen evidencias de que la carga parasitaria, tanto externa (Tchirren, et al. 2003) como sanguínea (Merino, et al. 2000) afecta a la respuesta inmunitaria de los hospedadores. Sin embargo, son escasos los trabajos que estudian las relaciones entre los parásitos intestinales y la respuesta inmunitaria en poblaciones salvajes. Además, no existen estudios en la actualidad que evalúen el efecto que tienen los parásitos intestinales sobre diferentes componentes de la eficacia biológica de los volantones o adultos, a pesar de que en condiciones de cautiverio, estos parásitos causan importantes daños a las aves rapaces (Forbes & Simpson, 1997; Upton, et al. 1990). Por último, existen trabajos que relacionan la calidad de los padres y diferentes variables de la descendencia sobre la respuesta inmunitaria, pero no existen evidencias sobre el efecto de la calidad o tamaño del huevo, entendido como el paso intermedio entre los padres y la descendencia.

## **2. OBJETIVOS CIENTÍFICOS**

---

El objetivo fundamental de la tesis que se presenta es contribuir al conocimiento científico, especialmente dentro del campo de la ecología reproductiva, utilizando tanto

métodos de estudio clásicos en este campo, como técnicas más modernas usadas en el estudio de la respuesta inmunitaria y del estrés. Además, se ha pretendido que los objetivos puedan trascender de aquellos puramente ornitológicos, partiendo de preguntas típicas del campo de la ecología evolutiva, especialmente en los aspectos relacionados con el período de crecimiento de los pollos que vienen determinados por la estrategia reproductiva de los padres. Los trabajos presentados en esta tesis podrían agruparse en dos grandes grupos: el primero de ellos sugiere diferentes estrategias por parte de la hembra que afectarían al desarrollo posterior de los pollos y un segundo bloque en el que se estudian los efectos que tienen estas estrategias medidos según diferentes indicadores de eficacia biológica.

El primer grupo de trabajos que exploran las estrategias reproductivas de la hembra, incluye 3 capítulos. El primero de ellos (**Capítulo I**), pretende estudiar las variaciones en el período embrionario de los huevos, tanto según su orden de puesta como en relación al sexo, ya que si los huevos que dan lugar a pollos macho o hembra dentro de una misma puesta pueden variar tanto su composición (Petrie et al., 2001) como su tamaño (Anderson et al., 1997; Cordero et al., 2001), y dado que estas variaciones pueden afectar al período embrionario, es razonable sugerir que el sexo puede influir en la duración del tiempo de incubación de los huevos macho o hembra, aspecto que, sorprendentemente, no había sido descrito anteriormente en la literatura científica. Posteriormente se estudia la jerarquía de tamaño de los huevos dentro de la puesta (**Capítulo II**) y el posible efecto mediador del sexo de los huevos. En este sentido y en relación a las

posibles implicaciones que pudieran tener los resultados del Capítulo I, se estudia cómo la variación del tamaño de los huevos mediado por el sexo influye en la jerarquía posterior de los pollos en el nido. Por último, en este grupo de trabajos se estudia experimentalmente la relación entre el tamaño y la calidad del huevo (**Capítulo III**) medida como el posible efecto que tenga el huevo de procedencia sobre la capacidad que tiene el pollo al final de su estancia en el nido de responder frente a la inyección de un antígeno.

En un segundo grupo de trabajos, se estudian cómo las estrategias anteriores afectan a los pollos durante su estancia en el nido. Primero, por primera vez en una población de rapaces en libertad se estudia el posible efecto de los parásitos intestinales (**Capítulo IV**) sobre los parámetros reproductivos y a la calidad de los volantones, medida adicionalmente como su capacidad de respuesta inmunitaria celular. El siguiente trabajo aborda la relación entre

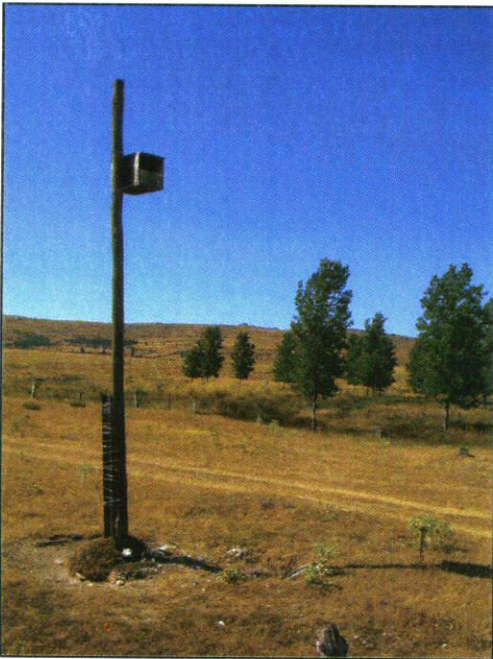


Foto 2. Caja nido

la jerarquía de tamaños creada por la asincronía de eclosión y el estrés de los pollos (**Capítulo V**), mediado por el sexo y la parasitación intestinal. El estrés de los pollos se evalúa a través de los niveles de HSPs, un reciente estimador de estrés ambiental. Por último, se estudia cómo la mortalidad favorecida por la asincronía de eclosión puede alterar la calidad de los volantones medida como su capacidad para montar una respuesta inmune celular (**Capítulo VI**). Asimismo, esta relación se estudia en relación al sexo de los volantones.

### 3. MÉTODOS GENERALES

#### 3.1. Especie de estudio.

El Cernícalo vulgar (*Falco tinnunculus*) es un Falcónido de pequeño-mediano tamaño (Village, 1990), siendo la rapaz más abundante de Europa. En Europa las poblaciones migratorias disminuyen a medida que se incrementa en latitud, estando la distribución invernal limitada por las regiones con nieve permanente en invierno (Village, 1990). Así, el Cernícalo Vulgar es migrador en latitudes norteñas, distribuyéndose por latitudes más templadas fuera de la época de cría, pudiendo llegar al África subsahariana (Araujo, 1973) y siendo migrador parcial en la Península Ibérica (Bernis, 1966), Gran Bretaña y Europa Central (Cavé, 1968; Cramp y Simmons, 1980). En el área de estudio, se comporta igualmente como migradora parcial con escasos efectivos invernales (Fargallo, 1999), aunque con una alta filopatría (obs. pers.; Fargallo, com. pers.). Se trata de una especie con dimorfismo sexual invertido en tamaño, es decir, las hembras son alrededor de un 20 % mayores que los machos. Estas diferencias sexuales empiezan a detectarse a

partir de los 11 días de vida, cuando las hembras son un 4% mayores que los machos, lo que puede implicar ventajas adaptativas en la competencia entre sexos por los recursos tróficos aportados por los padres al nido (Fargallo, et al. 2003). Como es común en rapaces (Newton, 1979), el macho alimenta a la hembra durante el cortejo. En el caso de formarse la pareja, el macho continúa alimentando a la pareja hasta que los pollos tienen una edad aproximada de dos semanas (Village, 1990).

El patrón general de emparejamiento es la monogamia, aunque puede darse la poliginia en un 5-20% en años de alta abundancia de alimento en Finlandia (Korpimäki, 1988). Este fenómeno ha sido detectado también en nuestra área de estudio (obs. pers.), donde parece infrecuente, si bien la escasez de ejemplares marcados no nos permite estimar su frecuencia de aparición. Se ha descrito que aunque el macho puede cubrir los huevos durante breves plazos de tiempo mientras la hembra se ausenta del nido, la totalidad de la incubación la realiza la hembra con diferentes patrones en el inicio de la incubación (Wiebe et al. 1998b).

Sin embargo, en nuestra área de estudio se han observado machos con placa incubadora bien desarrollada con cierta frecuencia, y la presencia de machos en el nido incubando en las últimas horas del día parece muy frecuente, circunstancia que hemos aprovechado para capturar a lo ejemplares de este sexo. Los volantones están en el nido alrededor de 30 días. Desde la salida del nido hasta su período de independencia, pueden transcurrir alrededor de 2-3 semanas, aunque son escasas las evidencias que lo pueden determinar con exactitud (Village, 1990).

### 3.2. Área de estudio.

El trabajo de campo se realizó durante las primaveras de los años 2000, 2001 y 2002 en la comarca agraria de Campo Azálvaro. Se localiza en la vertiente norte del Sistema Central comprendida entre las provincias de Segovia y Ávila (40°40'N, 4°20'O) y limitada al valle del Río Voltoya. Se trata de un área ganadera, en la que esta actividad agraria ha generado principalmente un pastizal de montaña a 1300 m.s.n.m. Únicamente un bosque mixto de encina (*Quercus ilex*) y roble (*Quercus pyrenaica*) cercano al área de estudio y pequeñas repoblaciones de chopos (*Populus sp.*), algún rodal de pino (*Pinus pinaster*) y árboles aislados conforman la cubierta arbórea.

Desde 1994 hasta 2002, nidales artificiales fueron dispuestos en el área de estudio paulatinamente (Fargallo, 2001), hasta el año 2002 en el que había instaladas un total de 54 cajas nido (Foto 2). Todas las manipulaciones y visitas fueron realizadas con el permiso de la Junta de Castilla y León, junto a los permisos necesarios expedidos por la Dirección General de Conservación de la Naturaleza.

### 3.3. Puesta y eclosión.

Antes del inicio de la puesta (finales de marzo), se realizó en todos los años una visita previa a los nidales para su reparación y relleno de tierra para evitar el riesgo de rotura de los huevos en posteriores manipulaciones. En la primera semana de abril se iniciaron las visitas a los nidos para determinar el inicio de la puesta. Antes y durante la puesta, los nidos se visitaron cada dos días, ya que el intervalo modal de puesta del Cernícalo vulgar es de dos días (Aparicio, 1999). Este ritmo de visitas,





**Foto 3.** Individualización del pollo recién eclosionado.

constante en el horario de visita diario, nos permitió conocer el orden de puesta de los huevos. Estos fueron marcados con un rotulador indeleble en el orden en que fueron encontrados, y dichas marcas permanecieron intactas hasta la eclosión. En el momento en el que los huevos fueron detectados, el largo y ancho máximos fueron medidos con calibre ( $\pm 0.01\text{mm}$ ) y fueron pesados con dinamómetros Pesola ( $\pm 0.25\text{g}$ ).

Entre una semana tras la finalización de la puesta y una semana antes del inicio de eclosión, se capturaron las hembras. Las hembras fueron capturadas en momentos en el que la climatología no fuera adversa con una red puesta en la entrada de la caja-nido mientras incubaba. Cuando fue posible, a cada hembra se le midió el ala y cola con regla ( $\pm 1\text{mm}$ ), el tarso con calibre ( $\pm 0.01\text{mm}$ ) y fueron pesadas con pesola ( $\pm 1\text{g}$ ). Todas ellas fueron anilladas con anillas de metal (DGCONA).

Transcurridos 25 días después de la puesta del primer huevo, se reiniciaron visitas diarias a cada nido. En el momento en que los primeros síntomas de eclosión fueron detectados (ligeras grietas en el

huevo o piar del pollo dentro del huevo) se visitaron los nidos dos veces al día. De este modo se pudo identificar a los pollos durante el proceso de eclosión antes de la eclosión total en un alto porcentaje de casos, mediante marcaje con rotuladores indelebles de colores en el diamante o pico (Foto 3). Esta marca se mantuvo durante toda la eclosión para una posterior identificación de los pollos con un rotulador en la cabeza. La asincronía de eclosión se cuantificó como la diferencia en horas de eclosión de cada huevo con respecto al primero eclosionado. Se consideró un nido asincrónico aquel en que la diferencia entre el primer y último nacimiento del nido superó las 24 h, y nidos sincrónicos aquéllos en que todos los huevos eclosionaron en menos de 24 horas (Stoleson & Beisinger, 1995). Consideramos como pollos de eclosión asincrónica aquellos que eclosionaron al menos 24 h después de que lo hiciera el primer huevo de ese nido. Cada 5 días, los nidos fueron revisitados para mantener las marcas en la cabeza y registrar datos de crecimiento de cada pollo: ala y cola con regla ( $\pm 1\text{mm}$ ), tarso con calibre ( $\pm 0.01\text{mm}$ ) y peso con pesola ( $\pm 0.5\text{g}$ ). En el momento en que los pollos estabilizaron su crecimiento óseo, cuando tenían alrededor de unos 16 días, fueron anillados. De este modo se pudo asegurar de qué huevo procedía cada uno de los pollos estudiados. No se hicieron más visitas al nido hasta que la edad del primer pollo eclosionado fue de 24 días.

### **3.4. Volantones.**

24 días después de la primera eclosión del nido, éste se visitó por penúltima vez (Foto 4). A esta edad, los pollos fueron medidos: ala ( $\pm 1\text{mm}$ ), tarso ( $\pm 0.01\text{mm}$ ) y pesados ( $\pm 0.5\text{g}$ ). Además, fueron medidas





Foto 4. Volantones de Cernícalo vulgar en el nido a una edad aproximada de 27 días.

las bandas negras terminales de la mitad derecha de la cola de cada pollo con calibre ( $\pm 0.01$ ). En esta visita se tomó una pluma del obispillo de cada pollo para posteriores análisis de color y porcentaje de gris. En esta misma visita se extrajo una muestra de sangre de la que se extrajo ADN para sexado molecular y se preparó un frotis para cada pollo. En cada frotis se buscaron parásitos sanguíneos y se realizaron conteos leucocitarios, con los que se calculó el ratio heterófilos/linfocitos (únicamente en 2002), considerado una buena medida de stress fisiológico (Hõrak, et al. 1998). En el año 2002, esta muestra de sangre puede emplearse para determinar niveles de HSPs, que se han revelado como un buen estimador de estrés ambiental en aves (Maxwell & Robertson, 1998).

En esta visita se procedió igualmente a la evaluación de la respuesta inmunitaria medida por Linfocitos T. Para ello, se empleo fitohematoglutinina (PHA), un antígeno ampliamente usado recientemente en estudios de ecología. La inyección local de este antígeno genera una acumulación local de linfocitos-T que puede ser cuantificada y que estima la calidad del sistema inmune celular de cada pollo. La

concentración empleada para esta evaluación fue de 3mg de PHA en 1ml de solución salina. La inyección se realizó en el patagio de cada pollo en las tres temporadas de campo. Pevio a la inyección, se mide el grosor del patagio con un espesímetro 3 veces ( $\pm 0.001$ ). A las 24h se midió nuevamente 3 veces el patagio del volantón en la zona inyectada, considerando la respuesta inmune celular como la diferencia de espesor entre las 2 medidas. Se tomaron 3 medidas antes y después de la inyección con la intención de minimizar el error de la medida ya que el espesor del patagio y la medición de la hinchazón son de escaso grosor en el primer caso y de escasa consistencia en el segundo. Se usaron por tanto los valores medios de las tres mediciones, y, no obstante, se hicieron análisis de repetibilidad que confirmaron la validez de las medias obtenidas de las medidas. Se trata pues de la obtención de la respuesta inmune a través del método reducido propuesto por Smits et al. (1999), que evita tiempo de manipulación de los pollos, minimizan el estrés de los mismos y disminuye el error de las medidas. Todas las medidas fueron tomadas por el autor de la tesis y siempre con el mismo espesímetro.

### 3.5. Alimentación suplementaria.

En el año 2002, se realizó un experimento de alimentación suplementaria a la hembra con objeto de mejorar su condición física antes del inicio de la puesta y estudiar los efectos que pudiera tener sobre la inversión en la descendencia. Para ello, a principios de marzo, cuando las primeras parejas empiezan a ocupar los nidos, se seleccionaron aleatoriamente 20 cajas-nodo. En el interior de éstas, se depositó una codorniz (*Coturnix coturnix*) de un peso aproximado de 150g cada dos

días. La ceba terminó en el momento en el que se inició la puesta (primer huevo puesto detectado). La alimentación suplementaria empezó al menos 18 días antes de la puesta, cubriendo completamente, por tanto, los 9 días requeridos para la formación del huevo en esta especie (Meijer, et al. 1989).

### 3.6. Estadística.

En todos los capítulos incluidos en esta tesis, se ha optado por trabajar con modelos mixtos. Estos modelos permiten emplear al pollo como unidad muestral evitando los problemas de pseudoreplicación que se derivan de usar a los pollos de un mismo nido. Para ello, se incluye en los modelos el nido como variable aleatoria, que permite controlar la varianza debida a las particularidades azarosas y que no pueden ser controladas de cada nido (incluyendo a los padres). Este tipo de análisis tiene además la ventaja de permitir trabajar con variables dependientes que pueden no ajustarse a una distribución normal, o bien con variables en las que sus muestras estén balanceadas. Todos los modelos mixtos fueron realizados con el paquete estadístico SAS (v8.0) con el macro específico para estos modelos GLIMMIX (Little, et al. 1996). El resto de análisis y gráficas fueron realizados con SPSS (v8.0).

## 4. RESULTADOS Y DISCUSIÓN

### 4.1. Diferencias sexuales en el período embrionario.

En el año 2000 se comparó la longitud del período embrionario de huevos de los que eclosionaron pollos macho o hembra (Capítulo I). Estos análisis detectaron que las hembras eclosionaban antes que los machos y demuestran por tanto que el período embrionario de éstas era más corto

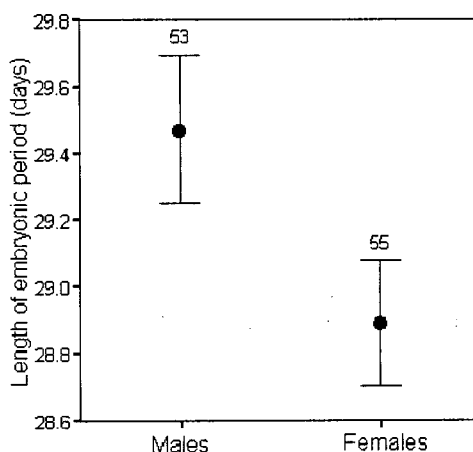


Figura 1. Variación en el período de incubación de huevos con embrión macho o hembra.

(Figura 1). En estos análisis se controló estadísticamente el posible efecto de otras variables que potencialmente podían afectar a la duración del período embrionario (ver Tabla 1 del Capítulo I). Asimismo, observamos que el período embrionario decreció con el tamaño de puesta y con el orden de puesta. En relación al orden de eclosión, se detectó que los huevos que eclosionaron dentro de un intervalo menor de 24 horas no lo hicieron siguiendo el patrón de puesta, como se ha sugerido en trabajos precedentes. El peso de los pollos eclosionados incrementó con el peso del huevo y disminuyó a medida que se incrementaba el período embrionario. El tamaño del huevo estuvo altamente relacionado con el peso del huevo, una vez controlado por las variables relacionadas. El período de incubación más corto de los huevos hembra induce un sesgo sexual en el orden de nacimientos, de forma que las hembras, nacidas antes, adquieren posiciones dominantes en la jerarquía, aunque no encontramos un sesgo en el sexo relacionado con el orden de puesta de los huevos. Por último, comprobamos que el



peso de los volantones, además de estar afectado por el dimorfismo sexual propio de la especie, era menor en pollos subordinados (nacidos tarde en la secuencia de eclosión) y estaba relacionado positivamente con el tamaño del huevo.

Estos resultados son la primera evidencia que demuestra que los embriones hembras eclosionan antes que los machos, al menos en una especie dimórfica como el Cernícalo vulgar. Este hecho sugiere que el crecimiento de las hembras en el huevo es más rápido, que las hembras nacen en un estadio de desarrollo ligeramente más temprano, o la interacción de ambos fenómenos. Nuestros resultados también muestran que la eclosión más temprana de las hembras les permite posicionarse de un modo ventajoso en la cúspide de la jerarquía de tamaños entre los hermanos, de forma que se produce un sesgo en la razón de sexos con el orden de nacimiento, aunque no existiera dicho sesgo con el orden de puesta. En este sentido un crecimiento más rápido puede ser favorable desde dos puntos de vista ópticas. Por un lado favorece la supervivencia, ya que se ha demostrado que los primeros pollos eclosionados tienen más probabilidades de sobrevivir (O'Connor, 1984; Ricklefs, 1993). Además, esta mayor supervivencia puede verse favorecida durante los primeros días de vida, ya que un menor período embrionario permitiría a los embriones eclosionar con mayores reservas y resistir frente a períodos tempranos de escasez de alimento (O'Connor, 1984; Ricklefs, 1993).

Por otro lado y unido a esta última idea, esta mayor reserva de nutrientes al nacer, podría ser favorecida por que un menor período de desarrollo requiere menos costes en el mantenimiento del metabolismo

relativo al crecimiento (Withow, 2000), por lo que el embrión con un desarrollo más rápido debería resultar en mayores reservas en la eclosión. En este sentido, un adelanto en la eclosión de los huevos puede implicar un mayor crecimiento o peso justo antes de la emancipación (Ricklefs, 1993), como apoyan nuestros resultados, lo cual significaría una ventaja competitiva respecto a sus hermanos, lo que tiene importantes implicaciones para la eficacia biológica del pollo, por ejemplo para la supervivencia tras la emancipación y el reclutamiento (Magrath, 1991). En este sentido, Ricklefs (1993) sugiere un compromiso entre tasa de crecimiento y requerimientos energéticos. En especies dimórficas, como las aves rapaces, se ha descrito que el sexo mayor puede sufrir inanición debido a sus altos requerimientos energéticos (Ricklefs, 1993; Bortolotti, 1986), por lo que una leve ventaja en la eclosión, podría favorecer al sexo mayor en condiciones de falta de alimento, especialmente en las etapas más tempranas de la vida del volantón.

Sin embargo, los mecanismos que están tras estas diferencias en la incubación, podrían ser o bien adaptaciones de los embriones hembras para mejorar su habilidad competitiva frente a sus hermanos machos, o bien una estrategia paternal, en la que la madre invierte en determinados recursos en el huevo que causan las diferencias en duración del desarrollo, o realiza una incubación diferencial de huevos machos y hembra si pudiera diferenciarlos de algún modo. En este sentido, existen recientes estudios que demuestran el papel de determinadas hormonas esteroides insertadas en el huevo por la hembra, que pueden influir tanto en el crecimiento del embrión, como del pollo tras la eclosión, así como en su capacidad competitiva en el

nido frente a sus hermanos (Schawbl, 1993, 1996; Sockman & Schawbl, 2000; Eising et al., 2001). Además, recientemente se ha demostrado que estas hormonas pueden ser administradas diferencialmente según el sexo de los embriones (Petrie et al., 2001). Estos resultados abren toda una nueva línea de investigación, ya que si las diferencias sexuales en la duración del período embrionario estuvieran más extendidas de lo que se conoce, constituirían un nuevo mecanismo evolutivo (parental o filial) en el establecimiento de jerarquías de tamaño y en el fenómeno de la competencia entre hermanos en especies nidícolas, en particular en especies con dimorfismo sexual en tamaño.

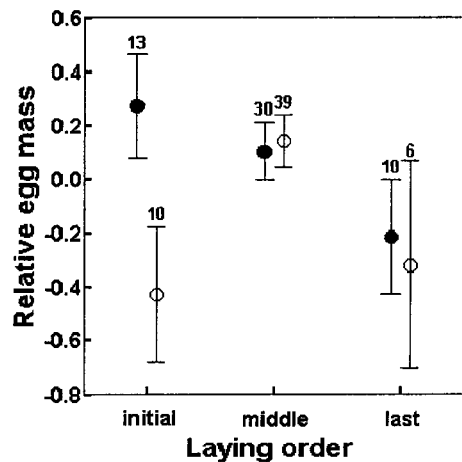
#### 4.2. Distribución de recursos en los huevos según el sexo y el orden de puesta.

En el año 2000, se estudió la relación entre el sexo de los huevos, el peso de estos, la secuencia de puesta y su relación con la jerarquía de los pollos antes del vuelo. Se detectó cómo el peso del huevo se relacionó con el orden de puesta y con la interacción entre ésta y el sexo del huevo, indicando que el peso del huevo disminuía con la secuencia de puesta y que el sexo sólo afectó al peso de los primeros huevos puestos. En este sentido se observó que los huevos hembra fueron menos pesados que los huevos macho, sólo en el primer huevo puesto. Sin embargo, este dimorfismo sexual tuvo implicaciones en el tamaño de los huevos siguientes de la puesta, en función del sexo del primer huevo. Así, aquellas hembras que iniciaron su puesta con un huevo del que eclosionaría un pollo macho, el peso de los huevos disminuyó con la secuencia de puesta, mientras que las hembras que iniciaron su puesta con un

huevo ‘hembra’, siguieron una estrategia opuesta en relación al peso del huevo (Figura 2). Igualmente, se detectó que los huevos que dan lugar a pollos macho fueron mayores y eclosionaron más tarde que los huevos de los que nacieron hembras.

Por último, se estudió cómo la inversión diferencial de acuerdo al sexo del primer huevo, pudo influir en el peso de esos mismos embriones en su época de volantón. En este sentido, se observó que el peso de los volantones decreció con el orden de eclosión en nidos que se iniciaron con puestas de huevos de los que eclosionaron machos mientras que no se encontraron variaciones en puestas iniciadas con huevos de los que eclosionaron hembras (Figura 3).

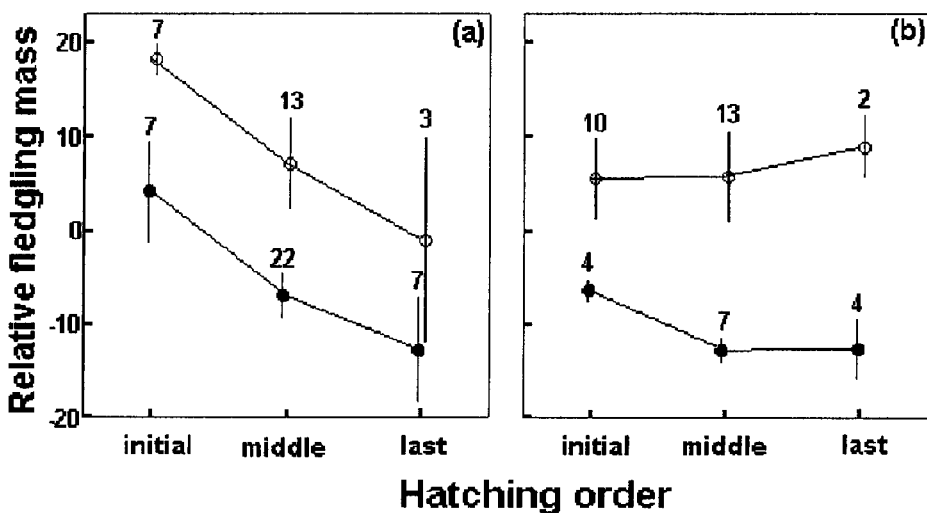
Estos resultados sugieren un control en la inversión en los huevos por parte de las hembras reproductoras en función del sexo y del orden de puesta, lo que no se ha descrito nunca antes aunque recientemente confirmado por Magrath et al. (2003), es



**Figura 2.** Dimorfismo sexual en los huevos de Cernicalo vulgar en relación al orden de puesta. Círculos rellenos indican machos y círculos vacíos indican hembras.

decir, diferencias sexuales sesgadas por el orden de puesta. Este fenómeno se ha descrito para otras especies (Mead, et al. 1987; Andersson *et al.* 1997; Viñuela 1997, Cordero *et al.* 2000, 2001; Cunningham & Russell 2001), pero esta es la primera evidencia de que dicha inversión diferencial puede depender del sexo del primer huevo puesto. En este sentido, los resultados sugieren que una inversión diferencial en los volantones puede ser modulada desde una inversión diferencial en los huevos y mediada por el sexo del primer huevo puesto. Sin embargo, mientras que limitaciones energéticas podrían explicar la disminución del tamaño del huevo con el orden de puesta o la influencia de la prolactina, desencadenante de la incubación (Leblanc 1987; Arnold 1991), éstas no explicarían un relativo menor tamaño del primer huevo puesto y cómo ésta se asociaría a un determinado sexo. Se sugiere, por tanto, que el mecanismo próximo que determina el tamaño del huevo inicial sería

una reabsorción parcial de los recursos inicialmente dedicados a embriones hembra en el paso por el oviducto, ya que la determinación del sexo del embrión es anterior a lo que la hembra invierte en ese determinado huevo (en yema no en clara). En el caso de los huevos macho, esta reabsorción no se llevaría a cabo en el paso por el oviducto. Parcialmente, los resultados muestran el mismo patrón anunciado en el **Capítulo I**, es decir, que los huevos de los que eclosionaron pollos hembra eclosionaron antes que aquellos de los que eclosionaron machos. La combinación de estos dos fenómenos sugieren la existencia de mecanismos de control maternal temprana de la inversión en el contexto de competencia entre hermanos y dimorfismo sexual en tamaño. La variación de la inversión en los huevos en función de que el primer huevo puesto sea macho o hembra podría ser un mecanismo para controlar la competencia de los pollos hembra eclosionados en primer lugar, ya que si la



**Figura 3.** Media  $\pm$  SE relativa del peso del volatón (peso del volatón menos la media de la pollos del nido) en relación al orden de eclosión y al sexo. En (a) puestas iniciadas con huevos de los que eclosionaron machos y (b) con hembras. Los números sobre las barras indican el tamaño de muestra.

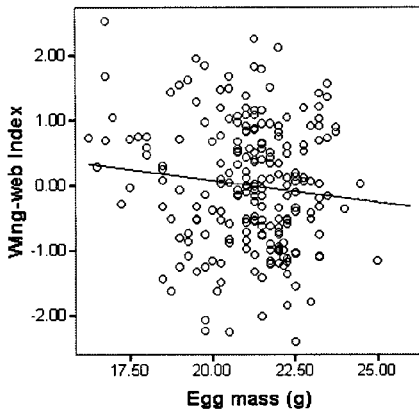
hembra eclosionara antes y además estuviera favorecida por un mayor tamaño del huevo, comprometería la supervivencia del resto de los pollos por su mayor capacidad competitiva. La opción contraria explicaría un mayor tamaño de los huevos de los que eclosionan machos. Por tanto, los resultados sugieren dos estrategias en relación la inversión en los huevos con objeto de controlar el grado de competencia entre hermanos. Por un lado, una estrategia de 'supervivencia' podría sugerirse para aquellas hembras que inician su puesta con huevos de los que eclosionan hembras, invirtiendo más en los huevos puestos en último lugar para evitar pérdidas en períodos de escasez de alimento. Sin embargo, una estrategia de 'reducción' sería adoptada por las hembras que inician su puesta con huevos de los que eclosionan machos, favoreciendo la eliminación de los últimos pollos eclosionados en períodos de escasez de alimento, aunque sin embargo la reducción de nidada es relativamente baja en nuestro área de estudio (**Capítulo VI**) o bien una inversión diferencial en los pollos. Por último, se observó que el sexo del primer huevo puede condicionar la jerarquía de tamaños de los pollos, ya que la jerarquía de tamaños (peso) decreció cuando el primer huevo puesto dio lugar a un macho y no presentó ninguna tendencia cuando fue una hembra. Debido a que el estudio fue observacional, no se determina si la hembra puede manipular el sexo de los huevos que pone en primer lugar, lo que sí sucedería es que de algún modo detecta el sexo de dicho huevo y en función de ello invierte diferencialmente en los huevos puestos a continuación. Este control sobre el peso del huevo influye en el peso del pollo eclosionado (Williams 1994; Christians 2002) y éste se traslada a la jerarquía de

tamaño de la puesta (Slagsvold *et al.* 1984; Viñuela 1997). Este control se centraría especialmente en el primer huevo eclosionado ya que el crecimiento y supervivencia de éste en una especie dimórfica podría comprometer o influir en la supervivencia de los hermanos (Bortolotti 1986; Blanco *et al.* 2002; Krebs *et al.* 2002).

Finalmente, las estrategias sugeridas podrían servir para (1) compensar las diferencias genéticas derivadas del dimorfismo sexual en la tasa de crecimiento o bien (2) manteniendo un cierto grado de variación en el tamaño de la descendencia, favorecer a los primeros pollos eclosionados. Por último, dado que no encontramos evidencias de un sesgo poblacional en la razón de sexos (aunque la muestra es limitada), nuestros resultados indican que pueden existir mecanismos sofisticados de control maternal de la inversión en huevos que permitan compensar las desventajas impuestas por el dimorfismo sexual en la competencia entre hermanos, y que pueden conducir a la producción de razones de sexos equilibradas.

#### **4.3. Compromiso entre calidad y tamaño del huevo.**

Debido al seguimiento detallado realizado desde la puesta a la eclosión, se pudo conocer en un alto porcentaje los huevos de los que procedía cada pollo ( $n = 332$  pollos, 86.2%). Por ello, se pudo relacionar las características de cada huevo con la respuesta inmune celular de los pollos eclosionados de esos mismos huevos. Se observó una relación negativa entre el peso del huevo y la respuesta inmune de los pollos aunque no significativa agrupando los años 2000 y 2001. Para ello, en el año 2002



**Figura 4.** Relación negativa y estadísticamente significativa entre el tamaño del huevo y la respuesta inmune de los pollos eclosionados de esos mismos huevos. Se consideraron los años 2000, 2001 y las puestas control del año 2002 (ver punto 3.3).

se realizó un experimento de ceba a la hembra antes de la puesta con la intención de ver si esta relación negativa marginalmente significativa estaba mediada por una limitación energética de la hembra.

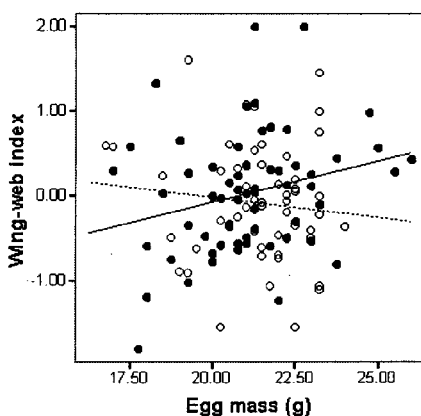
El experimento de ceba no afectó a ninguno de los parámetros reproductivos (fecha de puesta, tamaño de puesta, tamaño de los huevos, pollos volados, proporción de sexos, etc.), ni tampoco se detectó variación en la condición de la hembra durante la incubación, medida como la relación peso tamaño, entre nidos experimentales o control. Tampoco se detectaron variaciones en los tiempos de incubación de los huevos entre hembras cebadas y no cebadas o tiempos totales de incubación de la puesta. Sin embargo, considerando todas las puestas del año 2000, 2001 y las puestas controles de 2002, se observó que el tamaño del huevo se relacionó negativamente con la respuesta inmune de los pollos (Figura 4). Asimismo, el experimento mostró que la relación entre el peso del huevo y la

respuesta inmune de esos mismos huevos varió entre hembras suplementadas y controles. Así, hubo una relación negativa entre el peso del huevo de las hembras no cebadas y la respuesta inmune de esos pollos, mientras que en los huevos puestos por hembras suplementadas antes de la puesta, a medida que incrementaba el peso de los huevos también lo hacía la respuesta inmune de los pollos que eclosionaron de esos mismos huevos (Figura 5). Igualmente se observó un efecto del tamaño del huevo sobre el peso de los volantones y un incremento en la supervivencia de los pollos que procedieron de huevos de peso mayor.

Paralelamente, este capítulo arroja un hecho también novedoso sobre la respuesta inmune en poblaciones en libertad, ya que se observa un fuerte efecto del año en la respuesta inmune celular de los pollos, al igual que en el **Capítulo VI**, lo que sugiere que las condiciones particulares de cada año pueden alterar la respuesta inmune de los pollos y que debe ser controlada en los análisis, especialmente en trabajos no experimentales aunque también en aquellos experimentales debido a que ciertas condiciones ambientales (meteorología, por ejemplo) no son manipulables y que debe ser un factor a considerar de forma importante en futuras investigaciones.

Estos resultados sugieren que la hembra en condiciones normales, estaría limitada energéticamente para poder invertir en huevos grandes que favorecen la supervivencia de los pollos y además en embriones de mayor calidad, medida como la respuesta inmunitaria que desarrollarán esos embriones justo antes de volar.

Tradicionalmente se ha asociado el tamaño del huevo a una mayor calidad del mismo. Sin embargo, recientes estudios



**Figura 5.** Relación entre la respuesta inmune celular mediada por linfocitos T y el peso de los huevos de pollos eclosionados de hembras suplementadas (línea continua) antes de la puesta y hembras control (línea discontinua). El eje Y representa un índice de respuesta inmune del que fueron eliminados el efecto aleatorio del nido y el peso del volantón (ver Capítulo III).

sugieren que ambas características (peso y calidad) no tienen por qué estar unidas (Giron & Casas, 2003; Reynolds et al., 2003; Hipfner et al., 2003; Royle et al., 2003). Nuestros resultados apoyarían este hecho, si se asume como medida de calidad del huevo la respuesta inmune que los embriones de esos huevos son capaces de montar justo antes de la independencia. Por un lado, las hembras en condiciones normales invertirían en huevos mayores cuyos embriones generarían una respuesta inmune menor pero que se verían favorecidos por una mayor supervivencia, mientras que los huevos más pesados de las hembras cebadas podrían además adicionar recursos suplementarios para mejorar la respuesta inmune futura de esos huevos. Por tanto, las hembras cebadas suplirían una carencia alimenticia invirtiendo recursos adicionales en sus huevos, revelando un compromiso entre calidad y el peso del mismo.

Por tanto, cuando la hembra tiene una buena condición antes de iniciar la puesta, podría invertir más recursos en sus huevos. Esto hecho podría explicar por qué las hembras cebadas y control no difirieron ni en tamaño ni en condición, ya que estos recursos excedentarios podrían haberse invertido en los huevos. Estos resultados, sin embargo, no aclaran qué tipo de recurso podría la hembra invertir en sus huevos para que además de verse favorecido en tamaño, incremente su respuesta inmune celular justo antes de su independencia. Quizá, determinadas proteínas, vitaminas u hormonas puedan jugar un papel importante en este sentido.

#### **4.4. El papel de los parásitos intestinales en la reproducción y en la calidad de los volantones.**

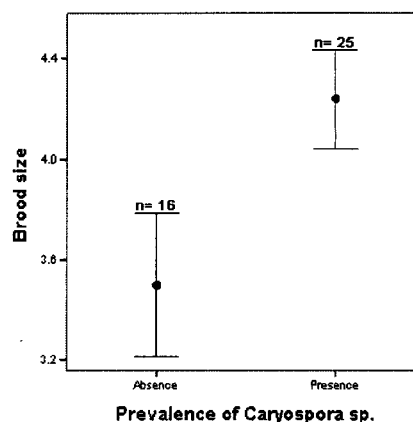
Se analizó en el año 2001 y 2002 una muestra aleatoria de heces de pollos de Cernícalo vulgar de edades comprendidas entre 24 y 26 días para estudiar sus parásitos intestinales. De un total de 71 pollos de los que se pueden colectar muestras fecales, 59.2% estuvieron infectados por ooquistes de parásitos intestinales con una media de intensidad de excreción de  $2991.36 \pm 5838.28$  ooquistes/g de muestra fecal. La única variable que se relacionó con la probabilidad de que al menos un pollo del nido estuviera infectado fue el tamaño de la nidada (Figura 6). Además, considerando la abundancia de excreción de ooquistes y el pollo como unidad muestral, el modelo mixto (ver detalles del modelo en **Capítulo IV**) mostró cómo la única variable relacionada fue el año. Además, estudiamos el efecto que pudo tener tanto la prevalencia como la abundancia de excreción en la respuesta inmunitaria celular. No se encontraron efectos significativos de la

infección por parásitos intestinales en la respuesta inmune celular.

A pesar de que los parásitos intestinales pueden causar un fuerte impacto en la salud de rapaces en cautividad (Forbes & Simpson, 1997; Upton, 1990), el conocimiento sobre su efecto en poblaciones naturales son escasos, excepto el caso de la muerte de un Cernícalo vulgar silvestre descrito recientemente (Krone, 2000). Por ello, este es el primer estudio que describe la infección de este tipo de parásitos en una población en libertad de una especie de ave de presa. Los resultados sugieren que las dos únicas variables que afectaron a la carga parasitaria intestinal fueron el año y el tamaño de la nidada. Por un lado las variaciones interanuales en las abundancias de parásitos pueden ser típicas, aunque es un aspecto muy poco estudiado, y con sólo dos años de estudio no se puede dilucidar ninguna causa de variación que la pudiera explicar. No obstante, el tamaño de nidada se relacionó positivamente con la probabilidad de infección en el nido. En el área de estudio, no se sabe realmente cuál podría ser el vector de los parásitos aunque es posible que el Topillo campesino (*Microtus arvalis*), pueda jugar un papel importante, ya que es la única especie del área de estudio descrita como hospedador (Volf, *et al.* 2001) del género *Caryospora*, y es una presa importante para los cernícalos de esta población (obs. pers.). En este sentido, sería razonable pensar que en puestas mayores, en las que el esfuerzo parental de caza aumenta (Dijkstra *et al.*, 1990), la probabilidad de captura de un topillo infectado sea mayor. Adicionalmente y de modo no excluyente, podría considerarse que un mayor número de pollos en el nido podría mejorar las condiciones de microhábitat en el nido para

el crecimiento y desarrollo de parásitos, así como una mayor competencia entre hermanos.

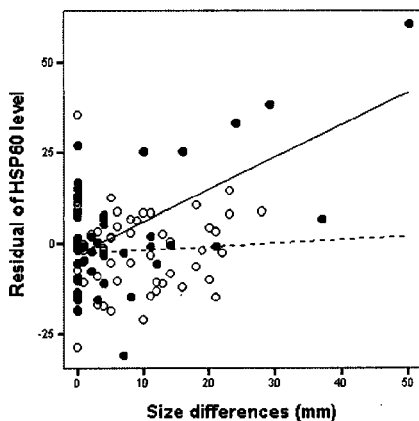
No se detectaron efectos de la parasitación en los parámetros considerados de calidad de los pollos, incluida la respuesta inmune celular. El hecho de que la infección no influyera en la respuesta inmune podría ser debido a que (1) los efectos de la infección se manifiesten en el período posterior a la salida de los pollos del nido, como muestra Krone (2000) o bien (2) que el método que empleamos para medir el efecto de estos parásitos en la respuesta inmune no sea el adecuado. Unido a que el mecanismo inmunitario frente a coccidios es poco conocido (Long, 1992), no se puede descartar un efecto negativo de estos parásitos sobre los cernícalos, aunque los resultados sugieren otros métodos para evaluar esta influencia y en otro momento del período reproductivo.



**Figura 6.** Media  $\pm$  SE del número de pollos en la nidada en relación a la probabilidad de infección por parásitos intestinales en la nidada. Los números sobre las barras de error indican los tamaños muestrales, according to infection of *Caryospora* spp.

#### 4.5. Efecto del sexo y la jerarquía de tamaños en el estrés de los pollos.

En el año 2002 se estudió el efecto del estrés de los pollos en relación al sexo y a la jerarquía de tamaños en el nido. El estrés se estimó como el nivel de proteínas de estrés o HSP (Heat Shock Protein) en sangre en volantones a la edad de 25 días. Adicionalmente, se compararon los resultados con otro estimador de estrés como es la relación heterófilos/linfocitos, junto al efecto de los diferentes parásitos presentes en los pollos a esa edad. Los resultados sugieren que los niveles de proteínas de estrés, estuvieron relacionados negativamente con el tamaño y crecimiento del ala, así como con el peso y el crecimiento en peso, además de positivamente relacionados con la diferencia en tamaño y peso del pollo con respecto a su hermano mayor. Sin embargo, cuando todas las variables fueron consideradas conjuntamente, sólo la diferencia en tamaño se mantuvo en el modelo, relacionándose negativamente con el nivel de HSP60.



**Figura 7.** Efecto de la interacción entre el sexo y las diferencias en tamaño respecto al hermano mayor en los niveles de HSP60 de pollos de Cernícalo vulgar. La línea continua indica hembras y la discontinua machos.

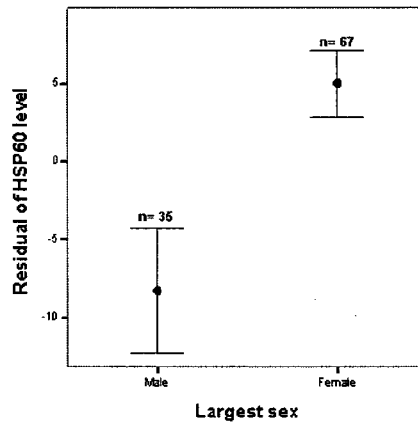
Además esta relación, estuvo mediada por el sexo de los volantones, de modo que las hembras con mayor diferencia en tamaño respecto a su hermano mayor sufrieron más estrés que los machos en esa misma situación (Figura 7). El modelo también detectó que en las nidadas en el que el pollo de mayor tamaño fue una hembra, el nivel de estrés de los pollos fue mayor (Figura 8), así como una variación positiva de los niveles de estrés con la fecha de puesta. Por último, considerando estas variables en el mismo modelo, se detectó que los parásitos intestinales incrementaron el nivel de estrés considerando únicamente presencia-ausencia de parasitación. No se encontraron parásitos sanguíneos en la población y tampoco relaciones estadísticamente significativas entre niveles e índices leucocitarios con los niveles de HSP60.

Nuestros resultados sugieren principalmente que las proteínas de estrés pueden ser un buen índice de estimación del grado de competencia entre hermanos en nidadas de aves, y especialmente las HSP60 pueden ser buenos indicadores del estrés nutricional, siendo consistentes con resultados precedentes que indican niveles mayores de estrés en condiciones de restricción alimenticia (Nuñez-de la Mora, *et al.* 1996; Kitaysky, Wingfield & Piatt 2001). Además, este índice se revela en poblaciones en libertad como un índice más sensible al estrés nutricional que el sugerido por la relación heterófilos/linfocitos. Estos niveles de estrés en la puesta podrían deberse a la competencia asociada para optar a una posición ventajosa en la ceba, especialmente en condiciones de abundancia de alimento desfavorables, como se ha sugerido para el cernícalo vulgar (Fargallo *et al.* 2003). Además el efecto de la fecha de puesta sobre los niveles de estrés, se sugiere



que puede ser debido a (1) un incremento de las temperaturas en la zona de estudio y su incidencia en la caja-nido, desde las primeras a las últimas parejas, o bien (2) debido a una disminución del alimento en la zona de estudio con la fecha de puesta.

Los resultados sugieren igualmente que los niveles de proteínas de estrés de los pollos más pequeños varían con el sexo de los pollos, de forma que los pollos hembra sufren un mayor estrés que los machos. A pesar de que en aves se ha apoyado la hipótesis de una mayor capacidad competitiva de la hembra bajo condiciones desfavorables (Fargallo *et al.* 2003; Fargallo *et al.* 2002; Arroyo, 2002; Anderson *et al.* 1993), otras hipótesis sugieren que el hecho de ser el sexo de mayor tamaño implica unos costes mayores asociados a mayores tasas de crecimiento y por tanto una desventaja frente al pollo de sexo menor (Velando, 2002; Torres & Drummond, 1997; Bortollotti, 1986). Nuestros resultados apoyarían la segunda opción, dado que una desventaja en tamaño dentro de la nidada impuesta por la asincronía de eclosión implicaría mayores costes para la hembra que para el macho. Adicionalmente, los resultados sugieren que cuando el pollo de mayor tamaño en la nidada es hembra, el resto de hermanos sufriría mayores niveles de estrés debido a una mayor capacidad competitiva de la hembra, como se ha demostrado tanto en Cernícalo vulgar (Fargallo, *et al.* 2003) como en Cernícalo americano (Anderson *et al.* 1993). Esta idea enlaza con los resultados mostrados en el punto 3.2. (ver además **Capítulo II**) en el que describíamos como cuando la primera eclosión es una hembra, los tamaños de los pollos al volar se homogenizan, no así cuando la primera eclosión es un macho. Quizá esta estrategia serviría para minimizar



**Figura 8.** Media ( $\pm$  SE) de los niveles de HSP60 en relación al sexo del pollo mayor de la puesta. Las cifras sobre las barras indican los tamaños muestrales.

los efectos de la mayor capacidad competitiva de la hembra.

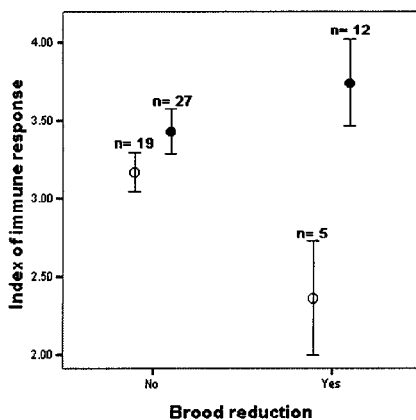
Adicionalmente, se observó que los parásitos intestinales incrementaron el nivel de estrés de los pollos infectados. Este es el primer resultado que muestra que los parásitos intestinales tienen un efecto fisiológico sobre una rapaz en libertad. Se ha descrito que en pollos de Cernícalo vulgar, la infección por cariosporosis incrementa la temperatura corporal (Macario, 1995) y que las proteínas de estrés incrementan debido a la infección parasitaria (Garbe, 1995). Por ello, este resultado puede explicarse por un incremento de la temperatura corporal, es decir, fiebre, como respuesta a la parasitación intestinal por *Caryospora sp.*

#### 4.6. Mortalidad en el nido y su efecto en la calidad de los hermanos.

Se estudió la asincronía de eclosión en los años 2000, 2001 y 2002 en el área de estudio, pudiéndose determinar con precisión esta variable en 86 puestas. Sin embargo, sólo se emplearon aquellas

pertenecientes a puestas modales que pudieron ser de eclosión asincrónica o sincrónica, siguiendo las recomendaciones de Wiebe y Bortolotti (1994). En las puestas analizadas se detectó que la asincronía de eclosión (medida como tiempo transcurrido entre la eclosión de cada pollo y el del primer pollo nacido en la nidada) es el principal factor explicativo de la probabilidad de morir en una nidada. Comparando puestas de eclosión sincrónica o asincrónica, no hubo efectos de la eclosión sobre la condición física de los pollos (medida como la relación peso-tamaño), el peso de los mismos, peso de los huevos o peso de los pollos en la eclosión. Tampoco hubo un efecto en las mismas variables entre aquellos nidos que se murió al menos un pollo y en los que no perecieron. Sin embargo, las puestas de eclosión asincrónicas fueron más tardías que las puestas de eclosión sincrónica. Considerando los tres años de estudio, los

modelos estadísticos mostraron que la relación entre la respuesta inmune celular que pueden generar los pollos y la asincronía de eclosión fue diferente, mediado por el efecto de la reducción de nidada. En este sentido, los pollos que eclosionaron en puestas asincrónicas, incrementaron su respuesta inmune celular cuando hubo reducción de nidada, mientras que los pollos que eclosionaron en puestas sincrónicas que sufrieron reducción de nidada, disminuyeron su respuesta inmune (Figura 9). No obstante, no se detectó un efecto de la asincronía de eclosión por sí sola o la reducción de nidada sobre la calidad de la respuesta inmune de los pollos. Tampoco se detectaron diferencias en la respuesta inmune en relación al orden de puesta o eclosión. Sin embargo se detectó que las puestas menores de 4 huevos siempre fueron sincrónicas y aquellas mayores de 6 siempre eclosionaron de modo asincrónico, lo que sugiere el tamaño de puesta puede ser un limitante para que la hembra pueda adoptar un tipo de eclosión u otro. No hubo diferencias sexuales en la respuesta inmune, una vez que se corrigió por el efecto del peso de los pollos. Sin embargo, la respuesta inmune de machos y hembras estuvo afectada por la reducción de nidada. De este modo, los machos se beneficiaron de ésta sin variar su respuesta inmune, mientras que las hembras se vieron perjudicadas por la reducción de nidada disminuyendo su respuesta inmune significativamente.



**Figura 9.** Media  $\pm$  SE de la respuesta inmune celular en relación a la asincronía de eclosión y la reducción de nidada. Círculos rellenos indican puestas de eclosión asincrónica y círculos vacíos indican puestas de eclosión sincrónica. Las cifras sobre las barras de error indican el tamaño muestral.

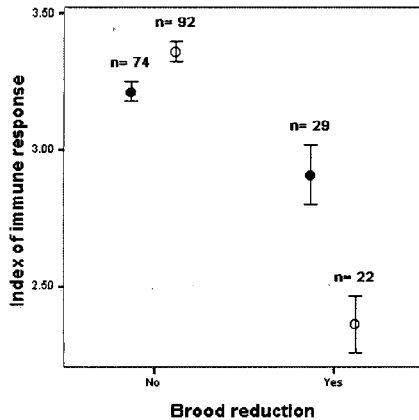
En todos los casos, el año resultó una variable altamente significativa, por lo que fue controlada en los análisis. Como se indicaba en el **Capítulo III**, el año es un factor muy a tener en cuenta en los estudios de respuesta inmune celular.

Los resultados muestran por tanto que a pesar de que la reducción de nidada favorecida por la asincronía de eclosión y por tanto por el patrón de inicio de incubación es baja en nuestra zona de estudio, sin embargo puede tener importantes efectos sobre la calidad de los pollos. Los resultados muestran que las puestas de eclosión asincrónica son favorecidas por la reducción de nidada y aquellas de eclosión sincrónica son perjudicadas cuando algún pollo perece. Estos resultados sugieren por tanto que una ausencia de jerarquía de tamaños puede perjudicar a la nidada bajo condiciones de restricción alimentaria, que es cuando alguno de los pollos es eliminado de la nidada (Wiebe & Bortolotti, 1994, Viñuela 1999). Estos resultados apoyan la hipótesis de Lack (1954) ya que una reducción de nidada favorece la calidad del resto de los hermanos aunque con indicadores de la calidad de los pollos, entendida ésta como la respuesta inmune celular, y no con variables clásicas como el peso de los volantones o el número de pollos volados. Sin embargo, estos resultados también pueden ajustarse a las predicciones de hipótesis similares, en particular la de reducción de la agresividad (Hahn 1981), que, de hecho, ha recibido más apoyo en la literatura reciente (revisión en Viñuela & Carrascal 1999), y sería necesario un estudio más detallado del comportamiento de los pollos en el nido para discernir entre ambas hipótesis.

Por tanto, la reducción de nidada, serviría para mejorar la calidad de los volantones (Slagsvold et al. 1995), sugiriendo un compromiso entre calidad y cantidad de pollos volados, ya que un incremento en la respuesta inmune puede ser también un buen indicador de supervivencia futura (Hörak et al. 1999;

Soler et al. 1999; Tella et al. 2000b). Las volantones eclosionados de puestas sincrónicas, que fueron más tempranas, sin embargo se verían favorecidas por unas condiciones más favorables en el momento de su independencia ya que se emanciparían al inicio de la temporada, momento más favorable por una mayor disponibilidad trófica. Por tanto, por un lado las puestas de eclosión asincrónica son más tardías y con la reducción de nidada los pollos mejorarían su eficacia biológica ("fitness") y supervivencia futura, en lo que puede ser una apuesta por calidad sobre número de volantones. En cambio, las puestas tempranas serían de eclosión más sincrónica, favoreciendo a la cantidad frente a la calidad de los pollos, quizás porque las condiciones más favorables para los pollos en su fase de emancipación del nido permiten compensar su peor calidad, medida al menos como su capacidad de respuesta inmune celular.

Los resultados en relación al sexo indicaron diferencias sexuales en la respuesta inmunitaria únicamente cuando hubo reducción de nidada (Figura 10). En contra de resultados anteriores que sugieren que las hembras tienen una mayor capacidad competitiva que el macho en condiciones de restricción de alimento, y esto se refleja en una mayor respuesta inmune (Fargallo, et al. 2002), nuestros resultados sugieren diferentes patrones. La respuesta inmunitaria disminuyó en nidos en los que hubo reducción de nidada, aunque no significativamente. Sin embargo, sí hubo un efecto diferencial en el crecimiento de los pollos según el sexo, ya que cuando hubo reducción de nidada, los machos ajustaron mejor su crecimiento y crecieron menos significativamente mientras que las hembras permanecieron en niveles similares de



**Figura 10.** Media  $\pm$  SE de la respuesta inmune celular de los pollos de Cernícalo vulgar en relación al sexo y la mortalidad del nido. Círculos completos indican hembras y círculos vacíos indican machos..

crecimiento en tamaño independientemente de si hubo o no restricción alimentaria, es decir, reducción de nidada. Este hecho pudo reflejar una menor flexibilidad y adaptación por parte de la hembra en su crecimiento y que unido a unos mayores costes de crecimiento y desarrollo por ser el sexo mayor, que pudo ser sustraído de los recursos que se derivaran a montar una respuesta inmune competitiva.

## 5. CONCLUSIONES Y PERSPECTIVAS

Esta tesis sugiere que el Cernícalo vulgar puede adoptar diferentes estrategias que podrían ir dirigidas a minimizar la competencia de la descendencia así como mejorar la calidad de su progenie. En este sentido, la hembra podría alterar el tamaño de los huevos en función del sexo de los mismos con objeto de minimizar la competencia de los pollos una vez eclosionados (**Capítulo II**), y podría comprometer la calidad del huevo frente al tamaño del mismo (**Capítulo III**). La

primera sugerencia, desde un punto de vista adaptativo, significaría que la hembra puede decidir la inversión en los huevos en función del sexo del primer huevo puesto con objeto de igualar la capacidad competitiva de los pollos en el nido según su sexo. No obstante, con los datos disponibles no hemos podido profundizar en cuáles son los factores próximos ni las causas ambientales que pudieran determinar la estrategia a seguir por la hembra. Nuestros resultados demuestran que las hembras ejercen cierto control de la inversión en su descendencia con estrategias aparentemente muy elaboradas. Investigaciones futuras deberían intentar conocer (1) si la hembra puede decidir el sexo de los huevos que pone en primer lugar, (2) si existe un efecto ambiental, como una mayor disponibilidad de alimento antes de la puesta, que pudiera modular o inducir a la hembra a invertir en un primer huevo macho o hembra y 3) hasta que punto nuestros resultados reflejan una estrategia de control parental o adaptaciones filiales.

Esta inversión diferencial en los huevos podría también estar modulada por la restricción alimentaria, ya que se sugiere un compromiso entre la calidad y el tamaño del huevo, donde primaría la segunda en condiciones control. Este hecho a nivel evolutivo sugeriría que la supervivencia de los pollos podría estar determinada en parte por las condiciones a las que esté expuesta la hembra antes de la puesta. En este sentido, los resultados mostrados sugieren que el tamaño del huevo tiene un importante efecto tanto sobre el tamaño del volantón (**Capítulo I y II**) como sobre la supervivencia de los pollos. Sin embargo, no parece que la calidad del huevo, medida como su respuesta inmune celular, esté relacionada con el tamaño del mismo. En

este sentido, ya que tanto el tamaño del huevo como la respuesta inmune celular se han sugerido como estimadores de supervivencia futura, sería necesario estudiar en qué tipo de componentes la hembra puede invertir sus recursos para mejorar la calidad de los huevos y cuáles pueden ser limitantes para la calidad de la descendencia. Este tipo de estudios, aportarían una información fundamental en el estudio de la ecología reproductiva, por que o bien se han estudiado cómo o qué componentes se invierten en el huevo, o bien cómo influye una determinada estrategia reproductiva en la calidad o cantidad de la descendencia. Sin embargo, no existe información de cómo los componentes que se invierten en el huevo afectan a diferentes componentes del fitness de la descendencia.

No obstante, en el Cernícalo vulgar en particular y en rapaces en general, la condición de la hembra antes de la puesta está íntimamente ligada a la calidad del macho. Es por ello, que futuras investigaciones deben explorar el efecto que tiene la calidad del macho sobre estos patrones y el papel de la selección sexual en este contexto (Wiehn & Korpimäki, 1997).

Además de que de los resultados mostrados se desprende que el sexo de la descendencia es un factor que puede modular la inversión maternal, también sugiere consecuencias en los pollos tanto en su etapa en el nido (**Capítulo V y VI**) como en el desarrollo embrionario (**Capítulo I**). En este sentido se mostró que las hembras en condiciones desfavorables, mostraron niveles más altos de estrés o bien sufrieron más la restricción alimentaria que los machos, medida esta como su capacidad de respuesta inmune celular. Estos resultados

contradicen la idea de que el sexo mayor tendría una ventaja competitiva bajo condiciones desfavorables y sin embargo, apoyaría la idea de que el sexo mayor tiene unos costes mayores para su crecimiento que serían más elevados en condiciones desfavorables. Es decir, el crecimiento mayor de la hembra es un condicionante genético y una mayor capacidad competitiva de la hembra en el nido frente a sus hermanos sería una consecuencia adaptativa. Desde esta perspectiva ambas posibilidades serían válidas, aunque no determina cuál sería más importante desde un punto de vista evolutivo. Quizá, estas diferencias entre resultados, especialmente con Cernícalo vulgar entre el norte de Europa (Fargallo, et al. 2001) y latitudes mediterráneas, puedan deberse a condicionantes ecológicos diversos en el que especialmente la disponibilidad u oscilación cíclica de alimento sugiera diferentes patrones adaptativos entre machos y hembras. Es decir, sería posible que los pollos machos o hembra sigan diferentes adaptaciones en función de la disponibilidad de alimento?. Los resultados obtenidos, sin embargo, apoyarían más la idea de un coste mayor para la hembra, el sexo mayor y una mayor flexibilidad de los machos para adaptarse a condiciones desfavorables, ya sea medida como estrés o como respuesta inmune celular. No obstante, los estudios que determinan el coste fisiológico de los pollos en función de su sexo son escasos, por lo que estudios experimentales con especies dimórficas en tamaño son determinantes para evaluar la importancia relativa de ambas ideas. Sin embargo, los resultados científicos recientes, sugieren que el macho en diferentes especies sufre más las condiciones desfavorables que las hembras,

medida ésta como su capacidad de respuesta inmune celular (Müller, et al., 2003; Tschirren et al., 2003; Fargallo et al, 2002), contrario a los mostrados en esta tesis (Capítulo VI). La escasez de trabajos que evalúen las diferencias sexuales en pollos en su capacidad de respuesta inmune celular, hace que más estudios sean llevados a cabo para clarificar estas diferencias, entre especies y dentro de especies.

Estas diferencias sexuales no sólo se detectaron durante la etapa post-eclosión sino que también se mostró que los embriones hembra tuvieron un período embrionario más corto que los embriones macho (**Capítulo I**). Este resultado apoyaría la idea señalada anteriormente. Un menor período embrionario de la hembra favorecería las condiciones post-eclosión y dirigiría a la hembra hacia una posición de, al menos, no en desventaja en tamaño frente a sus hermanos, especialmente durante los primeros días tras la eclosión ya que implicaría un mayor nivel de reservas en esos días en el caso de restricción alimenticia. Sin embargo, para evitar una jerarquía de tamaños demasiado marcada dentro de la nidada, que pudiera comprometer la supervivencia de la descendencia, la hembra modularía la inversión en sus huevos y favorecería una estrategia que no perjudique un sexo sobre otro (**Capítulo II**). En definitiva, estos resultados sugieren que las hembras de Cernícalo vulgar tienen un ajuste muy fino de la inversión en su descendencia, estando determinada por el sexo de los pollos y la calidad de los huevos. No obstante, estos resultados suscitan dos dudas importantes, que deberían ser la base de investigaciones futuras: 1) de nuevo, hasta que punto este fenómeno se trata de una estrategia parental o filial, y 2) tampoco conocemos las causas

“próximas” de esa variación en la duración del periodo embrionario como por ejemplo, inversión diferencial de la hembra en hormonas esteroides u otros elementos, o incubación diferencial de huevos macho y hembra si la hembra incubante puede reconocerlos de algún modo.

En definitiva, desde el punto de vista científico, esta Tesis sugiere que el sexo de los pollos y sus interacciones durante su etapa en el nido son factores que deben tenerse en cuenta en estudios de ecología evolutiva y dentro de ella a aquellos que aborden las estrategias vitales.

En relación a la asincronía de eclosión, se observó que podría favorecer un compromiso entre la calidad y cantidad de los volantones (**Capítulo VII**). Los resultados obtenidos sugieren una evidencia a favor de la hipótesis de reducción de nidada de Lack (1954) aunque podrían igualmente sugerir una evidencia más a favor de la hipótesis de la reducción de la competencia entre hermanos (Hahn, 1981) ya que los resultados obtenidos en diferentes capítulos (I y II, unidos a los resultados de los Capítulos V y VI), sugeriría que la hembra establece mecanismo muy ajustados para disminuir la competencia entre hermanos. Sin embargo, la hipótesis de la reducción de nidada es apoyada de un modo novedoso ya que no se estima la eficacia de la reducción de nidada en la cantidad de volantones, peso de los mismos o su crecimiento, si no que es apoyada considerando la calidad de los volantones. Es decir, la mortalidad asociada a la asincronía de eclosión favorece la calidad de los volantones ya que podría minimizar la competencia de los pollos de una nidada, lo que implicaría favorecer la eliminación de un pollo para mejorar la calidad de sus

hermanos. En diferentes estudios se ha sugerido que la asincronía de eclosión pudiera estar modulada por la variación cíclica de la abundancia de alimento, sin embargo, en la zona de estudio no se detectaron variaciones en este sentido al menos durante los tres años de campo, lo que indicaría que los resultados en relación a la asincronía de eclosión no estuvieron alterados por esta razón. No obstante, manipulaciones experimentales serían necesarias para explicar ambas posibilidades. Los resultados igualmente sugieren que el estudio del patrón de eclosión debe ser mucho más fino de lo que tradicionalmente se ha realizado en estudios científicos (ver por ejemplo Wiebe et al. 1998a; Wiebe & Bortolotti, 1994), ya que al menos debe ponerse en duda que el orden de puesta se relaciona con el orden de eclosión. Debido a que los huevos macho y hembra tienen períodos embrionarios diferentes, al menos en *Cernícalo vulgaris*, desde el punto de vista de las causas que determinen la asincronía de eclosión, debería considerarse qué efecto tiene proporción de sexos primaria con ésta. Así, podría esperarse que las puestas con igual proporción de machos y hembras sean más asincrónicas que las puestas con proporción de sexos sesgadas. En definitiva, los resultados obtenidos sobre el efecto de la asincronía de eclosión en la descendencia, sugerirían diferentes caminos para aclarar más, si es posible, las causas de este patrón de eclosión. Por un lado, (1) el estudio detallado de los patrones de incubación durante la puesta, que es cuando la hembra podría 'decidir' la estrategia a seguir, de la que existen escasos resultados. Por otro lado, (2) el estudio de la proporción de sexos de la puesta, ya que se asocia a diferentes períodos embrionarios. Además, si partimos de la idea de que el éxito real de

la reproducción no es la producción de la descendencia si no la reproducción futura de la descendencia, (3) es necesario conocer las consecuencias dinámicas que tiene para la población un patrón de eclosión sincrónica o asincrónica. Es decir, saber cómo se reproducen los pollos de eclosión tardía en comparación con los pollos que eclosionan más temprano. Así como (4) estudiar cómo de estables son los patrones de eclosión en la misma hembra durante los años para conocer si existe una limitación genética o ambiental en los patrones de inicio de incubación. En relación a la tradicional discusión sobre si la asincronía es una adaptación o una limitación, los resultados sugieren la primera opción aunque también apuntan que puede el tamaño de puesta podría limitar la estrategia seleccionada por la hembra a la hora de iniciar la incubación. La respuesta inmune celular es uno de los tres componentes más importantes del sistema inmune de defensa contra patógenos. En la literatura científica reciente, multitud de estudios emplean esta herramienta para validar sus hipótesis, ya sea en estudios experimentales u observacionales. Sin embargo, no existen en la actualidad estudios que empleen la respuesta inmune celular en diferentes años y observen su posible variación. Los resultados de esta Tesis sugieren que el año es una fuente de variación muy importante en la capacidad que tienen, al menos los pollos, de desarrollar una respuesta inmune celular. Por ello, y con objeto de mejorar la validez de los estudios de respuesta inmune celular, se sugiere que el factor año debe ser considerado especialmente en los estudios descriptivos. Además, si el efecto del año es importante en la respuesta inmune es necesario investigar qué causas pueden estar tras esta variación.

En relación a los parásitos, los pollos de Cernícalo vulgar en el área de estudio únicamente presentan ectoparásitos y parásitos intestinales. De los primeros, los únicos presentes son aquellos del género *Carnus* y no parecen producir efectos sobre los Cernícalos, también avalado por otros estudios (Roulin, et al. 2003; Dawson & Bortolotti, 1997). Debe destacarse también a aquellos de la familia Hippoboscidae de presencia muy baja en los pollos. Sin embargo, si se detectaron altas prevalencias de parásitos intestinales del género *Caryospora*, que afectaron positivamente a los niveles de estrés de los pollos infectados (**Capítulo V**) aunque no en otros componentes del fitness de los pollos (**Capítulo IV**), al menos en su etapa en el nido. Sin embargo, la alta presencia detectada por primera vez en una población de rapaces en libertad, indica la necesidad de más investigación sobre este tipo de parásitos. Por un lado en relación a la coloración de los pollos, ya que se ha descrito que estos parásitos afectan a esta característica, y su papel en la selección sexual, ya que únicamente se estudió la infección en los pollos. A pesar de que no se observó relación entre la parasitación intestinal y la respuesta inmune celular, no deben descartarse futuras asociaciones con otros componentes del sistema inmune.

Además, el estudio de la parasitación intestinal por coccidios sugiere una importante vía de estudio en relación al vector que lo transmite. En este sentido, parece que el topillo campesino (*Microtus arvalis*), junto a otros arvicolinos, puede jugar un importante papel, especialmente en la zona de estudio, ya que ésta es una presa de alta calidad y representando un 30% en biomasa en la dieta de los cernícalos. Esta relación parásito-hospedador podría ser más

estrecha en los países nórdicos, donde la dependencia de este tipo de roedores es muy alta. En definitiva, el estudio de la parasitación intestinal en rapaces en libertad puede tener importantes implicaciones en la conservación de éstas, por lo que la descripción de la parasitación y el estudio de sus efectos en poblaciones no amenazadas como las del Cernícalo vulgar, podrían sugerir importantes ideas desde el punto de vista de la ecología evolutiva, concretamente desde la relación parásito-hospedador y desde la óptica de la conservación.

Esta tesis sugiere además que las proteínas de estrés térmico o HSP's puede ser una importante indicador de estrés ambiental (**Capítulo V**) y que se está revelando como buena herramienta para el estudio en ecología evolutiva. En este sentido carece de las limitaciones propias del uso de hormonas de respuesta casi instantánea como la corticosterona o la testosterona. Sin embargo, sugiere incógnitas que desde el punto de vista de la ecología son difícilmente explicables y que necesitan de la colaboración de estudios de laboratorio. En este sentido, es destacable que parece que la HSP60 es un estimador más fino del estrés que la HSP70 y sabiendo que existen multitud de tipos de proteínas de estrés, sería necesario evaluar otros tipos de esta proteína en estudios de campo. Esta idea, no obstante es conservativa con los resultados obtenidos. Por otro lado, sería necesario más estudios con esta proteína que incrementaran su validez para estudios en ecología.

## 5. BIBLIOGRAFÍA

---

Alexander, R.D. 1974. The evolution of social behaviour. *Annual Review of Ecological Systematic*, 5: 325-384.



- Anderson, D.J., Budde, C., Apanius, V., Martinez Gomez, J.E., Bird, D.M. & Weathers, W.W. 1993. Prey size influences female competitive dominance in nestling American kestrels. *Ecology*, **74**: 367-376.
- Anderson, D.J., Reeve, J. & Bird, D.M. 1997. Sexually dimorphic eggs, nestling growth and sibling competition in American Kestrels *Falco sparverius*. *Functional Ecology*, **11**: 331-335.
- Anderson, D.J., Reeve, J. & Bird, D.M. 1997. Sexually dimorphic eggs, nestling growth and sibling competition in American Kestrels *Falco sparverius*. *Functional Ecology*, **11**: 331-335.
- Apanius, V. 1998. *Ontogeny of Immune function*. En: Avian growth and development. Evolution within the altricial-precocial spectrum (Starck, J.M. & Ricklefs, R.E. eds.). Oxford Ornithology Series. Oxford University Press. Oxford.
- Aparicio, J.M. 1999. Intraclutch egg-size variation in the eurasian kestrel: advantages and disadvantages of hatching from large eggs. *The Auk*, **116**: 825-830.
- Araujo, J. 1973. Falconiformes del Guadarrama occidental. *Ardeola*, **19**: 257 - 278.
- Arnold, T.W. 1991. Intraclutch variation in egg size of american coots. *Condor*, **93**: 19-27.
- Arnold, T.W., Rohwer, F.C. & Armstrong, T. 1987. Egg viability, nest predation, and the adaptive significance of clutch size in prairie ducks. *American Naturalist*, **130**: 643-653.
- Arroyo, B. 2002. Sex-biased nestling mortality in the Montagu's harrier *Circus pygargus*. *Journal of Avian Biology* **33**: 455-460.
- Barnard, C.J. & Behnke, J.M. 1990. *Parasitism and host behaviour*. Taylor and Francis. Londres.
- Beissinger, S.R. & Waltman, J.R. 1991. Extraordinary clutch size and hatching asynchrony of neotropical parrot. *Auk*, **108**: 863-871.
- Bennett, D.C., Whitehead, P.E. & Hart L.E. 1995. Growth and energy requirements of hand-reared Great Blue Heron (*Ardea herodias*) chicks. *Auk*, **112**: 201-209
- Bernis, F. 1966. *Aves Migradoras Ibéricas, Volumen 1*. Sociedad Española de Ornitología. Madrid. España.
- Bienz, M. 1984. *Xenopus hsp 70* genes are constitutively expressed in injected oocytes. *EMBO Journal*, **3**: 2477-2483.
- Blanco, G., Davila, J.A., Lopéz-Septiem, J.A., Rodríguez, R. & Martínez, F. 2002. Sex-biased initial eggs favours sons in the slightly size-dimorphic Scops Owl (*Otus scops*). *Biological Journal of the Linnean Society*, **76**:1-8.
- Bortolotti, G.R. 1986. Influence of sibling competition on nestling sex-ratios of sexually dimorphic birds. *The American Naturalist*, **127**: 495-507.
- Cavé, A.J. 1968. The breeding of the kestrel, *Falco tinnunculus*, in the reclaimed area Oostelik Flevoland. *Netherland Journal of Zoology*, **18**: 313 - 407.
- Charnov, E.L. 1982. *The theory of sex allocation*. Princeton : Princeton University Press.
- Christe, P., Møller, A.P. & de Lope, F. 1998. Immunocompetence and nestling survival in the house martin, the tasty chick hypothesis. *Oikos*, **83**: 175-179.
- Christians, J.K. 2002. Avian egg size: variation within species and inflexibility within individuals. *Biological Reviews*, **77**: 1-26.
- Clark, A.B. & Wilson, D.S. 1981. Avian breeding adaptations: hatching asynchrony, brood reduction, and nest failure. *Q. Review of Biology*, **56**: 253-277.
- Clutton-Brock, T.H 1991. *The evolution of parental care*. Princeton University Press.
- Clutton-Brock, T.H. 1986. Sex ratio variation in birds. *Ibis*, **128**: 318-329.
- Clutton-Brock, T.H., Albon, S.S. & Guinness, F.E. 1985. Parental investment and sex differences in juvenile mortality in birds and mammals. *Nature*, **313**: 131-133.
- Cordero, P., Griffith, S.C., Aparicio, J.M. & Parkin, D.T. 2000. Sexual dimorphism in house sparrow eggs. *Behavioral Ecology and Sociobiology*, **48**: 353-357.

- Cordero, P., Viñuela, J., Aparicio, J.M. & Veiga, J.P. 2001. Seasonal variation in sex ratio and sexual egg dimorphism favouring daughters in first clutches of the spotless starling. *Journal of Evolutionary Biology*, **14**: 829-834.
- Cramp, S. & Simmons, K.E.L. 1980. The birds of the Western Palearctic. Vol. 2. Hawks to bustards. Oxford University Press. Oxford. UK.
- Cunningham, E.J.A. & Russell, A.F. (2001). Reply to Petrie et al. *Nature*, **412**: 498.
- Darwin, C. 1871. *The descent of man and selection in relation to sex*. John Murray, London.
- Dawson, R.D. & Bortolotti, G.R. 2002. Experimental evidence for food limitation and sex-specific strategies of American kestrels (*Falco sparverius*) provisioning offspring. *Behavioral Ecology and Sociobiology*, **52**: 43 – 52.
- Dawson, R.D. & Bortolotti, G. 1997. Ecology of parasitism of nestling American kestrels by *Carnus hemapterus* (Diptera: Carnidae). *Canadian Journal of Zoology*, **75**: 2021-2026.
- Dijkstra, C., Daan, S. & Buker, J.B. 1990. Adaptive seasonal variation in the sex ratio of kestrel broods. *Functional Ecology*, **4**: 143-147.
- Drummond, H., Osorno, J.L., Torres, R., García Chavelas, C. & Larson, H.M. 1991. Sexual size dimorphism and sibling competition, implications for avian sex ratios. *American Naturalist*, **138**: 623 – 641.
- Eising, C.M., Eikenaar, H. Schwabl H. & Groothuis T.G.G. 2001. Maternal androgens in black-headed gull (*Larus ridibundus*) eggs: consequences for chick development. *Proceedings of the Royal Society of London B*, **268**: 839-846.
- Emlen S.T. 1997. When mothers prefer daughters over sons. *Trends in Ecology and Evolution*, **12**: 291-292.
- Fair, J.M., Hansen, E.S. & Ricklefs, R.E. 1999. Growth, developmental stability and immune response in juvenile Japanese quails (*Coturnix coturnix japonica*). *Proceedings of the Royal Society of London B*, **266**: 1735 – 1742.
- Fargallo, J.A., Laaksonen, T., Korpimäki, E., Pöyri, V., Griffith, S.C. & Valkama, J. 2003. Size-mediated dominance and begging behaviour in Eurasian kestrel broods. *Evolutionary Ecology Research*, **5**: 549-558.
- Fargallo, J.A., Blanco, G., Potti, J. & Viñuela, J. 2001. Nest boxes provisioning in a rural population of Eurasian Kestrels: breeding performance, nest predation and parasitism. *Bird Study*, **48**: 236-244.
- Fargallo, J.A. 1999. Efecto del cernícalo vulgar sobre la abundancia de topillo campesino: un caso de manejo de poblaciones naturales. *Colección Naturaleza y Medio Ambiente*. Caja Segovia.
- Fargallo, J.A., Laaksonen, T., Pöyri, V. & Korpimäki, E. 2002. Inter-sexual differences in the immune response of Eurasian kestrel nestlings under food shortage. *Ecology Letters*, **5**: 95-101.
- Fisher, R.A. 1930. The genetical theory of natural selection. Oxford University Press. Oxford.
- Folstad, I. & Karter, A.J. 1992. Parasites, bright males and the immunocompetence handicap. *The American Naturalist*, **139**: 603-622.
- Forbes, N.A. & Simpson, G.N. 1997. *Caryospora neofalconis*: an emerging threat to captive-bred raptors in the United of Kingdom. *Journal of Avian Medicine Surgery*, **11**: 110-114.
- Garbe, T.R. 1992. Heat shock proteins and infection: Interactions of pathogen and host. *Experientia*, **48**: 635-639.
- Giron, D. & Casas, J. 2003. Mothers reduce egg provisioning with age. *Ecology Letters*, **6**: 273-277.
- González, G., Sorci, G., Møller, A.P., Ninni, P., Haussy, C. & de Lope, F. 1999. Immunocompetence and condition-dependent sexual advertisement in male house sparrow (*Passer domesticus*). *Journal of Animal Ecology*, **68**: 1225 – 1234.
- Greig-Smith, P. 1985. Weight differences, brood reduction, and sibling competition among nestling Stonechats *Saxicola torquata* (Aves: Turdidae). *Journal of Zoology*, **205**: 453-465.

- Hahn, D.C. 1981. Asynchronous hatching in the Laughing Gull: cutting losses and reducing rivalry. *Animal Behaviour*, **29**: 421-427.
- Hamilton, W.D. & Zuk, M. 1982. Heritable true fitness and bright birds: A role for parasites?. *Science*, **218**: 384-387.
- Hart, B.L. 1990. Behavioral adaptations to pathogens and parasites: five strategies. *Neuroscience and Biobehavioral Reviews*, **14**: 273-294.
- Hart, B.L. 1994. Behavioral defense against parasites: interactions with parasite invasiveness. *Parasitology*, **109**: 139-151.
- Hipfner, J.M., Gaston, A.J., Herzberg, G.R., Brosnan, J.T. and Storey, A.E. 2003. Egg composition in relation to female age and relaying: constraint on egg production in Thick-billed murre (Uria lomvia). *Auk*, **120**: 645-657.
- Hörak, P., Tegelmann, L., Ots, I. & Møller, A.P. 1999. Immune function and survival of great tit nestling in relation to growth conditions. *Oecologia*, **121**: 316-322.
- Hörak, P., Ots, I & Muramägi, A. 1998. Haematological health state indices of reproducing great tits: A response to brood size manipulation. *Functional Ecology*, **12**: 750-756.
- Hussel, D.J.T. 1972. Factors affecting clutch size in Arctic passerines. *Ecological Monographs*, **42**: 317-364.
- Ingolia, T.D., Graig, E.A. & McCarthy, B.J. 1980. Sequence of three copies of the gene for the major Drosophila heat shock induced protein and their flanking regions. *Cell*, **21**: 669-679.
- Jackson, W.M. 1993. Causes of conspecific nest parasitism in the northern masked weaver. *Behavioral Ecology and Sociobiology*, **32**: 119-126.
- Johnsen, A., Andersen, V., Sunding, C. & Lifjeld, J.T. 2000. Female bluethroats enhance offspring immunocompetence through extra-pair copulation. *Nature*, **406**: 296 – 299.
- Karch, F., Torok, I. & Tissieres, A. 1981. Extensive regions of homology in front of the two hsp70 heat shock variant genes in Drosophila melanogaster. *Journal of Molecular Biology*, **148**: 219-230.
- Kitaysky, A.S. Wingfield, J.C. & Piatt, J.F. 2001. Corticosterone facilitates begging and affects resource allocation in the black-legged kittiwake. *Behavioral Ecology*, **12**: 619-625.
- Kolman, W. 1960. The mechanism of natural selection for the sex ratio. *American Naturalist*, **94**: 373 - 377
- Korpimäki, E. 1988. Factors promoting polygyny in European birds of prey – a hypothesis. *Oecologia*, **77**: 287-293.
- Korpimäki, E., May, C.A., Parkin, D.T., Wetton, J.H. & Wiehn, J. 2000. Environmental- and parental condition-related variation in sex ratio of kestrel broods. *Journal of Avian Biology*, **31**: 128-134.
- Krebs, E.A., Green, D.J., Double, M.C. & Griffiths, R. 2002. Laying date and laying sequence influence the sex ratio of Crimson Rosella broods. *Behavioral Ecology and Sociobiology*, **51**: 447-454.
- Krone, O. 2002. Fatal Caryospora infection in a free-living juvenile Eurasian kestrel (*Falco tinnunculus*). *Journal of Raptor Research*, **36**: 84-86.
- Laaksonen, 2003. *Life history strategies of birds of prey in a variable environment*. PhD Tesis.
- Lack, D. 1954. *The natural regulation of animal numbers*. Oxford University Press.
- Leblanc, Y. 1987. Intraclutch variation in egg size of Canada Geese. *Canadian Journal of Zoology*, **65**: 3044-3047.
- Leppä, S. & Sistonen, L. 1997. Heat shock response – Pathophysiological implications. *Annals of Medicine*, **29**: 73-78.
- Lindquist, S. 1986. The heat shock response. *Annual Review of Biochemistry*, **55**: 1151-1191.
- Littell, R.C., Milliken, G.A., Stroup, W.W. & Wolfinger, R. D. 1996. *SAS system for mixed models*. SAS Institute. Cary. New York.
- Lochmiller, R.L. & Deerenberg, C. 2000. Trade-offs in evolutionary immunology: just what is the cost of immunity?. *Oikos*, **88**: 87-98.
- Long, P. 1992. *Avian coccidiosis*. In: Kreier, JP (ed). Parasitic protozoa. Vol. 4. Academic Press, London, pp: 1 – 75.

- Macario, A.J.L. 1995. Heat-shock proteins and molecular chaperones: Implications for pathogenesis, diagnostics, and therapeutics. *International Journal of Clinical & Laboratory Research*, **25**: 59-70.
- Magrath, M.J.L., Brouwer, L. & Komdeur, J. 2003. Egg size and laying order in relation to offspring sex in the extreme sexually dimorphic brown songlark *Cincloramphus cruralis*. *Behavioral Ecology and Sociobiology*, **54**: 240-248.
- Magrath, R.D. 1991. Nestling weight and juvenile survival in the blackbird, *Turdus merula*. *Journal of Animal Ecology*, **60**: 335-351.
- Magrath, R.D. 1990. Hatching asynchrony in altricial birds. *Biological Review*, **95**: 587-622.
- Magrath, R.D. 1988. Hatching asynchrony in altricial birds: nest failure and adult survival. *American Naturalist*, **131**: 893-900.
- Massemin, S., Korpimäki, E. & Wiehn, J. 2000. Reversed sexual dimorphism in raptors: evaluation of the hypotheses in kestrels breeding in a temporally changing environment. *Oecologia*, **124**: 26-32.
- Maxwell, M.H. & Robertson, G.W. 1998. The avian heterophil leucocyte: A review. *World's Poultry Science Journal*, **49**: 34-43.
- Mead, P.S. & Morton, M.L. 1985. Hatching asynchrony and in the Mountain White-crowned Sparrow (*Zonotrichia leucophrys oriantha*): selected or incidental trait?. *Auk*, **102**: 781-792.
- Mead, P.S., Morton, M.L. & Fish, B.E. 1987. Sexual dimorphism in egg size and implications regarding facultative manipulation of sex in Mountain White-crowned Sparrows. *Condor*, **89**: 798-803.
- Meijer, T., Masman, D. & Daan, S. 1989. Energetics of reproduction in female kestrels. *Auk*, **106**: 549-559.
- Merino, S., Moreno, J., Sanz, J.J. & Arriero, E. 2000. Are avian blood parasites pathogenic in the wild? A medication experiment in blue tits (*Parus caeruleus*). *Proceedings of the Royal Society of London B*, **267**: 2507-2510.
- Merino, S. & Potti, J. 1995. Mites and blowflies decrease growth and survival in nestling pied flycatchers. *Oikos*, **73**: 95-103.
- Mock, D.W. & Parker, G.A. 1997. *The evolution of sibling rivalry*. Oxford, Oxford University Press.
- Mock, D.W. & Ploger, B.J. 1987. Parental manipulation of optimal hatch asynchrony in cattle egrets: an experimental study. *Animal Behaviour*, **35**: 150-160.
- Mock, D.W. & Schwagmeyer, P.L. 1990. The peak load reduction hypothesis for avian hatching asynchrony. *Evolutionary Ecology*, **4**: 249-260.
- Moore, J. 2002. *Parasites and the behaviour of animals*. Oxford University Press, Oxford.
- Moreno, J., Sanz, J.J. & Arriero, E. 1999. Reproductive effort and T-lymphocyte cell-mediated immunocompetence in female pied flycatcher *Ficedula hypoleuca*. *Proceedings of the Royal Society of London B*, **266**: 1105-1109.
- Müller, W., Dijkstra, C. & Groothuis, T.G.G. En prensa. Inter-sexual differences in T-cell-mediated immunity of black-headed gull chicks (*Larus ridibundus*) depend on the hatching order. *Behavioral Ecology and Sociobiology*.
- Newton, I. 1979. *Population Ecology of Raptors*. T&AD Ediciones.
- Núñez-de la Mora, A., Drummond, H. Wingfield, J.C. 1996. Hormonal correlates of dominance and starvation-induced aggression in chicks of the Blue-footed booby. *Ethology*, **102**: 748-761.
- O'Connor R.J. 1984. *The growth and development of birds*. Chichester, Wiley and Sons.
- Olsen, P.D. & Cockburn, A. 1991. Female-biased sex allocation in peregrine falcons and other raptors. *Behavioral Ecology and Sociobiology*, **28**: 417-423.
- Oring, L. 1982. Avian mating systems. En: *Avian Biology*, vol. 6 (D.S. Farner, J.R. King & Parkes, K.C. eds). Academic Press, New York, pp: 1-92.

- Pen, I. 2000. Sex allocation in a Life History Context. Tesis doctoral. Rijksuniversiteit Groningen. The Netherlands.
- Petrie, M., Schawbl, H., Brande-Lavridsen, N. & Burke, T. 2001. Sex differences in avian yolk hormone levels. *Nature*, **412**: 498.
- Reynolds, S.J., Schoech, S.J. & Bowman, R. 2003. Nutritional quality of prebreeding diet influences breeding performance of the Florida scrub-jay. *Oecologia*, **134**: 308-316.
- Reznik, D. 1992. Measuring the costs of reproduction. *Trends in Ecology & Evolution*, **7**: 42-45.
- Ricklefs, R.E. 1993. *Sibling competition, hatching asynchrony, incubation period, and lifespan in altricial birds*. Pp 199-276 in D. M. Power, ed, Current Ornithology.
- Riedstra, B., Dijkstra, C. & Daan, S. 1998. Daily energy expenditure of male and female Marsh harrier nestlings. *Auk*, **115**: 635-641.
- Ritossa, F. 1962. A new puffing pattern induced by temperature shock and DNP in *Drosophila*. *Experientia*, **18**: 571-573.
- Roulin, A., Brinkhof, M.W.G., Bize, P., Richner, H., Jungi, T.W., Bavoux, C. Boileau, N. & Burneleau, G. 2003. Which chick is tasty to parasites? The importance of host immunology vs. parasite life history. *Journal of Animal Ecology*, **72**: 75-81.
- Royle, N.J., Suray, P.F. & Hartley, I.R. 2003. The effect of variation in dietary intake on maternal deposition of antioxidants in zebra finch eggs. *Functional Ecology*, **17**: 472-481.
- Saino, N., Calza, S. & Møller, A.P. 1998. Effects of a dipteran ectoparasites on immune response and growth trade-offs in barn swallow, *Hirundo rustica*, nestlings. *Oikos*, **81**: 217-228.
- Schawbl, H. 1993. Yolk is a source of maternal testosterone for developing birds. *Proceedings of the National Academy of Science USA*, **90**: 11446-11450.
- Schawbl, H. 1996. Maternal testosterone in the avian egg enhances postnatal growth. *Comparative Biochemistry and Physiology A*, **114**: 271-276.
- Schlesinger, M.J. 1990. Heat shock proteins. *Journal of Biological Chemistry*, **265**: 12111-12114.
- Sheldon, B.C., Merilä, J., Lindgren, G. & Ellegren, H. 1998. Gender and environmental sensitivity in nestling collared flycatchers. *Ecology*, **79**: 1939-1948.
- Slagsvold, T. 1990. Fisher's sex ratio theory may explain hatching patterns in birds. *Evolution*, **44**: 1009-1017.
- Slagsvold, T. 1986. Asynchronous versus synchronous hatching in birds: experiments with the Pied Flycatcher. *Journal of Animal Ecology*, **55**: 1155-1134.
- Slagsvold, T. & Lifjeld, J.T. 1990. Hatching asynchrony in birds: the hypothesis of sexual conflict over parental investment. *American Naturalist*, **134**: 239-253.
- Slagsvold, T., Sandvik, J., Rofstad, G., Lorentsen, O. & Husby, M. 1984. On the adaptive value of intra-clutch egg-size variation in birds. *The Auk*, **101**: 685-697.
- Slagsvold, T., Røskoft, E. & Engen, S. 1986. Sex ratio, differential cost of rearing young, and differential mortality between the sexes during the period of parental care: Fisher's theory applied to birds. *Ornis Scandinavica*, **17**: 117-125.
- Smits, J.E., Bortolotti, G.R. & Tella, J.L. 1999. Simplifying the phytohemagglutinin skin-testing technique in studies of avian immunocompetence. *Functional ecology*, **13**: 567-572.
- Sockman, K.W. & Schawbl, H. 2000. Yolk androgens reduce offspring survival. *Proceedings of the Royal Society of London B*, **267**: 1451-1456.
- Sorci, S., Soler, M. & Møller, A.P. 1997. Reduced immunocompetence of nestlings in replacement clutches of the European magpies (*Pica pica*). *Behavioral Ecology*, **10**: 281 - 286.
- Stenning, M.J. 1996. Hatching asynchrony, brood reduction and other rapidly reproducing hypotheses. *Trends in Ecology and Evolution*, **11**: 243-246.

- Stinson, C.H. 1979. On the selective advantage of fratricide in raptors. *Evolution*, **33**: 1219-1225.
- Stoleson, S. H. & Beissinger, S. R. 1995. Hatching asynchrony and the onset of incubation in birds, revisited. When is the critical period?. *Current Ornithology*, **12**: 191-270.
- Tella, J.L., Bortolotti, G., Dawson, R.D. & Forero, M.G. 2000. The T-cell-mediated immune response and return rate of fledgling American kestrel are positively correlated with parental clutch size. *Proceedings of the Royal Society of London B.*, **267**: 891-895.
- Tella, J.L., Scheuerlein, A. & Ricklefs, R.E. 2002. Is cell-mediated immunity related to the evolution of life-history strategies in birds?. *Proceedings of the Royal Society of London B*, **269**: 1059-1066.
- Torres, R. & Drummond, H. 1997. Female-biased mortality in nestling of a bird with size dimorphism. *Journal of Animal Ecology*, **66**: 859-865.
- Trivers, R.L. 1974. Parent-offspring conflict. *American Zoologist*, **14**: 249-265.
- Tschirren, B., Fitze, P.S. & Richner, H. 2003. Sexual dimorphism in susceptibility to parasites & cell-mediated immunity in great tit nestlings. *Journal of Animal Ecology*, **72**: 839 – 845.
- Upton, S. J., Campbell, M., Weigel, M. & McKnown, R. D. 1990. The Eimeriidae (Apicomplexa) of raptors: review of the literature and description of new species of the genera *Caryospora* and *Eimeria*. *Can. J. Zool.*, **68**: 1256 – 1265.
- Veiga, J.P., Salvador, A., Merino, S. & Puerta, M. 1998. Reproductive effort affects immune response and parasite infection in a lizard: a phenotypic manipulation using testosterone. *Oikos*, **82**: 313-318.
- Velando, A. & Alonso-Álvarez, C. 2003. Differential body condition regulation by males and females in response to experimental manipulation of brood size and parental effort in the blue-footed booby. *Journal of Animal Ecology*, **72**: 846-856.
- Velando, A. 2002. Experimental manipulation of maternal effort produces differential effects in son and daughters, implications for adaptive sex ratios in the blue-footed boobies. *Behavioral Ecology*, **13**: 443 – 449.
- Velando, A., Lessells, C.M. & Márquez, J.C. 2001. The function of female and male ornaments in the Inca tern, evidence for link between ornament expression and both adult condition and reproductive performance. *Journal of Avian Biology*, **32**: 311-318.
- Village, A. 1990. *The Kestrel*. Editado por T&AD Poysser Ediciones.
- Viñuela, J. 2000. Opposing selective pressures on hatching asynchrony, egg viability, brood reduction and nestling growth. *Behavioral Ecology and Sociobiology*, **48**: 333-343.
- Viñuela, J. & Carrascal, L. M. 1999. *Hatching patterns in nonprecocial birds: a preliminary comparative analysis*. En: Adams, N. J. & Slotow, R. H. (eds). Proc. 22 Int. Ornithol. Congr., Durban: 584-599.
- Viñuela, J. 1999. Sibling aggression, hatching asynchrony, and nestling mortality in the black kite (*Milvus migrans*). *Behavioral Ecology and Sociobiology*, **45**: 33-45.
- Viñuela, J. 1997. Adaptation vs constraint: intraclutch egg-mass variation in birds. *Journal of Animal Ecology*, **66**: 781-792.
- Volf, J., Modrý, D. & Koudela, B. 2001. Experimental transmission of *Caryospora kutzery* (Aplicomplexa: Eimeriidae) by rodent hosts. *Folia parasitologica*, **48**: 11- 14.
- Welch, W.J. 1992. Mammalian stress response: cell physiology, structure/function of stress proteins and implications for medicine and disease. *Physiological Reviews*, **4**: 1063-1081.
- Whittow, G.C. (Ed.) 2000. *Sturkie's Avian Physiology*. Academic Press, New York.
- Wiebe, K.L. & Bortolotti, G. .1994. Energetic efficiency of reproduction, the benefits of asynchronous hatching for American kestrels. *Journal of Animal Ecology*, **63**: 551 – 560.
- Wiebe, K.L., Korpimäki, E. & Wiehn, J. 1998a. Hatching asynchrony in Eurasian kestrels in relation to the abundance and predictability of cyclic prey. *Journal of Animal Ecology*, **67**: 908 – 917.
- Wiebe, K.L., Wiehn, J. & Korpimäki, E. 1998b. The onset of incubation in birds: can females

control hatching patterns?. *Animal Behaviour*, **55**: 1043-1052.

Wiehn, J., Petteri, I., Korpimäki, E., Pakkala, M. & Wiebe, K. 2000. Hatching asynchrony in the Eurasian kestrel *Falco tinnunculus*, an experimental test of the brood reduction hypothesis. *Journal of Animal Ecology*, **69**: 85-95.

Wiehn, J. & Korpimäki, E. 1997. Food limitation on brood size: experimental evidence in the Eurasian kestrel. *Ecology*, **78**: 2043-2050.

Williams, T.D. 1994. Intraspecific variation in egg size and egg composition in birds: effects on offspring fitness. *Biological Reviews*, **68**: 35 – 59.

Zuk, M. & Stoehr. 2002. Immune defence and host life history. *The American Naturalist*, **160**: S9-S22.







**NOTA:** Los siguientes Capítulos reproducen el texto íntegro de los manuscritos originales de que consta la Tesis, cada uno en diferentes estados de publicación. Por esta razón, se han presentado en el idioma en que fueron publicados o enviados para su publicación. No obstante, cada Capítulo va acompañado de un resumen en castellano.



# CAPÍTULO I

## **Primera evidencia de diferencias sexuales en el período embrionario de aves: consecuencias para la competición entre hermanos en aves dimórficas.**

Este capítulo reproduce íntegramente el texto del siguiente manuscrito:

**Guillermo Blanco, Jesús Martínez-Padilla, José A. Dávila, David Serrano and Javier Viñuela.** 2003. First evidence of sex differences in the length of avian embryonic period: consequences for sibling competition in sexually dimorphic birds. *Behavioral Ecology*, 14(5): 702-706.

### **Resumen del Capítulo.**

La Teoría de las historias vitales predice que los padres pueden favorecer determinados volantes sobre otros con el objeto de maximizar el fitness total. En este sentido, este favoritismo diferencial de los padres en aves debería ser mejorado si los padres pudieran controlar alguno de las características de los huevos que influyen en la ontogenia del embrión durante el período de incubación. El tamaño del huevo y su composición pueden influenciar el tiempo de incubación de los embriones, así como los períodos de eclosión, pudiendo variar en tamaño y composición a aquellos huevos que dan lugar a embriones de diferentes sexos. Por tanto, el sexo de los embriones podría también influir en su ontogenia antes de la eclosión. Nosotros consideramos esta predicción, analizando las diferencias en el tiempo de período embrionario de diferentes sexos en Cernícalo vulgar (*Falco tinnunculus*), un ave rapaz el que las hembras adultas pueden llegar a ser un 20% mayores que los machos adultos. Así, encontramos la primera evidencia en diferencias sexuales en el tiempo de desarrollo embrionario de huevos de aves donde embriones hembra tuvieron un desarrollo embrionario más corto que los embriones machos, lo que permite a las hembras eclosionar antes en el orden de eclosiones y asumir un rango mayor en los embriones hembras que los machos en la jerarquía de tamaños de los pollos dentro del nido. Aquellos embriones con un rápido crecimiento y desarrollo resultaron en pollos eclosionados con mayores reservas energéticas y por tanto mayor peso, lo que sugiere que un período embrionario más corto requiere un menor mantenimiento del metabolismo para crecer en las primeras fases del crecimiento tras la eclosión. Nuestros resultados también indican que una temprana eclosión en relación a sus hermanos puede ser una ventaja con el objeto de posicionarse en un alto rango en la secuencia de tamaños dentro de la nidada independientemente del efecto del sexo en el peso del volantón. Por último, las diferencias sexuales en la ontogenia de huevos de aves puede por tanto ser un factor a tener en cuenta en los rasgos de las historias vitales asociados con un control parental de la competición entre hermanos, lo que debería ser considerado en futuros trabajos sobre inversión reproductiva.



# First evidence of sex differences in the length of avian embryonic period: consequences for sibling competition in sexually dimorphic birds.

GUILLERMO BLANCO<sup>1</sup>, JESÚS MARTÍNEZ-PADILLA<sup>1</sup>, JOSÉ A. DÁVILA<sup>1</sup>, DAVID SERRANO<sup>2</sup> and JAVIER VIÑUELA<sup>1</sup>. <sup>1</sup>*Instituto de Investigación en Recursos Cinegéticos (C.S.I.C.-U.C.L.M.), Ronda de Toledo s/n, 13005 Ciudad Real, Spain, and* <sup>2</sup>*Department of Applied Biology, Estación Biológica de Doñana (C.S.I.C.). Avda. Maria Luisa s/n, 41013 Sevilla, Spain*

## ABSTRACT.

Life-history theory predicts that parents may favour some offspring over others to maximise overall fitness. Parental favouritism in birds would be enhanced if parents can control any egg feature influencing the ontogeny of the embryo during incubation. Egg size and composition may influence the length of incubation and hatching periods, and eggs bearing embryos of different sex may differ in size and composition. Therefore, the sex of the embryo could also influence its ontogeny before hatching. We tested this prediction by searching for differences in the length of the embryonic period of different-sex embryos in the Eurasian kestrel (*Falco tinnunculus*), a sexually dimorphic raptor in which adult females are ca.20% heavier than adult males. We found the first evidence of sex differences in the length of the embryonic period in avian eggs. Female embryos had a shorter embryonic period than male embryos, which allowed females to hatch earlier in the hatching sequence and assume a higher rank than males in the intra-brood size hierarchy. Embryos with a fast growth and development resulted in hatchlings with greater residual reserves and thus larger mass, which suggest that a shorter embryonic period requires less maintenance metabolism relative to growth. Our results also indicated that early hatching may be advantageous in order to gain a high rank in the size hierarchy within the brood independently of the effect of sex on fledgling mass. Sex differences in avian egg ontogeny may hence be a factor shaping life-history traits associated with parental control of sibling competition, which should be addressed in any future work on optimal reproductive investment.

Key words: *avian egg, embryo ontogeny, parental favouritism, sex differences.*

## INTRODUCTION

---

Life-history theory predicts that parents may favour some offspring over others to maximise overall fitness (Clutton-Brock, 1991). In birds, the adjustment of reproductive investment would be enhanced if parents can control any egg feature influencing the ontogeny of the embryo during incubation. For instance, egg size and composition may influence the length of incubation and hatching periods (Sockman and Schwabl, 2000; Eising et al., 2001).

Given that eggs bearing embryos of different sex may differ in size (Anderson et al., 1997; Cordero et al., 2001) and composition (Petrie et al., 2001), the sex of the embryo could also influence its ontogeny before hatching. The recent finding of sex differences in avian yolk hormone levels (Petrie et al., 2001) is remarkable, because it suggests possible sex differences in pre-hatching ontogeny, as yolk hormones may influence embryonic growth rate and the onset of hatching (Ricklefs, 1993; Sockman and Schwabl,

2000; Eising et al., 2001). However, sex differences in embryo ontogeny have not, to our knowledge, directly addressed so far by any investigation.

Parent birds may adjust reproductive investment efficiently by controlling the hatching patterns and the size of chicks at hatching, which in turn affect the relative survival of offspring within a brood due to the establishment of within-brood size hierarchies (Wiebe et al., 1998; Viñuela, 2000). In sexually dimorphic species, a particular sex may be more costly to produce for parents, so the costs for parental fitness and offspring survival may not be independent of the gender composition of the brood (Fisher, 1930; Bortolotti, 1986; Clutton-Brock, 1991; Oddie, 2000). More specifically, the larger size sex may suffer higher mortality during the nestling period, because its higher demand of resources, and this may cause sex-biased mortality and sex ratio at fledging when resources are limited (Clutton-Brock et al., 1985; Oddie, 2000). However, available evidence suggests that in most cases sex ratio at fledging in non-precocial birds do not differ from unity, even in highly dimorphic species (Clutton-Brock, 1986).

Thus, apparently parent bird may compensate the disadvantage of higher resource demand by the larger sex in some way, but the mechanisms to achieve that are poorly known (Oddie, 2000). One possible mechanism could be sex-biased hatching order, because earlier hatched chicks have better access to resources, and thus depending on the sex sequence the probability of survival or later-hatched chicks may be enhanced or reduced (Bortolotti, 1986; Blanco et al., 2002). Embryonic growth may determine mass at

hatching and duration of incubation, thus affecting the delay in hatching time with respect to earlier hatched siblings, and overall hatching asynchrony (Viñuela, 1997). Hatching asynchrony and hatchling mass are clear determinants of individual competitive ability within a brood, and of the probability of survival in a critical stage, because most mortality in non-precocial avian broods affect to chicks of short age (O'Connor, 1984; Gibbons, 1987; Amundsen and Slagsvold, 1991; Williams, 1994; Viñuela, 2000). Thus, strong selective pressures on growth rate during incubation period may be expected (Skutch, 1974), even more in species with sexual dimorphism in size.

Differences in growth dynamics between sons and daughters in sexually dimorphic birds are well known (O'Connor, 1984; Bortolotti, 1986). In these species, sex also could affect growth rate of the embryo and duration of embryonic period, but, to our knowledge, there is no evidence showing sex-biased duration of embryonic period in birds. In this paper, we tested the prediction that the sex of the embryo may influence its ontogeny before hatching in the Eurasian kestrel (*Falco tinnunculus*). Thus, we searched for differences in the duration of the embryonic period of different-sex embryos and assessed their consequences for hatchlings and fledglings.

## **MATERIAL AND METHODS**

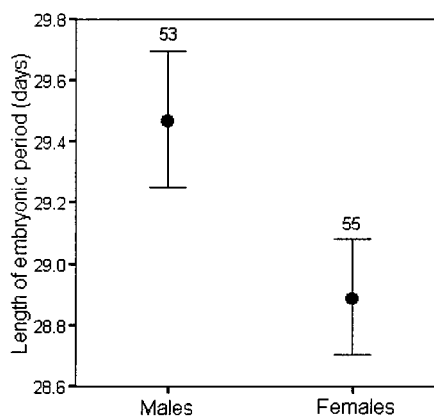
---

We monitored breeding in the Eurasian kestrel, a sexually dimorphic raptor in which adult females are ca.20% heavier than adult males. The study was carried out during the breeding season of 2000 in Campo Azálvaro region, a montane grassland area in central Spain (Fargallo et al., 2001). Nest boxes were monitored daily during egg laying and

eggs were marked as they were laid, so laying sequence was accurately known. Eggs were weighed on the day of laying with an electronic balance to the nearest 0.25 g. During hatching, nests were visited at least twice each day to assign each marked egg to its corresponding nestling. Hatchlings were identified by marking them with indelible ink in the hatching tooth in the case of eggs found during hatching process, and later with ink on the head until banding. The embryonic period of each particular egg was defined as the number of days between the start of incubation and the day of hatching. There is some variation in the onset of incubation, but females typically incubated 95-100% of the time (full incubation) on, or on the day after, the penultimate egg was laid (Wiebe et al., 1998; unpub. own data). Given that some embryo development could have occurred before full incubation started (Wiebe et al., 1998), we considered that incubation began on the day before the penultimate egg was laid, when partial incubation has occurred in all nests (see also Wiebe et al., 1998). Nestlings were weighed to the nearest 0.25 g on the day of hatching and 24 days after hatching (just prior to fledging). Nestling sex was determined by molecular procedures (Fridolfsson and Ellegren, 1999) using DNA extracted from blood obtained by brachial venipuncture. Nestlings that died were sexed by the same method using body tissues as a source of DNA.

Generalized linear mixed models with normal error and identity link function (GLIMMIX macro of SAS, Littell et al., 1996) were used to investigate the effect of sex on the length of the embryonic period, and the effects of sex and length of the embryonic period on hatching and fledging mass. The nest was included as a random

term in the manner of a randomised complete block design to avoid pseudoreplication (Hurlbert, 1984). Additionally, by introducing this random term we ensured that the effects of the fixed variables were not influenced by characteristics of the parents or the nest. Statistic tests associated with random terms denote significant nest variation in the three response variables examined (embryonic period,  $Z = 3.47$ ,  $P = 0.0003$ ; hatching mass,  $Z = 1.76$ ,  $P = 0.0394$ , fledging mass:  $Z = 2.86$ ,  $P = 0.0021$ ). We included other possible influencing variables as explanatory terms in the models (see Table 1). Some of the explanatory variables could covary, so we fitted their effects to the observed data following a forward stepwise procedure, testing the significance of each variable and adding only the variable that resulted in a better fit of the model. The significance of the remaining variables were tested again until no additional variable or interaction reached significance. The result is the most adequate model for explaining



**Figure 1.** Mean  $\pm$  SE duration (in days) of the embryonic period of male and female eurasian kestrel eggs. Numbers above bars are sample sizes. Note this is an univariate comparison (see Table 1 for statistics).

Response term	Explanatory term	Rejected term	Estimate	SE	F	df.	P	
Embryonic period	Sex (male)		0.08439	0.03798	4.94	1,78	0.0292	
	Laying order		-0.07214	0.01233	34.24	1,78	<0.0001	
	Clutch size		-0.29650	0.14140	4.40	1,78	0.0392	
		Laying date		-0.02380	0.01195	3.80	1,77	0.0548
		Egg mass		0.01869	0.02327	0.65	1,77	0.4242
Hatching mass	Embryonic period		-0.01814	0.00589	9.49	1,76	0.0029	
	Egg mass		0.05442	0.00449	147.23	1,76	<0.0001	
	Clutch size		-0.04572	0.01528	8.95	1,76	0.0037	
	Laying date		-0.00477	0.00132	13.09	1,76	0.0005	
		Sex (male)		-0.01671	0.01146	2.13	1,74	0.1491
		Hatching order		0.00273	0.00394	0.48	1,73	0.4901
		Laying order		0.00114	0.00539	0.04	1,72	0.8336
Fledging mass	Sex (male)		-0.09321	0.01611	33.49	1,72	<0.0001	
	Egg mass		0.02178	0.00737	8.72	1,72	0.0042	
	Hatching order		-0.01288	0.00533	5.84	1,72	0.0182	
		Clutch size		0.02204	0.20380	0.69	1,72	0.4104
		Brood size		-0.02283	0.01495	2.33	1,72	0.1310
		Laying order		0.00781	0.00697	1.25	1,71	0.2666
		Laying date		0.00069	0.00234	0.09	1,67	0.7696
		Hatching mass		0.00296	0.00827	0.13	1,67	0.7217
		Embryonic period		0.00170	0.00944	0.03	1,66	0.8606

**Table 1.** Factors affecting the length of the embryonic period, and hatching and fledging mass. Explained deviance: embryonic period (32.56%), hatching mass (18.24%), fledging mass (60.70%). All interactions between the explanatory terms were not significant ( $P > 0.05$ ).

the variability in the response variable, where only the significant explanatory variables are retained. All tests are two-tailed.

## RESULTS

We compared the length of the embryonic period (from incubation start to hatching) of different-sex embryos and found that female embryos hatched sooner than male embryos (Fig. 1), after controlling for other possible confounding variables (Table 1). As in other studies (Vince 1968; Viñuela 1997) we also found that the length of the embryonic period decreased with laying order and clutch size (Table 1). To examine whether these differences influenced the sex sequence at hatching, we conducted a log-

linear analysis with laying order, hatching order and sex as factors (three-way interaction, NS; fit of the model  $G = 11.45$ ,  $df = 25$ ,  $p = 0.90$ ). Results showed that eggs generally did not hatch in the order they were laid (laying order x hatching order,  $G = 110.74$ ,  $df = 25$ ,  $p < 0.0001$ ) and that, although laying order was not sex-biased (laying order x sex,  $G = 7.83$ ,  $df = 5$ ,  $p = 0.17$ ), females hatched earlier than males (hatching order x sex,  $G = 12.17$ ,  $df = 5$ ,  $p = 0.033$ ).

Hatching mass increased with egg mass (Table 1) and decreased as the duration of embryonic period increased (Table 1) after controlling for hatchlings sharing nests (Table 1), but there was no effect of hatchling sex (Table 1). In addition,



hatchling mass decreased as clutch size and laying date increased (Table 1). After controlling for parental and nest effects, we found that fledgling mass was higher in females than males (Table 1), increased with egg mass (Table 1), and decreased with hatching order (Table 1).

## DISCUSSION

---

We compared the length of the embryonic period of different-sex embryos and found that female embryos hatched sooner than male embryos. This result is, to our knowledge, the first evidence of sex differences in the length of the embryonic period in avian eggs, and suggests that female embryos grew faster, hatched at an earlier stage of development than male embryos, or both. The shorter embryonic period of female embryos allowed them to hatch earlier in the hatching sequence and assume a higher rank than males in the intra-brood size hierarchy.

It has been demonstrated that early-hatched chicks have survival advantages over their nest mates (O'Connor, 1984; Ricklefs, 1993). If earlier hatching is the result of a faster ontogeny at the egg stage, we would expect earlier-hatched offspring to have more yolk or fat reserves. Presumably, hatchlings with larger energy reserves (e.g. a larger yolk sac) can withstand more prolonged food restriction (O'Connor, 1984; Ricklefs, 1993). Our results showed that mass at hatching increased with egg mass and decreased as the duration of embryonic period increased. In addition, hatchling mass decreased as clutch size and laying date increased. The effects of larger clutch and later laying date on this relationship may be due to higher temperatures in small clutches later in the

season owing to incubation efficiency, as higher temperatures may result in higher respiration compared to tissue accumulation (Withow, 2000).

This is the first study to our knowledge showing that hatchling mass may vary according to the length of the embryonic period after controlling for parental and nest effects, egg mass, and other influencing variables. Presumably, a shorter development period requires less maintenance metabolism relative to growth (Withow, 2000) and then embryos with a fast growth and development should result in hatchlings with greater residual reserves. Some internal egg feature, such as shape and composition, or an external factor susceptible of female control, such as incubation pattern and temperature, may be behind this association.

Early hatching may place female nestlings at the top of within-brood competitive feeding hierarchies induced by hatching asynchrony and mass at hatching. A higher competitive rank in the brood sequence may result in faster growth rate or greater fledgling mass (Ricklefs, 1993), which is likely to have important implications for offspring fitness, as it has been found to be related to postfledging survival and recruitment (reviewed in Magrath, 1991). In this study, we showed that fledgling mass was higher in females than males, probably because of the genetically determined adult sexual dimorphism. Fledgling mass also increased with egg mass, as predicted by the egg-size hypothesis (Clutton-Brock, 1991; Styrsky et al., 1999), and decreased with hatching order. These results indicate that early hatching may be advantageous in order to gain a high rank in the size hierarchy within

the brood independently of the effect of sex on fledgling mass, and that intrabrood competition may be a strong selective factor affecting duration of incubation of birds, as suggested by Skutch (1974). A trade-off between growth rate and energy requirements has been suggested to govern the evolution of post-hatching ontogeny (Ricklefs, 1993). In sexually dimorphic species, the larger sex may be more susceptible to starvation due to its higher food demands (Ricklefs, 1993; Bortolotti, 1986). Therefore, selective forces that act on embryo ontogeny in sexually dimorphic birds should favour more rapid embryonic development and greater residual reserves to enhance survival of the larger sex when food is scarce just after hatching, or to enhance competition between siblings of the large sex within broods.

The relatively small difference in hatching time in favour of females may be sufficient to compensate for their higher requirements compared with males if food is scarce just after hatching. The difference we found in duration of embryonic period of males and females could be a mechanism to compensate the disadvantage of the larger sex when resources are scarce, thus avoiding possible biases in sex ratio at fledging. Sex differences in the length of embryonic period may be a form of parental favouritism if they are influenced by internal egg features or differential incubation. Alternatively, sex differences may be strictly a function of embryo adaptations to enhance competitive ability. Distinguishing between these possibilities may provide fruitful research avenues for understanding the evolution of parental control of sibling competition. There is growing evidence that steroid hormones are involved in the control of both embryonic and post-hatching growth

(Schawbl, 1993, 1996; Sockman and Schawbl, 2000; Eising et al., 2001), and recent evidence of sex differences in avian yolk hormone levels (Petrie et al., 2001).

Therefore, egg hormones are good candidates to be involved in differential ontogeny of different-sex embryos. However, more research is needed, because there is contradictory information about the effect of steroids on growth of birds (Whitlow, 2000). Furthermore, while interspecific variation in incubation period of eggs of birds seems to be well explained by differences in egg size, eggshell structure, environmental temperatures, incubation behaviour, or predation pressure (Skutch, 1976; Starck and Ricklefs, 1998; Whitlow, 2000), little is known about these relationships within a species (Christensen et al., 2001). There are intriguing differences in the intraspecific variation of incubation. Thus, in some species, but not in others, incubation period is affected by laying order, laying date, clutch size or egg size. This may be partly due to differences in the length of embryonic period depending on the sex of embryos that, up to now, had not been detected in birds, and that should be examined in future studies of variation in incubation period.

## **ACKNOWLEDGEMENTS**

---

We thank the Finat family for kindly allowing us to conduct the study in their property, and J. San Teodoro for collaboration in fieldwork. We thank J. Moreno and J. Potti for their support to conduct the study and R. E. Ricklefs for comments on the manuscript.

## **REFERENCES**

---

Amundsen, T. and T. Slagsvold. 1991. Hatching

- asynchrony: facilitating adaptive or maladaptive brood reduction?. Pp. 1707-1719 in N. J. Adams and R. H. Slotow, eds. Proc. 20<sup>th</sup> Int. Ornithol. Congr., Durban, Johannesburg, BirdLife South Africa.
- Anderson, D. J., J. Reeve, and D. M. and Bird. 1997. Sexually dimorphic eggs, nestling growth and sibling competition in American kestrels *Falco sparverius*. *Funct. Ecol.* **11**: 331-335.
- Blanco, G., J. A. Dávila, J. A. López Septiem, R. Rodríguez, and F. Martínez. 2002. Sex-biased initial eggs favours sons in the slightly size-dimorphic Scops owl (*Otus scops*). *Biol. J. Linn. Soc.* **in press**.
- Bortolotti, G. R. 1986. Influence of sibling competition on nestling sex-ratios of sexually dimorphic birds. *Am. Nat.* **127**: 495-507.
- Christensen, V. L., J. L. Grimes, M. J. Wineland, L. G. Bagley. 2001. Effects of turkey breeder hen age, strain, and length of the incubation period on survival of embryos and hatchlings. *J. Appl. Poultry Res.*, **10**: 5-15.
- Clutton-Brock, T.H. 1986. Sex ratio variation in birds. *Ibis*, **128**: 317-329.
- Clutton-Brock, T.H. 1991. *The evolution of parental care*. Princeton University Press.
- Clutton-Brock, T.H., S. D. Albon, and F. E. Guinness. 1985. Parental investment and sex differences in mortality in juvenile birds and mammals. *Nature*, **313**: 131-133.
- Cordero, P., J. Viñuela, J. M. Aparicio, J. P. Veiga. 2001. Seasonal variation in sex ratio and sexual egg dimorphism favouring daughters in first clutches of the spotless starling. *J. Evol. Biol.*, **14**: 829-834.
- Eising, C. M., C. Eikenaar, H. Schwabl, and T. G. G. Groothuis. 2001. Maternal androgens in black-headed gull (*Larus ridibundus*) eggs: consequences for chick development. *Proc. R. Soc. Lond. B.*, **268**: 839-846.
- Fargallo, J. A., G. Blanco, J. Pott, and J. Viñuela, J. 2001. Nestbox provisioning in a rural population of Eurasian Kestrels: breeding performance, nest predation and parasitism. *Bird Study*, **48**: 236-244.
- Fisher, R. A. 1930. *The genetical theory of natural selection*. Oxford University Press, Oxford, UK.
- Fridolfsson, A-K., and H. Ellegren. 1999. A simple and universal method for molecular sexing of non-ratite birds. *J. Avian Biol.*, **30**: 116-121.
- Gibbons, D. W. 1987. Hatching asynchrony reduces parental investment in the Jackdaw. *J. Anim. Ecol.*, **56**: 403-414.
- Hurlbert, S.H. 1984. Pseudoreplication and the design of ecological field experiments. *Ecol. Monogr.*, **54**: 187-211.
- Littell, R. C., G. A. Milliken, W. W. Stroup, and R. D. Wolfinger. 1996. *SAS system for mixed models*. Cary, NY. SAS Institute.
- Magrath, R. D. 1991. Nestling weight and juvenile survival in the blackbird, *Turdus merula*. *J. Anim. Ecol.*, **60**: 335-351.
- O'Connor R. J. 1984. *The growth and development of birds*. Chichester, Wiley and Sons.
- Oddie, K. R. 2000. Size matters: competition between male and female great tit offspring. *J. Anim. Ecol.*, **69**: 903-912.
- Petrie, M., H. Schawbl, N. Brande-Lavridsen, and T. Burke. 2001. Sex differences in avian yolk hormone levels. *Nature*, **412**: 498.
- Ricklefs, R. E. 1993. Sibling competition, hatching asynchrony, incubation period, and lifespan in altricial birds. Pp 199-276 in D. M. Power, ed, *Current Ornithology*. Vol.11. New York, Plenum Press.
- Schwabl, H. 1993. Yolk is a source of maternal testosterone for developing birds. *Proc. Natl. Acad. Sci. USA*, **90**: 11446-11450.
- Schwabl, H. 1996. Maternal testosterone in the avian egg enhances postnatal growth. *Comp. Biochem. Physiol. A.*, **114**: 271-276.
- Skutch, A. F. 1974. *Parent birds and their young*. University of Texas Press, Austin.
- Sockman, K. W., and H. Schawbl. 2000. Yolk androgens reduce offspring survival. *Proc. R. Soc. Lond. B.*, **267**: 1451-1456.

- Starck, J. M. and R. E. Ricklefs. 1998. *Avian Growth and Development: Evolution within the Altricial-Precocial Spectrum*. Oxford. Univ. Press, New York.
- Styrsky J. D., K. P. Eckerle, and C. F. Thompson. 1999. Fitness-related consequences of egg mass in nestling house wrens. *Proc. R. Soc. Lond. B.*, **266**: 1253-1253.
- Vince, M. A. 1968. Retardation as a factor in the synchronization of hatching. *Anim. Behav.*, **16**: 332-335.
- Viñuela, J. 1997. Laying order affects incubation duration in the Black Kite (*Milvus migrans*): counteracting hatching asynchrony? *Auk*, **114**: 192-199.
- Viñuela, J. 2000. Opposing selective pressures on hatching asynchrony: egg viability, brood reduction and nestling growth. *Behav. Ecol. Sociobiol.*, **48**: 333-343.
- Whittow, G. C. (Ed.) 2000. *Sturkie's Avian Physiology*. Academic Press, New York.
- Wiebe, K. L., J. Wiehn, and E. Korpimäki. 1998. The onset of incubation in birds: can females control hatching patterns?. *Anim. Behav.*, **55**: 1043-1052.
- Williams, T. D. 1994. Intraspecific variation in egg size and egg composition in birds: effects on offspring fitness. *Biol. Rev.*, **68**: 35-59.

## CAPÍTULO II

### **Inversión en peso a diferentes huevos dentro de la secuencia de puesta: consecuencias para el ajuste del esfuerzo parental en un ave sexualmente dimórfica en tamaño.**

Este capítulo reproduce íntegramente el texto del siguiente manuscrito:

**Blanco, G., Martínez-Padilla, J., Serrano, D., Dávila, J. A., and Viñuela, J.** 2003. Mass provisioning to different-sex eggs within the laying sequence: consequences for adjustment of reproductive effort in a sexually dimorphic bird. *Journal of Animal Ecology*, 72: 831-838.

#### **Resumen del capítulo.**

En aves, la habilidad potencial de la madre para ajustar los recursos a diferentes huevos dentro de la puesta puede tener importantes efectos en la esperanza de supervivencia de pollos determinados o incluso de puestas enteras. En este capítulo, evaluamos cómo una especie con dimorfismo sexual invertido en tamaño como el Cernícalo vulgar (*Falco tinnunculus*) puede ajustar su esfuerzo reproductivo adoptando diferentes estrategias de la inversión en su descendencia en relación al sexo de los pollos y al orden de puesta. En este sentido, se observa que los primeros huevos de la puesta que dieron lugar a pollos macho fueron más pesados que aquellos iniciales de los que eclosionó una hembra. Sin embargo, no hubo diferencias en el peso del huevo en relación al sexo en los huevos puestos posteriormente en la puesta. Además, en puestas donde el primer huevo fue macho, la inversión en peso de los huevos posteriores de la puesta decreció, mientras que en puestas iniciadas con un huevo hembra se detectó la tendencia contraria. Este hecho sugiere una inversión diferencial de recursos a los huevos en los primeros huevos puestos en relación al sexo que permitiría tomar o eliminar recursos para los huevos posteriores puestos por la hembra. Paralelamente, las hembras eclosionadas de los primeros huevos eclosionaron antes que los machos de primeros huevos, siendo consistente con los resultados del anterior capítulo, lo que permitiría incrementar las posibilidades de supervivencia de los pollos más pequeños eclosionados más tarde. Estas estrategias, por tanto, en relación a la inversión en los huevos y el patrón de eclosión dependiendo del sexo del primer huevo puesto fueron asociadas respectivamente con marcadas jerarquías de tamaño y ausencia de la mismas en los pollos a la edad de 25 días (justo antes de su salida del nido), en puestas iniciadas con huevos macho o hembra.

En definitiva, los parejas reproductoras de Cernícalo vulgar, pueden distribuir su esfuerzo reproductivo promoviendo un favoritismo hacia pollos de eclosión temprana o evitando ningún tipo de favoritismo produciendo pollos de cada sexo con similares pesos. Este patrón podría manipularlo la hembra dependiendo de su habilidad para identificar el sexo de los huevos antes de la puesta y absorber parcialmente o diferencialmente invertir recursos a sus huevos para ajustar su inversión reproductiva. Este puede ser un mecanismo clave para el control de la competencia entre hermanos en aves con dimorfismo sexual en tamaño.



# Mass provisioning to different-sex eggs within the laying sequence: consequences for adjustment of reproductive effort in a sexually dimorphic bird.

GUILLERMO BLANCO<sup>a</sup>, JESÚS MARTÍNEZ-PADILLA<sup>a</sup>, DAVID SERRANO<sup>b</sup>, JOSÉ A. DÁVILA<sup>a</sup>, and JAVIER VIÑUELA<sup>a</sup>

<sup>a</sup>*Instituto de Investigación en Recursos Cinegéticos (C.S.I.C.-U.C.L.M.), Ronda de Toledo s/n, 13005 Ciudad Real, Spain, and* <sup>b</sup>*Department of Applied Biology, Estación Biológica de Doñana (C.S.I.C.), Avda. María Luisa s/n, 41013 Sevilla, Spain.*

## ABSTRACT

1. In birds, the potential maternal ability to adjust resource allocation to different eggs in the clutch might have a major effect on the survival expectancies of particular nestlings or entire broods. We assessed whether sexually size-dimorphic Eurasian kestrels *Falco tinnunculus* (Linnaeus) are able to adjust their reproductive effort by adopting different strategies of egg mass provisioning according to egg sex and laying order.

2. Initial eggs bearing male embryos were heavier than initial eggs bearing female embryos, but no differences in egg mass associated to sex were detected for eggs laid subsequently. Furthermore, in clutches started with a male egg, egg mass declined in subsequent eggs, while in clutches started by a female egg the opposite trend in within-clutch egg-mass variation was found. This suggests differential deposition of resources invested in initial eggs of different sex leading to saved or depleted resources for subsequent eggs.

3. Daughters from initial eggs hatched earlier than sons from initial eggs, which may enhance survival of smaller siblings hatched later. These contrasting strategies of egg provisioning and hatching patterns depending on the sex of the first-laid egg were associated respectively with marked mass hierarchies and a lack of mass hierarchies at fledgling in broods initiated with eggs bearing sons and daughters.

4. Parental kestrels may allocate reproductive effort by promoting favouritism towards early hatched chicks or by avoiding any favouritism by producing siblings of each sex with similar mass. This may be achieved depending on the female ability both to identify egg sex and to partially reabsorb or differentially allocate resources to eggs accordingly to adjust reproductive investment. This may be a key mechanism to control sibling competition in birds with sexual dimorphism in mass.

*Key-words: brood hierarchies, sexual egg dimorphism, differential resource allocation.*

## INTRODUCTION

Life-history theory predicts that if offspring fitness is affected by sex, reproductive effort should concentrate investment in a particular sex or brood which sex composition deals with the highest fitness expectations per unit of

parental investment (Stearns 1992; Bortolotti 1986). This may be enhanced if parents have some control on the sex composition of the progeny or if parents may identify offspring sex and then invest accordingly (Emlen 1997), which should be made at an early stage of propagule production to minimise costs and maintain

the investment control (Stearns 1992). In birds, numerous studies have assessed the reproductive value of eggs of different mass within the laying sequence according to different parental strategies of resource allocation (Slagsvold *et al.* 1984). These strategies have been argued to be adopted by different species depending on adaptations and constraints characterizing their life-histories (Slagsvold *et al.* 1984; Viñuela 1997). For instance, some species lay a small final egg to enhance the hierarchy imposed by hatching asynchrony, whereas others lay a relatively large final egg to enhance the survival of last hatched chicks (Slagsvold *et al.* 1984; Viñuela 1997; 2000). Reproductive effort has been suggested to be also potentially adjusted through sex-biased laying orders (Bortolotti 1986) and differential provisioning to different-sex eggs (Anderson, Reeve & Bird 1997). However, the value of different egg-mass allocation strategies for adjusting reproductive effort according to sex composition and sequence of the brood have been not directly addressed so far by any investigation.

In sexually dimorphic species, the offspring of the larger size sex generally have faster growth and increased metabolic rates, and thus a higher demand of resources, so they are usually more expensive to rear (Bortolotti 1986; Anderson *et al.* 1993). The establishment of a competitive brood hierarchy due to egg provisioning biased by laying order may promote that the larger sex further increases its competitive head start if hatched in first order (Bortolotti 1986; Bednarz & Hayden 1991; Blanco *et al.* 2002; Krebs *et al.* 2002). Therefore, the costs for parental fitness and offspring survival may be not independent of the gender composition and sequence of

the brood (Bortolotti 1986). Sex-biased laying order may be a valuable mechanism of parental control of sibling competition, because the access to resources and then the survival probability of particular chicks over their siblings may thus be enhanced or reduced (Bortolotti 1986; Blanco *et al.* 2002). By logical extension, this potential control of sibling competition may be translated to the control of offspring number, quality and sex ratio in the brood. Females may further adjust reproductive investment by allocating resources to eggs according to the sex of the embryo because egg mass and composition may influence hatching success, hatchling mass, and nestling growth, and thus survival and recruitment (Schawbl 1996; Williams 1994; Styrsky, Eckerle & Thompson 1999; Eising *et al.* 2001).

The potential maternal ability to adjust the resources allocated to different-sex eggs laid in different orders might have a major relevance in the life expectancies of particular nestlings or entire broods, although this has never been documented. Alternatively, a biased provisioning according to laying order and sex might not be the result of female control, but rather an epiphenomenon due to female behavioural and physiological changes from before to during laying. In the latter case, differential investment on different-sex eggs according to laying order might not cause any advantage to chicks depending on the eggs from which they hatched. To determine whether sexual egg dimorphism and sex-biased laying order are adaptive traits or non-adaptive consequences of an underlying process, it may be necessary to investigate the consequences of such bias for particular nestlings and entire broods. A study of sexual egg dimorphism in American kestrels



(*Falco sparverius* L.) suggested that eggs producing sons were larger than eggs producing daughters in each laying order to help parents to manipulate mass relationships between their offspring (Andersson *et al.* 1997). Other recent studies of sexual egg dimorphism have, however, not further explored the consequences of such dimorphism for nestlings because embryos were sexed after killing them before hatching (Cordero *et al.* 2000, 2001). In this paper we assessed whether Eurasian kestrels *Falco tinnunculus* are able to adjust their reproductive effort by adopting different strategies regarding the control of mass provisioning to different-sex eggs laid in different orders. In birds that lay multiple-egg clutches, first hatched chicks may influence growth and survival of their smaller siblings but chicks hatched later in the sequence generally do not affect to their larger siblings (Viñuela 2000; Blanco *et al.* 2002). In addition, first-laid eggs are often only significantly sex-biased, or sex bias is more marked than in eggs laid later in the laying sequence (Bortolotti 1986; Bednarz & Hayden 1991; Leroux & Bretagnole 1996; Arnold, Griffith & Goldizen 2001; Blanco *et al.* 2002). Therefore, we predict an important role of the combination of sex and mass of initial eggs in the adjustment of reproductive effort in sexually dimorphic birds. To explore this prediction we assessed whether starting the clutch with a male or female egg, and to differentially allocate resources accordingly, may be different parental strategies to enhance the optimal investment in offspring.

## **METHODS**

---

### **Field procedures**

We monitored breeding of Eurasian kestrels (a sexually size-dimorphic raptor),

nesting in nest boxes during the breeding season of 2000 in Campo Azálvaro grasslands, central Spain (Fargallo *et al.* 200, Blanco *et al.* in press). Nest boxes were monitored during egg laying and eggs were marked as they were laid (one egg each two days, see also Wiebe, Wiehn & Korpimäki 1998) so laying date and laying sequence were accurately known. Eggs were weighed to the nearest 0.25g on the day of laying. Before and during hatching, nests were visited at least twice each day to assign each marked egg to its corresponding nestling. Hatchlings were identified by marking them with indelible ink in the hatching tooth in the case of eggs found during hatching process, and later with ink on the head until banding. We visited the nests each five days after hatching of the last chick in an attempt to collect dead nestlings in order to sex them. Nestlings were weighed to the nearest 0.25g the day of hatching and 24 days after hatching (just prior to fledging). Nestling sex was determined by molecular procedures using DNA extracted from a drop of blood obtained by brachial venipuncture. Nestlings that died and did not disappear from the nests ( $n = 5$ ) were sexed by the same method using body tissues as a source of DNA.

### **Statistical Analyses**

The overall population sex ratio was analyzed using the binomial test. Factors influencing brood sex-ratios were examined by fitting generalized linear models (GLM) with binomial error distribution and logistic link function (GENMOD procedure of SAS), where the response variable was number of males in a brood over brood size. Explanatory variables included laying date, clutch size, number of lost eggs and number of lost chicks. We performed generalized

linear mixed models (GLMM) where log-transformed egg and chick mass were employed as response variables using a normal distribution of errors and an identity link function (GLIMMIX macro of SAS). To test for variables influencing egg mass, we considered egg sex and laying order as fixed factors and clutch size and laying date as covariates while controlling by the influence of the nest which was treated as a random factor in the manner of a randomized complete block design to avoid pseudoreplication. Laying order was encoded in three categories (i.e. first, middle and last egg laid), which may potentially have different reproductive values according to different within-clutch egg-mass hierarchies and because there were different clutch sizes. We first confirmed that there was no difference in mass between eggs laid in different orders within the middle category (mixed model using the CONTRAST procedure available in SAS;

all  $P$ -values  $> 0.05$ ).

Some of the explanatory variables could covary, so we fitted their effects to the observed data following a forward stepwise procedure, testing the significance of each variable and adding only the variable that resulted in a better fit of the model. The significance of the remaining variables was tested again until no additional variable or interaction reached significance. Quadratic and cubic terms were also tested in the models to account for potential non-linear relationships. The result is the most adequate model for explaining the variability in the response variable, where only the significant explanatory variables are retained. All tests are two-tailed.

## RESULTS

### Sex ratio and laying order

We sexed 136 hatchlings (65 males, 71 females) from 31 broods, and we did not find any bias in overall sex ratio of chicks (binomial test  $P = 0.67$ ). This sample included five chicks that died by starvation (four males and one female), while other ten chicks, probably also starved, disappeared rapidly from the nests, so they could not be sampled in order to sex them. Sex-ratio at fledgling (61 males, 70 females from 31 broods) did not significantly differ from a binomial distribution, both considering all broods (47 % males, binomial test,  $P = 0.49$ ) and broods where all eggs laid produced a fledgling (50 % males, binomial test,  $P = 1.00$ ,  $n = 64$  chicks from 13 clutches). The sex ratio of eggs laid in each order did not differ from parity (binomial tests, all  $P$ -values  $> 0.31$ ). Similar results were obtained when egg order was encoded as initial ( $P = 0.68$ ), middle ( $P = 0.28$ ) or last laid ( $P =$

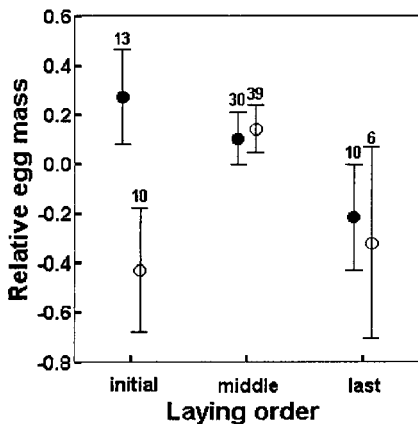


Fig. 1. Sexual dimorphism in mass of Eurasian kestrel eggs in relation to laying order. Egg mass was expressed as mean  $\pm$  SE relative egg mass (egg mass minus the clutch mean) to represent intraclutch variation. Full symbols represent males and open symbols represent females. Numbers above bars are sample sizes.

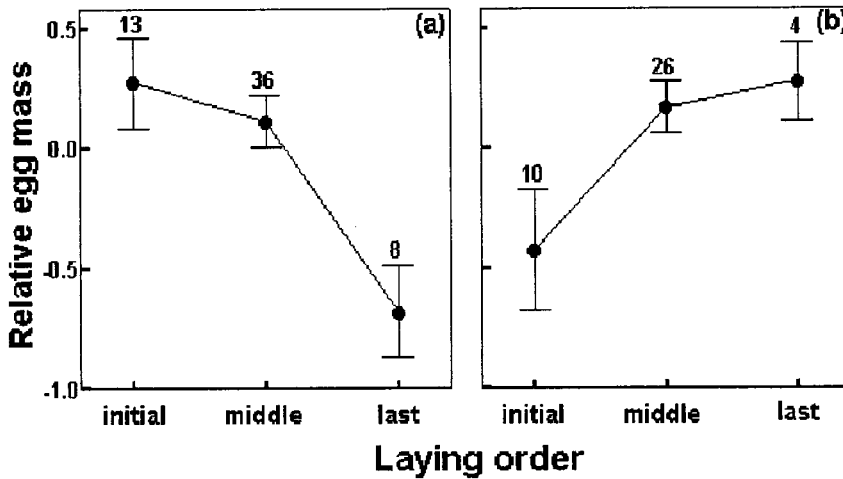


Fig. 2. Mean  $\pm$  SE relative egg mass (egg mass minus the clutch mean) according to laying order in (a) clutches initiated with an egg bearing a son or (b) a daughter. Numbers above bars are sample sizes. Unsexed eggs within the categories “middle” and “last” eggs have been included.

0.45). Brood sex ratios were not related to clutch size, laying date and number of eggs or chicks lost when all broods were considered (GLM, all  $P$ -values  $> 0.32$ ). Similarly, sex-ratio of broods where all eggs produced a fledgling was not related to laying date or clutch size (all  $P$ -values  $> 0.66$ ).

### Sexual dimorphism in egg mass

We were able to assign 108 marked eggs to its corresponding nestling (53 males, 55 females) from 28 broods, to compare the mass of eggs bearing embryos of different sex laid in different orders. Results of the mixed model showed a significant effect of laying order ( $F_{2,74} = 5.01$ ,  $P = 0.0091$ ), a marginally significant effect of egg sex ( $F_{1,74} = 3.64$ ,  $P = 0.060$ ), and a significant effect of the interaction between both factors on egg mass ( $F_{2,74} = 3.63$ ,  $P = 0.031$ ).

These results indicated that egg mass tended to decline along laying sequence, and that sex affected mass only in first-laid eggs (tests of effect slices, first-laid eggs:  $F_{1,74} = 9.39$ ,  $P = 0.0030$ ; middle-laid eggs:  $F_{1,74} = 0.01$ ,  $P = 0.9395$ ; last-laid eggs:  $F_{1,74} = 0.01$ ,  $P = 0.9395$ ). Thus, first-laid eggs bearing a female embryo were lighter than first-laid eggs with a male embryo, and of similar mass to that of last-laid eggs (Fig 1), but there was no sexual dimorphism in the mass of eggs laid in middle or last orders (Fig. 1).

In addition, egg mass decreased significantly as laying date increased ( $F_{1,74} = 10.59$ ,  $P = 0.0017$ ), but there was no significant effect of clutch size on egg mass ( $F_{1,74} = 0.07$ ,  $P = 0.79$ ). This model explained 22.55% of the original deviance without considering the deviance explained by parental and nest characteristics, which were controlled for by including the nest as a random term ( $Z = 3.33$ ,  $P = 0.0004$ ).

Variables	Parameter estimate (SE)	F	d.f.	P
Sex of initial egg (male)	-0.00207 (0.03727)	1.84	1,69	0.1793
Laying order (initial)	-0.03936 (0.02141)	1.42	2,69	0.2475
Laying order (middle)	-0.00885 (0.01982)			
Sex of initial egg (male) x laying order (initial)	0.08549 (0.02663)	5.50	2,69	<b>0.0061</b>
Sex of initial egg (male) x laying order (middle)	0.04781 (0.02420)			
Laying date	-0.00440 (0.00216)	4.16	1,69	<b>0.0452</b>

**Table 1.** GLMM with normal error and identity link function on mass of Eurasian kestrel eggs, where sex of the first laid egg in each nest and laying order (first egg laid, middle and last egg laid) were included as fixed factors. This model explained a 24.84% of the original deviance, without considering the deviance explained by parental and nest characteristics which were controlled for by including the nest as a random term ( $Z = 2.98$ ,  $P = 0.0015$ ). Other potentially influencing variables such as clutch size and egg sex, and the remaining interactions between the variables, were not significant ( $P > 0.88$ ). Parameter estimates and *SE* for the levels of fixed factor were calculated considering a reference value of zero for last order level in the variable ‘laying order’ and for the female level in the variable ‘sex of initial egg’.

### Consequences of clutch initiation with eggs bearing sons and daughters

Given that only initial eggs were sexually dimorphic, we explored which possible consequences for broods could have that females started the clutch with an egg bearing a son or a daughter. We have also explored whether starting the clutch with an egg bearing a son or a daughter influenced the mass of eggs laid subsequently. For these analyses, we excluded five clutches where the sex of the initial egg was unknown because we failed to assign it to their corresponding hatchling. Given that no sexual dimorphism was found for middle or late orders, we have included in the analyses all eggs measured within those categories, even those unsexed (larger sample sizes in Fig. 2 with respect to Fig. 1). There was no significant difference in laying date ( $t_{21} = 0.16$ ,  $P = 0.87$ ) and clutch size (Mann-Whitney U-test,  $Z = 0.14$ ,  $P = 0.93$ ) between nests initiated with eggs bearing sons ( $n = 13$ ) and daughters ( $n = 10$ ).

We conducted a GLMM where log-transformed egg mass was the response variable, egg sex, laying order, and the

variable ‘sex of the initial egg in each nest’ were fixed factors, and considering the effect of other potentially influencing variables (Table 1). Results showed a significant interaction between ‘sex of initial egg’ and laying order (Table 1), suggesting that the relationship between egg mass and laying order was different for clutches initiated with eggs bearing sons or daughters. Thus, females starting the clutch with an egg bearing a son decreased the mass of the eggs laid subsequently in the laying sequence (Fig. 2a), while the opposite strategy was adopted by females initiating the clutch with an egg bearing a daughter (Fig. 2b). In addition, we found that egg mass decreased with laying date (Table 1). Hatching asynchrony, i.e. the number of days that elapsed between the hatching of the first and the last chick, did not differ between clutches initiated with eggs bearing sons ( $n = 13$ ) and daughters ( $n = 10$ ) (Mann-Whitney U-test,  $Z = 0.032$ ,  $P = 0.97$ ). There was no significant difference in the number of eggs lost (Mann-Whitney U-test,  $Z = 0.41$ ,  $P = 0.74$ ), number of chicks hatched ( $Z = 0.37$ ,  $P = 0.74$ ), nestlings lost ( $Z = 0.41$ ,  $P = 0.74$ ) or number of chicks fledged ( $Z = 0.60$ ,  $P = 0.56$ ) between nests

Variables	Parameter estimate (SE)	F	d.f.	P
Sex of initial egg (male)	-0.14330 (0.05014)	4.10	1,63	<b>0.0471</b>
Fledgling sex (male)	-0.08794 (0.01704)	26.63	1,63	<b>&lt;0.0001</b>
Hatching order (initial)	-0.01104 (0.04037)	2.18	2,63	0.1217
Hatching order (middle)	-0.00966 (0.03744)			
Sex of initial egg (male) x hatching order (initial)	0.13550 (0.05248)	3.33	2,63	<b>0.0420</b>
Sex of initial egg (male) x hatching order (middle)	0.09311 (0.05033)			
Egg mass	0.01637 (0.00796)	4.23	1,63	<b>0.0440</b>

**Table 2.** GLMM with normal error and identity link function on mass of Eurasian kestrel fledglings, where fledgling sex, sex of the first laid egg in each nest and hatching order (first hatched, middle and last hatched) were included as fixed factors. This model explained a 46.78% of the original deviance, without considering the deviance explained by parental and nest characteristics which were controlled for by including the nest as a random term ( $Z = 2.47$ ,  $P = 0.0068$ ). Other potentially influencing variables such as laying date, clutch size, laying order, hatching mass, number of brothers and sisters, and the remaining interactions between the variables, were not significant ( $P > 0.23$ ). Parameter estimates and *SE* for the levels of fixed factor were calculated considering a reference value of zero for last order level in the variable 'laying order', female level in the variable 'sex of initial egg', and for the female level in the variable 'fledgling sex'.

initiated with eggs bearing sons ( $n = 13$ ) and daughters ( $n = 10$ ). The number of sons ( $2.85 \pm 0.90$ ) and daughters ( $1.77 \pm 1.01$ ) fledged from clutches initiated with an egg bearing a male were, respectively, higher ( $t_{21} = 3.65$ ,  $P = 0.002$ ) and similar ( $t_{21} = 1.54$ ,  $P = 0.14$ ) than those fledged from clutches initiated with an egg bearing a female (sons:  $1.50 \pm 0.85$ , daughters:  $2.50 \pm 1.27$ ). However, sex ratio at fledging in nests initiated with eggs bearing sons (61% males,  $n = 59$ ) and daughters (38% males,  $n = 40$ ) did not differ from parity, although opposite trends were apparent (binomial test,  $P = 0.12$  and  $P = 0.16$ , respectively). Given that eggs of different sex laid in different orders might differ in laying and hatching order in nests initiated with eggs bearing sons or daughters, we performed a mixed model (logistic link function, binomial error) where the sex of the eggs was the response variable. In this form, we assessed simultaneously whether laying and hatching order were sex-biased in both kind of nests. We controlled by the influence of the nest, which was treated as a random factor. This analysis revealed that the sex of the eggs was not biased by laying order

( $F_{1,72} = 0.72$ ,  $P = 0.35$ ), but that females hatched earlier than males ( $F_{1,72} = 7.25$ ,  $P = 0.009$ ). In addition, there was a significant effect of the variable 'sex of initial egg in the clutch' showing that the number of hatched chicks of each sex differed between broods from clutches initiated with eggs of each sex ( $F_{1,72} = 7.84$ ,  $P = 0.007$ ). That is, there were more sons among nestlings hatched in nests initiated with a male egg, and more daughters in nests initiated with eggs bearing females. In addition, sex ratio of chicks hatched in each order did not differ from parity in broods from clutches initiated with an egg bearing a son or a daughter (binomial test, all  $P$ -values  $> 0.13$ ), except for first hatched eggs from clutches initiated with eggs bearing daughters, which were female biased (76% females,  $n = 17$ , binomial test  $P = 0.049$ ).

### Fledgling mass in broods initiated with eggs bearing sons and daughters.

We explored whether the observed differential investment in egg mass according to sex of initial eggs and laying order influenced the mass of particular

fledglings. Therefore, the mass of fledglings of each sex from nests initiated with an egg bearing a male or a female embryo was compared by conducting a GLMM where fledgling sex and 'sex of the initial egg in each nest' were fixed factors, and also considering other potentially influencing variables (Table 2). Hatching order was included as a factor with three levels (first, middle and last order) because of the predictable differences in fledgling mass with hatching order according to brood-survival or brood-reduction strategies potentially adopted by different females. Results showed that fledgling mass was higher in females than males (Table 2) as expected due to reversed sexual dimorphism, and that fledgling mass increased as did egg mass (Table 2).

In addition, there was a significant effect of the interaction between hatching order and the variable 'sex of initial egg' (Table 2). This interaction indicated that fledgling

mass decreased from first to last hatched chicks in nests initiated with eggs bearing sons (Fig 3a), while fledgling mass of chicks hatched in different orders did not differ in nests initiated with eggs bearing daughters (Fig. 3b).

## DISCUSSION

### Sexual dimorphism in egg-mass biased by laying order

We found that females may potentially adjust reproductive investment by differentially allocating resources to eggs according to the sex of the embryo. To our knowledge, only other five studies have reported sexual size-dimorphism in size of avian eggs (Mead, Morton & Fish 1987; Andersson *et al.* 1997; Cordero *et al.* 2000, 2001; Cunningham & Russell 2001). Furthermore, we found evidence that female kestrels may allocate resources to eggs differentially depending simultaneously on

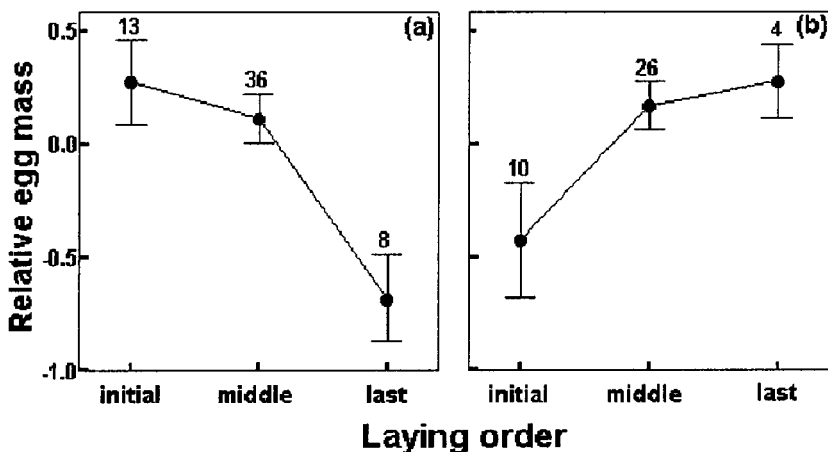


Fig. 3. Mean  $\pm$  SE relative fledgling mass (fledgling mass minus the brood mean) according to hatching order and sex (open symbols are females, full symbols are males) in (a) clutches initiated with an egg bearing a son or (b) a daughter. Numbers above bars are sample sizes. Note that there was male and female chicks hatched in first order in both nests initiated with eggs bearing sons and daughters because laying and hatching order of particular eggs may differ (see results).

sex and laying order. That is, initial eggs bearing a male embryo were heavier than initial eggs bearing a female embryo, but no differences in mass associated to sex were found for eggs laid later in the laying sequence. To our knowledge, this striking result provides the first evidence of sexual egg dimorphism biased by laying order. Parental favouritism expressed in differential resource allocation to different-sex eggs may be a mechanism to adjust allocation for both the primary and secondary sex-ratio of clutches in order to increase parental and offspring fitness (Cordero *et al.* 2000, 2001).

We found that differential investment in eggs bearing sons or daughters was not related with the proportion of the sexes in the first *versus* subsequent eggs, and that sex-ratio was not biased by laying order.

Other studies have shown that first-laid eggs are the only significantly sex-biased or more biased than later-laid eggs (Emlen 1997). Laying order may be biased towards the larger (Leroux & Bretagnole 1996; Arnold *et al.* 2001) or the smaller sex (Bednarz & Hayden 1991; Blanco *et al.* 2002) in sexually size-dimorphic birds. Furthermore, opposite trends have been found in different populations of the same species (Clotfelter 1996; Kilner 1998) and between years with contrasting food conditions (Dzus, Bortolotti & Gerrard 1996).

Our results suggest that differential investment in offspring may be also reached by differential provisioning of eggs according to the sex of the first-laid egg, rather than biasing sex ratios of initial eggs, although we can not discard that female kestrels could be able to choose the sex of first laid eggs.

### **Sex of initial eggs and egg-mass hierarchies within clutches**

Intraclutch egg-mass variation may result from physiological or nutritional constraints on the laying female, but it may also be an adaptive response to enhance the parental control of sibling competition (Slagsvold *et al.* 1984; Viñuela 1997). These alternatives are not mutually exclusive for explaining within-clutch egg mass variation between species, as it may depend on how the resources for laying are gathered (Viñuela 1997). Different strategies of intraclutch egg-mass variation might also occur within species if individuals differ in how and how many resources for laying are gathered and allocated within the clutch, as suggested by experimental food supplementation before and during laying altering egg-mass hierarchies within clutches (Simmons 1994; Wiebe & Bortolotti 1996; Aparicio 1999). Our results indicate that females starting the clutch with an egg bearing a daughter increased mass investment on eggs laid subsequently in the clutch, while females laying a first egg bearing a son invested a decreasing amount of nutrients in subsequent eggs. This is the first evidence suggesting that different individuals of a given population may adopt different strategies of intraclutch egg-mass variation depending on the sex of initial eggs. Constraints such as 'gearing up' physiologically for starting egg production may explain relatively small initial eggs (Parsons 1976; Leblanc 1987), while prolactin influence on developing follicles during incubation or depletion of reserves at the end of laying may explain relatively small last laid-eggs (Leblanc 1987; Arnold 1991). However, these proximate mechanisms can not explain why a relatively large or small initial egg should be

associated with a particular sex and furthermore, why this association should influence the pattern of investment on subsequent laid eggs in the clutch. Our results suggest that resources invested on initial eggs bearing daughters would be partially reabsorbed or differentially provisioned when passing through the oviduct to result in relatively small eggs, and saved resources should be then invested in relatively larger eggs laid subsequently in the laying sequence independently of their sex. On the other hand, resources invested in relatively large initial eggs bearing sons would be not restricted and then reserves should be increasingly reduced due to their depletion during the laying period.

#### **The dilemma of the initial-egg sex and its consequences for offspring**

Different strategies of resource allocation according to egg sex and laying order may be different forms of investment in the progeny by influencing the degree of sibling competition. According to the 'brood-survival' hypothesis, females may allocate a relative large amount of resources on eggs laid in last order to decrease the probability that the last-hatched nestling will starve. Alternatively, females may invest a relatively larger amount of nutrients on initial eggs to enhance some mortality due to the hierarchy imposed by hatching asynchrony under unpredictable food shortages ('brood-reduction' hypothesis). In our study, different patterns of intraclutch egg-mass variation were not found being translated to differences in egg losses, hatching asynchrony or offspring mortality. Brood reduction in this population of kestrels is relatively rare (<10% of hatched chicks died), probably due to high food availability and optimal hunting habitat, and

thus the possible effects of intra-clutch egg mass variation on brood reduction may be difficult to detect. Alternatively, our results suggest that parents may be able to invest in their progeny through different egg-mass hierarchies, depending on the sex of the first laid eggs, and this may allow to reduce possible non-adaptive mortality induced by increasing sexual dimorphism in the mass of chicks along growth (Bortolotti 1986).

Regarding brood composition, we found that clutches initiated with an egg bearing a male fledged more sons than those initiated with an egg bearing a female. The opposite trend, albeit not reaching significance, was found for the number of daughters. Even if these trends influenced the distribution of sexes among fledglings from clutches initiated with different-sex eggs, sex-ratios at fledgling did not differ from parity in both kinds of nests, and perhaps these non-significant results could be caused by the relatively small sample size in these analyses (see West & Sheldon 2002).

The small difference between the number of sons and daughters raised in both kinds of nests may be probably a simple effect of considering the sex of the initial egg as the grouping variable. Alternatively, these patterns may be due to differences in environmental or parental features, such as condition, age, breeding experience or the onset of incubation start, influencing the ability to raise broods with different sex ratios. We lack data on parental characteristics to test these predictions, but a negative relationship between female condition and proportion of male offspring (the cheaper sex) have been found in this species (Korpimäki *et al.* 2000) and in the closely related American kestrel (Wiebe & Bortolotti 1992).



Therefore, it remains possible but untested that different sex allocation strategies associated with different egg-mass hierarchies and sex of initial eggs depend on parental characteristics. A fine-tuned egg mass versus laying sequence strategy has been suggested unlikely to evolve in American kestrels because females seem not to have the ability to always hatch eggs in the same order they are laid (Bortolotti & Wiebe 1993). Eurasian kestrels have been suggested to be able to hatch eggs in the order they were laid (Wiebe *et al.* 1998). However, the possibility that the lack of matching between laying and hatching orders would enhance the adjustment of parental effort when eggs of different sex are provisioned differently has been not addressed before. We found that daughters from initial eggs, which were relatively lighter than the remaining eggs in the clutch, hatched earlier than sons from initial eggs, which were relatively heavier than the remaining eggs in the clutch, probably due to differences in the duration of incubation associated to sex (Blanco *et al.* in press). Therefore, females may be adjusting egg-mass provisioning to initial eggs of different sex in an attempt to control sex-sequence at hatching and then possible mass hierarchies within the brood (Bortolotti 1986; Blanco *et al.* 2002; Krebs *et al.* 2002).

Thus, first laid eggs bearing daughters would be relatively lighter to compensate for faster growth rate of female chicks if hatched in first order (see Blanco *et al.* 2002). In this case, daughters from initial eggs may not take advantage in the brood hierarchy due to the mass of the eggs from which they hatch. In contrast, initial eggs bearing sons would be relatively heavier in an attempt to compensate for their lower growth rate and delayed hatching.

Different strategies of egg provisioning and hatching patterns were associated respectively with mass hierarchies and the lack of mass hierarchies without differential mortality in broods initiated with eggs bearing sons and daughters. Thus, fledgling mass decreased with hatching order in nests initiated with eggs bearing sons, while fledging mass of chicks hatched in different orders did not differ in nests initiated with eggs bearing daughters. Overall, mean mass of both male and female fledglings did not differ between both kinds of broods. However, fledgling mass differences due to hatching order in nest initiated with a male egg could result in differential mortality during breeding seasons with a food shortage or after fledgling. We do not know if females were able to control the sex of initial eggs, but we have showed that they were apparently able to identify their sex and provision them accordingly to presumably maintain the subsequent control of investment. This control may mainly be achieved due to the clear influence that egg mass have on hatchling mass (Williams 1994; Christians 2002), and then the potential influence that egg-mass allocation within the clutch may have on the brood mass hierarchy (Slagsvold *et al.* 1984; Viñuela 1997). In addition, this control may be achieved through the influence that first-hatched chicks may have on growth and survival of their siblings in sexually dimorphic species with variable degree of hatching asynchrony (Bortolotti 1986; Blanco *et al.* 2002; Krebs *et al.* 2002).

In conclusion, the combination of small initial eggs and the larger mass of subsequent laid eggs in the clutch may smooth intra-brood mass hierarchy when the first egg laid bears an embryo of the larger-size sex. In contrast, relatively heavier

initial eggs, and the lightest mass of final eggs in the clutch enhances intra-brood mass hierarchy when the first egg laid bears an embryo of the smaller-size sex. These strategies may potentially be adopted to (1) compensate differences in mass induced by genetically determined sexual differences in growth rate, thus avoiding possible maladaptive intrabrood mass hierarchies induced by asynchronous hatching, while (2) maintaining a given degree of intrabrood mass hierarchy favouring early hatched chicks. However, the outcome of these strategies may probably vary depending on environmental or parental conditions, or both, which need be further investigated. The main conclusion of this study reinforces the evidence that egg mass may potentially be controlled depending on the female ability both to identify egg sex and to differentially allocate resources accordingly to adjust reproductive investment. More research is needed on the role of the combination of sex and mass of eggs laid in particular orders, especially initial eggs, in the adjustment of reproductive effort in birds with marked sexual dimorphism in mass.

## ACKNOWLEDGEMENTS

We thank J. Moreno, J. Potti and J. A. Fargallo for their support to conduct the study. Two anonymous referees improved the manuscript.

## REFERENCES

- Anderson, D.J., Reeve, J., Martínez, J.E., Weathers, W.W. & Bird, D.M. (1993) Sexual size dimorphism and food requirements of nestlings birds. *Canadian Journal of Zoology* **71**, 2541-2545.
- Anderson, D.J., Reeve, J. & Bird, D.M. (1997) Sexually dimorphic eggs, nestling growth and sibling competition in American Kestrels *Falco sparverius*. *Functional Ecology* **11**, 331-335.
- Aparicio, J.M. (1999) Intraclutch egg-size variation in the eurasian kestrel: advantages and disadvantages of hatching from large eggs. *The Auk* **116**, 825-830.
- Arnold, T.W. (1991) Intraclutch variation in egg size of american coots. *Condor* **93**, 19-27.
- Arnold, K.E., Griffith, S.C. & Goldizen, A.W. (2001) Sex-biased hatching sequences in the cooperatively breeding Noisy Miner. *Journal of Avian Biology* **32**, 219-223.
- Bednarz, J.C. & Hayden, T. (1991) Skewed brood sex ratio and sex-biased hatching sequence in Harris's hawks. *The American Naturalist* **137**, 116-132.
- Blanco, G., Davila, J.A., López-Septiem, J.A., Rodríguez, R. & Martínez, F. (2002). Sex-biased initial eggs favours sons in the slightly size-dimorphic Scops Owl (*Otus scops*). *Biological Journal of the Linnean Society* **76**, 1-8.
- Blanco, G., Martínez, J. Dávila, J. A., Serrano, D. & Viñuela, J. First evidence of sex differences in the duration of avian embryonic period: consequences for sibling competition in sexually dimorphic birds. *Behavioral Ecology*, in press.
- Bortolotti, G.R. (1986) Influence of sibling competition on nestling sex-ratios of sexually dimorphic birds. *The American Naturalist* **127**, 495-507.
- Bortolotti, G.R. & Wiebe, K.L. (1993) Incubation behaviour and hatching patterns in the American Kestrel *Falco sparverius*. *Ornis Scandinavica* **24**, 41-47.
- Christians, J.K. (2002) Avian egg size: variation within species and inflexibility within individuals. *Biological Reviews* **77**, 1-26.
- Clotfelter, E.D. (1996) Mechanisms of facultative sex ratio variation in Zebra Finches (*Taenopygia guttata*). *The Auk* **113**, 441-449.

- Cordero, P., Griffith, S.C., Aparicio, J.M. & Parkin, D.T. (2000) Sexual dimorphism in house sparrow eggs. *Behavioral Ecology and Sociobiology* **48**, 353-357.
- Cordero, P., Viñuela, J., Aparicio, J.M. & Veiga, J. P. (2001) Seasonal variation in sex ratio and sexual egg dimorphism favouring daughters in first clutches of the spotless starling. *Journal of Evolutionary Biology* **14**, 829-834.
- Cunningham, E.J.A. & Russell, A.F. (2001). Reply to Petrie *et al.* *Nature* **412**, 498.
- Dzus, E. H., Bortolotti, G.R. & Gerrard, J.M. (1996) Does sex-biased hatching order in bald eagles vary with food resources?. *Écoscience* **3**, 252-258.
- Eising, C.M., Eikenaar, C., Schwabl, H. & Groothuis, T.G.G. (2001) Maternal androgens in black-headed gull (*Larus ridibundus*) eggs: consequences for chick development. *Proceedings of the Royal Society of London B* **268**, 839-846.
- Emlen S.T. (1997) When mothers prefer daughters over sons. *Trends in Ecology and Evolution* **12**, 291-292.
- Fargallo, J.A., Blanco, G., Potti, J. & Viñuela, J. (2001) Nestbox provisioning in a rural population of Eurasian Kestrels: breeding performance, nest predation and parasitism. *Bird Study* **48**, 236-244.
- Kilner, R. (1998) Primary and secondary sex ratio manipulation by zebra finches. *Animal Behaviour* **56**, 155-164.
- Korpimäki, E., May, C.A., Parkin, D.T., Weton, J.H. & Wiehn, J. (2000) Environmental- and parental condition-related variation in sex ratio of kestrel broods. *Journal of Avian Biology* **31**, 128-134.
- Krebs, E.A., Green, D.J., Double, M.C. & Griffiths, R. (2002) Laying date and laying sequence influence the sex ratio of Crimson Rosella broods. *Behavioral Ecology and Sociobiology* **51**, 447-454.
- Leblanc, Y. (1987). Intraclutch variation in egg size of Canada Geese. *Canadian Journal of Zoology* **65**, 3044-3047.
- Leroux, A. & Bretagnolle, V. (1996) Sex ratio variations in broods of Montagu's Harriers *Circus pygargus*. *Journal of Avian Biology* **27**, 63-69.
- Mead, P.S., Morton, M.L. & Fish, B.E. (1987) Sexual dimorphism in egg size and implications regarding facultative manipulation of sex in Mountain White-crowned Sparrows. *Condor* **89**, 798-803.
- Parsons, J. (1976) Factors determining the number and size of eggs laid by the Herring Gull. *Condor* **78**, 481-492.
- Schawbl, H. (1996) Maternal testosterone in the avian egg enhances postnatal growth. *Comparative Biochemistry and Physiology A* **114**, 271-276.
- Simmons, R.E. (1994) Supplemental food alters egg size hierarchies within harrier clutches. *Oikos* **71**, 341-348.
- Slagsvold, T. Sandvik, J. Rofstad, G., Lorentsen, O. & Husby, M. (1984) On the adaptive value of intra-clutch egg-size variation in birds. *The Auk* **101**, 685-697.
- Stearns, S.C. (1992). *The Evolution of Life Histories*. Oxford University Press, Oxford.
- Styrsky, J.D., Eckerle, K. P. & Thompson, C.F. (1999) Fitness-related consequences of egg mass in nestling House Wrens. *Proceedings of the Royal Society of London B* **266**, 1253-1253.
- Viñuela, J. (1997) Adaptation vs constraint: intraclutch egg-mass variation in birds. *Journal of Animal Ecology* **66**, 781-792.
- Viñuela, J. (2000) Opposing selective pressures on hatching asynchrony: egg viability, brood reduction and nestling growth. *Behavioral Ecology and Sociobiology* **48**, 333-343.
- West, S. A. & Sheldon B. C. (2002). Constraints in the evolution of sex ratio adjustment. *Science* **295**: 1685-1687.

Wiebe, K. & Bortolotti, G.R. (1992) Facultative sex ratio manipulation in American Kestrels. *Behavioural Ecology and Sociobiology* **30**, 379-386.

Wiebe, K. & Bortolotti, G. R. (1996) The proximate effects of food supply on intraclutch egg-size variation in American Kestrels. *Canadian Journal of Zoology* **74**, 118-124.

Wiebe, K. L., Wiehn, J. & Korpimäki, E. (1998) The onset of incubation in birds: can females control hatching patterns?. *Animal Behaviour* **55**, 1043-1052.

Williams, T.D. (1994) Intraspecific variation in egg size and egg composition in birds: effects on offspring fitness. *Biological Reviews* **68**, 35-59.

## CAPÍTULO III

### **La condición de la hembra antes de la puesta influye en la asociación entre el peso del huevo y la inmunidad de los volantones en el Cernícalo vulgar.**

Este capítulo reproduce íntegramente el texto del siguiente manuscrito:

**Jesús Martínez-Padilla.** *En revisión.* Prelying maternal condition mediates the association between egg mass and Nestling immunity in the Eurasian kestrel. *Evolutionary Ecology Research.*

#### **Resumen del Capítulo**

Los modelos teóricos de cuidado parental predicen una inversión diferencial en relación al fitness esperado de los volantones. Debido a que las aves deben invertir todos los recursos en un mismo paquete energético, la inversión maternal juega un papel fundamental en las historias vitales. Por ello, es predecible que las madres puedan influir en la inversión de su descendencia variando la composición o el tamaño de los huevos, como recientes estudios sugieren. En este contexto, la defensa inmunitaria medida como la capacidad de generar una respuesta inmune celular, se está revelando como una herramienta adecuada para medir la calidad de los volantones. Sin embargo, el enlace entre la calidad de los volantones medida de este modo y la inversión que hace la hembra en los huevos, es poco conocida. Sin embargo, se sabe poco sobre esta interacción en relación al peso de los huevos, que también ha sido sugerido como un estimador del fitness del volantón, aunque con resultados controvertidos. En este capítulo, se estudia la relación entre el tamaño del huevo y la respuesta inmune de volantones de Cernícalo vulgar (*Falco tinnunculus*) durante tres años de estudio. Dado el potencial efecto materno en la inmunidad de los volantones, se hizo un experimento de alimentación suplementaria en el que se mejoró la condición de la hembra antes de la formación de los huevos. El efecto de la alimentación suplementaria en la condición de la hembra desapareció antes del final del período de incubación. Los resultados indicaron que la respuesta inmune se relacionó negativamente con el peso del huevo considerando los tres años de estudio, aunque la alimentación suplementaria no influyó en la respuesta inmune. Sin embargo, la interacción entre el tratamiento experimental y el peso del huevo fue significativa, indicando que para hembras suplementadas en el año experimental, la respuesta inmune se incrementó con el peso del huevo. Este resultado sugiere que la condición de la hembra antes de la formación de los huevos puede mediar la relación entre el tamaño del huevo y la futura respuesta inmune de sus volantones. Se sugiere, por tanto, que la hembra está limitada energéticamente para mejorar la futura respuesta inmune de su descendencia debido a un compromiso en la distribución de recursos en su descendencia determinado por la condición de la hembra antes de la formación de los huevos.



## CAPÍTULO III

### **Prelaying maternal condition mediates the association between egg mass and nestling immunity in the Eurasian kestrel**

JESÚS MARTÍNEZ-PADILLA AND JAVIER MILLÁN. *Instituto de Investigación en Recursos Cinegéticos (IREC, CSIC - UCLM - JCCLM), PO Box 535, E-13080 Ciudad Real, Spain.*

#### **ABSTRACT**

Theoretical models of parental care predict differential allocation in relation to the expected fitness of the offspring. As birds must allocate all the resources required for embryonic development in self-contained packages, maternal egg allocation takes a central role in avian life histories. It is predicted that mothers can influence offspring performance by varying the size or composition of their eggs. In this context, immune defence measured as a T-cell mediated response, has been revealed as a valuable tool to evaluate fledgling fitness. However, little is known about its relationships to egg mass, which has also been suggested controversially as an estimator of fledgling fitness. In this paper we explore the association between egg mass and immune response of Eurasian kestrel fledglings (*Falco tinnunculus*) during 3 years. Given the potential maternal effects on offspring immunity, we improved female condition prior to egg formation in certain nests through food supplementation. The effect of supplementation on female condition disappeared before the end of the incubation period. I found that fledgling immune response decreases negatively at egg mass increases throughout the 3 years of study, although food supply did not influence immune response. However, the interaction between food supply and egg mass was significant. For supplemented females I found that fledgling immune response increases with egg mass. This result suggests that female body condition prior to egg formation could mediate the relationship between egg mass and future immune response of their offspring. Females may be constrained in improving the future immune response of their offspring due to resource allocation trade-offs determined by their prelaying condition.

*Key words: Egg quality, female condition, food supplementation, maternal effects, Falco tinnunculus.*

#### **INTRODUCTION**

---

Parental investment theory predicts that breeding strategies should have evolved to allow differential allocation of investment in relation to the expected fitness of the offspring (Clutton-Brock, 1991). Oviparous animals in general, and birds in particular, must allocate all the resources required for embryonic development in discrete units at the time of laying, and thus maternal

allocation of resources to eggs takes a central role in avian life history evolution. Mothers can control their investment at laying by varying the size or composition of their eggs (Mousseau and Fox, 1998), and this distribution of resources may affect offspring fitness (Einum and Fleming, 2000; Roff, 2002). On the other hand, the allocation of resources to eggs during the short period of laying may imply costs to parents in terms of survival or future

reproductive success (Clutton-Brock, 1991; Stearns, 1992).

Offspring fitness may be related to egg size (Clutton-Brock, 1991), because larger eggs produce structurally larger chicks or with larger energy reserves at the time of hatching, and this may enhance their future survival prospects (Williams, 1994). Egg size could be an estimator of fitness at least in early development or growth (Williams, 1994), and several results support that egg size may be related to hatching success (Croxall, et al. 1992; Magrath, 1992), early nestling survival (Ankney, 1980; Bolton, 1991), fledgling survival (Blomqvist, et al. 1997) or fledgling size (Blanco et al. 2003a; Blanco, et al. 2003b), and thus that it may be an important estimator of offspring fitness (Williams, 1994, Smith and Bruun, 1998). Recently, it has been shown that the performance of the immune system in fledglings may be a relevant estimator of offspring fitness, because it may be correlated with future survival (Christe et al. 1998; Hõrak, et al. 1999; Soler et al. 1999; Tella, et al. 2000a). Recent studies of immune function in nestling birds have focused on studying how it may be affected by different factors such as sex (Fargallo, et al. 2002; Tschirren, et al., 2003), inheritance (Tella et al. 2000b; Soler, et al. 2003), brood size (Sorci et al. 1997; Hõrak et al. 1999), secondary sexual traits (González et al. 1999), paternity (Johnsen et al. 2000), fluctuating asymmetry (Fair, et al. 1999) or intensity of parasitism (Saino, et al. 1998; Christe et al. 1998). On the other hand, there is increasing evidence that maternal effects through egg composition may affect offspring immunity (Gasparini et al. 2002; Saino, et al. 2002). In addition, recent studies suggest a relationship between prelaying conditions and differential

allocation of components to eggs laid, mediated by egg size or laying sequence. Within this context, maternal diet rich in lipids and proteins prior to laying could alter the proportion of these components allocated to eggs according to the laying sequence (Reynolds et al. 2003). In addition, females could allocate different resources differentially to eggs, as carotenoids (Blount et al. 2002) and different relationships between albumen and yolk varied with egg mass (Hipfner, et al. 2003). Therefore, if egg mass is linked to egg composition, we could expect an association between egg mass and maternally-determined offspring immunity that has not been previously explored. A heavy investment in nutritional resources for the embryo affecting egg size could negatively affect the maternal capacity to allocate certain resources to the egg, determining a negative association between both fitness traits.

Both the amount of resources invested in egg formation by females and the quality of fledgling immune response could be affected by resource availability for females and nestlings during and after laying (Viñuela 1997; unpublished data). Thus, the effects of egg size and fledgling immunity on offspring fitness may be mediated by food availability, and it is important to control for this crucial environmental variable. In this study I explore the relationship between egg mass and fledgling immune response in Eurasian kestrels (*Falco tinnunculus*). The results obtained by observational procedures suggested the need to perform an experiment controlling the effect of food availability before laying. In the last year of my study, I carried out a food supplementation experiment prior to egg formation in order to control resource availability for females and evaluate the



immune response of fledglings hatched from those eggs laid by supplemented and control females. I predicted that (1) fledglings hatched from eggs laid by supplemented females could mount a stronger immune response than fledglings hatching from eggs laid by control females and, (2) that the association between egg mass and offspring immune response could interact with maternal condition prior to egg formation.

## **MATERIAL AND METHODS**

---

### **Nest monitoring and egg variables**

The study was conducted in a Eurasian kestrel (*Falco tinnunculus*, kestrel hereafter) population breeding in nest boxes during the breeding seasons of 2000-2002 in the Campo Azálvaro area (40°40'N, 4°20'W, 1300m.a.s.l.), an homogeneous mountain grassland in central Spain (see Fargallo *et al.* 2001 for more details).

I monitored nest-boxes every day during the prelaying period to control laying order. Eggs were marked as they were laid, so laying sequence was accurately known. At the first visit that I found an egg, it was weighed (to the nearest  $\pm 0.25$ gr) and measured (length and breadth to the nearest  $\pm 0.001$ mm). Nests were visited daily since 4 - 5 days before estimated date of hatching (x days after laying of the last egg), and 2 - 3 times every day when I detected the first evidences of hatching (sounds inside eggshells). In order to assign each egg to its corresponding nestling, hatchlings were marked with indelible and harmless ink in the hatching tooth when eggs were found during the hatching process, and later every 4 - 5 days with ink on the head until banding. Hatchlings were weighed with Pesola balances to the nearest  $\pm 0.25$ gr, and their tarsus and wing lengths measured to

the nearest 0.01mm at the first visit they were found hatched. Nestlings 24 days old were weighed with Pesola balances to the nearest  $\pm 1$ gr and their tarsus and wing length measured to the nearest  $\pm 1$ mm. Additionally, I estimated the length of the embryonic period of each particular egg because it has been suggested that the duration of the embryonic period may be related to the immune response in birds of prey (Tella *et al.* 1998). We estimated this variable as the number of days elapsed between the start of incubation and the day of hatching (Blanco, *et al.* 2003a). Given that some embryo development could have occurred before full incubation started (Wiebe *et al.*, 1998), I considered that incubation began on the day before the penultimate egg was laid, when partial incubation has occurred in all nests (see also Wiebe *et al.*, 1998; Blanco, *et al.* 2003a). Only nests that did not suffer any predation event have been included in the analyses. Nestling sex was determined by molecular procedures (Fridolfsson and Ellegren 1999) using DNA extracted from a drop of blood obtained by brachial venipuncture. All measurements were taken by JM, so we assumed the same error for each measure.

### **Immune response**

To evaluate the efficiency of the immune system, a mitogen was injected to generate a local proliferation of circulating T lymphocytes. Twenty four days after hatching, kestrel chicks were injected intradermally in the left wing web with 0.1ml of a solution of PHA (3mg of PHA in 1ml of PBS) after measuring its thickness at the point of injection. 24h after injection I measured again wing web thickness and I considered the difference between initial and final measurement as the T-cell

mediated immune response (Smits, *et al.* 1999). Three measures were taken with a digital spessimeter (Mitutoyo Absolute ID-112B) to the nearest 0.01mm before and after injection. The repeatabilities of initial ( $F_{122,246} = 275.45$ ,  $p < 0.0001$ ) and final ( $F_{122,246} = 281.58$ ,  $p < 0.0001$ ) measurements were high, so we used mean values of the three measurements. All measurements were taken by JM with the same spessimeter.

### **Food supplementation experiment**

To control for the possible effect of food availability before laying on egg size and immune response of chicks, I performed a food supplementation experiment prior to the onset of laying in 2002. Each experimental pair ( $n = 20$ ) was supplemented every two days with 150gr of common quail at the beginning of the breeding season, when pairs were occupying nest boxes. In all cases, food supplementation started at least 18 days before egg laying, thus covering completely the period required to form eggs in this species (9 days; see Meijer, *et al.* 1989). Supplementation ceased on the day when the first egg was laid.

Females were captured at the nest boxes while incubating, between a week after laying and a week before hatching, in order to compare female body condition at the same stage in their breeding period after the cessation of food supplementation. Females were weighed with Pesola balances to the nearest g and their tarsus and wing length measured to the nearest mm.

### **Statistical procedures**

Since eggs, hatchlings and fledglings from the same nest had probably more similar mass than those from different nests

due to the influence of sharing common parents and rearing environments, they were not considered independent samples. Therefore, I performed generalized linear mixed models (GLMM) using a normal distribution of errors and an identity link function, and to explore the effect of the experiment I used binomial distribution of errors and logit function (GLIMMIX macro of SAS, Littell *et al.* 1996). In all models and analyses, we only use those fledglings for which we could determine from which egg they were hatched.

GLIMMIX was performed to explore the effect of experimental manipulation, allowing me to control the random effect of nest. Mixed model evaluates the random effect of the nest in the model, allowing us to treat chicks as sample unit while avoiding pseudoreplication (Littell *et al.* 1996). A second GLIMMIX was performed with immune response as dependent variable. Fledgling sex, experimental treatment, nest hatching asynchrony, brood reduction and laying order were introduced in the model as factors. I considered nest with asynchronous hatching those clutches that all eggs hatched in more than 24h between the first and the last hatchling in a brood (Stoleson and Beisinger, 1995). I considered brood reduction when at least one chick died in brood after hatchling. Laying order was encoded as first, middle or last laid egg, allowing me to compare differences in egg mass within the laying sequence between different clutch sizes. I considered sex ratio as the proportion of males in a brood. In the same way, I encoded hatching order. Additional variables were introduced as covariates in the model, including fledgling mass. A third GLIMMIX was performed in order to evaluate the effect of egg mass on immunity during three different years with

the same variables indicated above and including for 2002 only control nests. The same nest or breeding parents in different years are a source of pseudoreplication. To control for this effect, I nested nest within year. Finally, a fourth GLIMMIX was performed in order to evaluate the effect of experiment on incubation period of each particular egg where laying date and egg mass was introduced as covariate, treatment as factor and nest nested in year as random variable.

Some of the explanatory variables could covary, so I fitted their effects to the observed data following a forward stepwise procedure, testing the significance of each variable and adding only the variable that resulted in a better fit of the model. The significance of the remaining variables was tested again until no additional variable or interaction reached significance. The result is the most adequate model for explaining the variability in the response variable, where only the significant explanatory variables are retained. All tests are two-tailed.

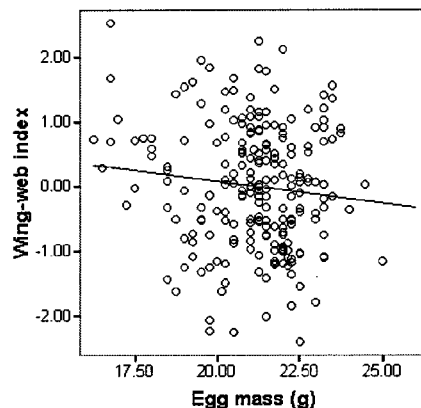
## RESULTS

### Egg mass and immune response

There was a negative association between egg mass and fledgling immune response when only considering unsupplemented pairs and controlling for year and fledgling mass ( $p = 0.2012$ ; year and fledgling mass as covariates in the model, both  $p < 0.0001$ ). This was especially so for modal clutches with 4 and 5 eggs ( $p = 0.0537$ ; year and fledgling mass as covariates in the model, both  $p < 0.0001$ ) which represent 80 % ( $n = 96$ ) in our kestrel population.

### Food supplementation in relation with egg mass and fledgling immune response.

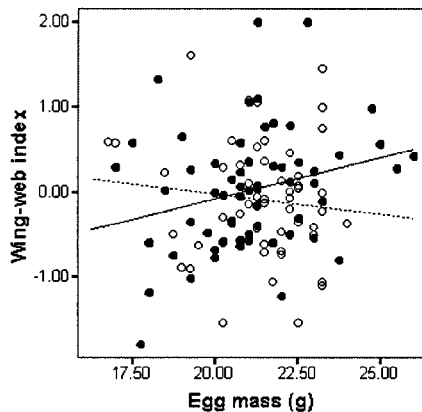
Twenty kestrel pairs were originally supplemented, but two of them did not breed, one suffered clutch predation, and another suffered total brood loss for unknown causes. Thus, 16 kestrel pairs that were supplemented produced fledglings, and 17 randomly selected unsupplemented pairs were considered as control pairs. GLIMMIX suggested that laying date, clutch size, egg mass, fledgling mass and number of fledglings were not affected by the experimental treatment (Table 1). Female body mass and body condition during incubation were not affected by food supply ( $n = 26$ , 12 control and 15 supplemented, Table 1), considering the residuals of the regression of female mass on wing length as an estimate of body condition. In addition, a mixed model showed that egg mass in the laying sequence was not related to food supplementation (interaction between experiment and laying order:  $F_{2,93} = 0.50$ ,  $p =$



**Figure 1.** Linear regression between egg mass and immune response. Y axis represent the index of immune response that were obtained eliminating the influenced variables (random effect of nest, fledgling mass and year). See results and Table 2 for statistics.

		<i>n</i>	Supplemented	<i>n</i>	<i>d.f.</i>	F	<i>p</i>
	<b>Control</b>						
Laying date	65.46 ± 8.93	18	65.59 ± 10.81	17	1,33	0.002	0.969
Clutch size	4.67 ± 0.77	18	4.71 ± 0.77	17	1,33	0.023	0.881
Egg mass (g)	21.23 ± 1.64	18	21.03 ± 1.60	17	1,33	0.139	0.712
First laid egg	21.75 ± 1.37	12	20.89 ± 1.58	11	1,21	1.974	0.175
Middle laid eggs	21.45 ± 1.47	39	21.31 ± 1.91	41	1,78	0.130	0.719
Last laid egg	20.72 ± 1.58	15	20.52 ± 2.01	14	1,27	0.088	0.768
Brood size	3.28 ± 1.18	18	3.76 ± 1.35	17	1,33	1.299	0.263
Chicks died	0.67 ± 1.09	18	0.19 ± 0.54	16	1,32	2.547	0.120
Fledgling mass (g)	211.43 ± 18.44	17	209.12 ± 15.07	16	1,31	0.155	0.697
Female mass (g)	246.88 ± 17.71	12	241.27 ± 22.44	15	1,25	0.499	0.486
Female condition	-4.63 ± 18.55	7	2.31 ± 20.84	14	1,19	0.554	0.466
Sex ratio	0.50 ± 0.39	17	0.53 ± 0.33	16	1,31	0.060	0.808

**Table 1.** Effects of food supplementation on reproductive traits of Eurasian kestrels. Female parent body condition was corrected by wing length as covariate. Adjusted means ± SD are shown.



**Figure 2.** Relationship between immune response and egg mass for fledglings hatched from eggs laid by supplied (continuous line) and unsupplied females (broken line) in 2002. Y axis represent the index of immune response that were obtained eliminating the random effect of nest and the effect of fledgling mass. See results and Table 2 for statistics.

0.609; laying date as covariate:  $p = 0.003$ ). However, we found that eggs mass was strongly correlated with fledgling mass ( $R = 0.23$ ,  $F_{1,314} = 18.048$ ,  $p < 0.0001$ ), and a mixed model showed that nestling survival increased with egg mass ( $F_{1,228} = 5.33$ ,  $p = 0.022$ ).

### Interaction of food supplementation with egg mass.

Considering all control pairs monitored during the three study years I found a negative relationship between immune response and egg mass (Figure 2;  $F_{1,161} = 4.44$ ;  $p = 0.0368$ , nest nested in year as random variable:  $Z = 3.70$ ,  $p = 0.0001$ ; fledgling size as covariate:  $F_{1,161} = 35.49$ ,  $p < 0.0001$ ). However, in 2002 I found that egg mass was not correlated with fledgling immune response, but this relationships turned to be significant when the interaction with treatment was introduced into the model (Table 2, Figure 2). This result suggests that the effect of egg mass on immune response could vary depending on the prelaying condition of the female parent. Treatment in the model only was significant when the interaction between egg mass and treatment was introduced. Selecting only chicks of supplemented females in 2002, immune response increased with egg mass ( $F_{1,43} = 8.74$ ,  $p = 0.005$ , estimate = 0.1602; considering nest as random variable and fledgling mass as covariate), while in chicks from control females there was a negative relationship between immune response and egg mass ( $F_{1,37} = 4.64$ ,  $p = 0.0379$ , estimate

Response term	Explanatory term	Rejected term	Estimate	F	Df.	P	
Immune Response	Egg mass		0.0807	0.42	1,81	0.5194	
	Treatment <sup>†</sup>		5.1217	5.02	1,81	0.0278	
	Treatment <sup>†</sup> *Egg mass		-0.2375	4.91	1,81	<b>0.0295</b>	
	Fledgling mass		0.0137	13.69	1,81	<b>0.0004</b>	
		Clutch size		-0.2622	3.90	1,81	0.0601
		Egg length		0.0932	1.66	1,80	0.2015
		Egg width		0.4371	2.73	1,79	0.1026
		Brood size		-0.1849	2.35	1,78	0.1289
		Hatching asynchrony of nest <sup>†</sup>		-0.4493	2.90	1,77	0.0927
		Incubation period		0.0375	0.95	1,76	0.3331
		Fledgling sex <sup>†</sup>		-0.1154	0.49	1,70	0.4843
		Laying date		-0.0048	0.14	1,69	0.7052
		Laying order <sup>†</sup>		-0.1194	0.36	2,67	0.6989
		Brood reduction <sup>†</sup>		-0.0323	0.01	1,66	0.9411
		Hatching order <sup>†</sup>		-0.0205	0.03	2,64	0.9718

**Table 2.** GLMM with normal error and identity link function on intensity of T-cell dependent immune response of Eurasian Kestrel fledglings. The model explained 38.1% of original variance, without considering the deviance explained by parental and nest characteristics which were controlled for by including the nest as a random term ( $Z = 2.23$ ,  $P = 0.0130$ ). Other potentially influencing variables and the remaining interactions between variables, were not significant ( $P > 0.065$ ). Parameter estimates and *SE* for the levels of fixed factor (<sup>†</sup>) were calculated considering a reference value of zero. In the final model, egg mass was maintained to explore interactions.

= - 0.2165; considering nest as random variable and fledgling mass as control variable). Other variables related to egg size or egg structure had no effect on fledgling immune response (Table 2). In addition, fledgling sex, laying date, laying order, hatching order or hatching asynchrony of chicks were not related to immune response in the model (Table 2). GLIMMIX showed that incubation periods of eggs laid by supplied and control females were similar ( $F_{1,81} = 0.001$ ,  $p = 0.916$ ). In addition, in the same model, the interaction between egg mass and treatment was not significant ( $F_{1,80} = 0.72$ ,  $p = 0.400$ ).

## DISCUSSION

I found that immune response increased with egg mass only when females were supplemented prior to the onset of laying, while in nests where food was not provided the opposite trend was found after removing

statistically the effect of other factors that could influence immune response in our kestrel population. I suggest that food supply could improve female condition prior to the onset of egg formation allowing them to increase the resources allocated when egg size increases. Increasing evidences suggest that females could allocate resources differentially in eggs in relation to laying order (Sockman and Schwabl, 2000), sex (Petrie, et al. 2001) or egg size (Hipfner et al., 2003). In addition, a recent study suggest that breeding birds improved with diet rich in proteins and lipids prior laying, could increase the amount of proteins allocated to eggs, increasing with egg size (Reynolds, et al. 2003). Therefore, I suggest that food supplementation could eliminate a nutritional trade-off between resources allocated to eggs that could increase future immune response and other resources that could increase egg size. In agreement with this, a similar body condition was found

between supplemented and control females as supplemented females could allocate extra resources to their eggs without suffering costs. In this context, results show that nestling survival increases with egg mass, as suggested by previous studies (Bolton, et al. 1991; Magrath, 1992; Blomqvist, et al. 1997; Amat, et al. 2001), and that fledgling mass is strongly related to egg size (see also, Blanco et al. 2003a; Blanco et al. 2003b). In addition, fledgling immune response could be a good estimator of survival (Hörak et al. 1999; Soler et al. 1999; Tella et al. 2000a). Therefore, larger eggs laid by unsupplemented females could be favoured by their higher probability of survival, while larger eggs laid by supplemented females could be favoured by their size and by their better future immune response. In addition, small eggs from control females could favour nestling survival by increasing their future immune response. Therefore, our results confirm that females may adaptively adjust egg composition in order to increase benefits for their offspring in terms of growth and survival in the nest (Williams, 1994; Bernardo, 1996).

Supplemented females are able to improve offspring immunity without sacrificing the advantages determined by egg size. However, this is only evident for females laying large eggs. Supplemented females laying small eggs do not benefit their offspring by investing in their future immune system, as control females with small eggs apparently do. The small eggs laid by some supplemented females could be the expression of inherited constraints on egg size (Christians, 2002; Potti, 1999; Potti, 1993; Boag and van Noordwijk, 1987; Hailman, 1986) and not of nutritional constraints as for some control females. It is

striking that egg composition as expressed by effects on offspring is more dependent on the prelaying condition of the female than egg size (for review, see Christians, 2002), at least in our population. Therefore, it seems that egg size and egg quality may not be necessarily correlated as other authors suggest (Giron and Casas, 2003).

I suggest that better female prelaying condition could increase fledgling fitness, by inducing a higher resource allocation to their eggs. As supplemented females had more resources available for allocating to eggs than control females, these resources could be critical in allowing nestlings to develop a stronger immune system. Because I quantified the proliferation of circulating T-lymphocytes, resources aimed at the development of organs related to lymphocyte proliferation such as spleen, thymus and bursa of Fabricius could be involved in our of the food supplementation experiment. However, the question remains of which resource is allocated to eggs that could improve this trait, especially taking into account that egg mass was not significantly affected by the experiment. In this respect, the effects that different macronutrients have on fledgling fitness or improved embryo development have led to controversial conclusions. Parsons (1970) suggested that yolk reserves may be fundamental to long-term survival, Tullet and Burton (1982) proposed that hatchling mass depends on the water content of the egg and Klasing (2002) indicated that vitamins are likely to influence embryonic development. In addition, it has been suggested that proteins could be a crucial factor in the development of embryo tissues (Starck and Ricklefs, 1998; Nisbet, 1978). Our food supplementation experiment does not allow us to determine which component

allocated by females to the eggs affects offspring immunity. We suggest that proteins could play an important role in differential allocation, because Reynolds et al. (2003) suggest that in eggs with similar sizes, females supplied with a rich diet of protein and lipids, could allocate more proteins than control females. I propose a similar mechanism, where supplied females could eliminate an energetic constraint increasing egg components in bigger eggs, may be proteins, although egg size was unaffected. Therefore, further research is needed in order to elucidate how different components allocated to eggs affect fledgling fitness, as precedent authors suggest (Deeming, 2001; Christians, 2002; Reynolds, et al. 2003) using non-destructive techniques for the measurement of egg composition (Sinervo and Huey, 1990).

In conclusion, results suggest that egg size may be determined by adaptive allocation patterns (Blanco et al. 2003b), while egg quality, measured as immune response mounted by resulting nestlings, may be constrained by female prelaying condition. In addition, this study suggests that females may experience a trade-off between allocating resources to eggs favouring offspring immune system organs and those leading to benefits derived from large egg size. More research should be devoted to clarifying the basis for this allocation trade-off.

## ACKNOWLEDGMENTS

We thank J. Moreno (BOS 2001-0587-MCyT project), J. Viñuela and G. Blanco for finance and supporting this study and improving the original manuscript. We thank the Finat family for kindly allowing us to conduct the study in their property, and

C. Marqués, L. Bolonio, J. San Teodoro for their collaboration in fieldwork. Referees J. Redondo, A. Sáez and R. Pérez repaired JMP's computer, thereby saving data and allowing me to continue my researches.

## REFERENCES

- Amat, J.A., Fraga, R.M. and Arroyo, G.M. 2001. Intraclutch egg-size variation and offspring survival in the Kentish Plover *Charadrius alexandrinus*. *Ibis*, **143**: 17-23.
- Ankey, C. D. 1980. Egg weight, survival and growth of lesser snow goose goslings. *J. Wildlife Manage.*, **44**: 174-182.
- Bernardo, J. 1996. The particular maternal effects of propagule size, especially egg size: patterns, models, quality of evidence and interpretations. *Am. Zool.*, **36**: 216-236.
- Blanco, G.; Martínez-Padilla, J.; Dávila, J. A.; Serrano, D. and Viñuela, J. 2003a. First evidence of sex differences in the duration of avian embryonic period: consequences for sibling competition in sexually dimorphic birds. *Behav. Ecol.*, **14**: 702-706.
- Blanco, G.; Martínez-Padilla, J.; Serrano, D.; Dávila, J. A. and Viñuela, J. 2003b. Mass provisioning to different-sex eggs within the laying sequence: consequences for adjustment of reproductive effort in a sexually dimorphic bird. *J. Anim. Ecol.*, **72**: 831-838.
- Blomqvist, D.; Johansson, O. C. and Götmark, F. 1997. Parental quality and egg size affect chick survival in a precocial bird, the Lapwing *Vanellus vanellus*. *Oecologia*, **110**: 18-24.
- Blount, J. D.; Surai, P. F.; Nager, R. G.; Houston, D. C.; Møller, A. P.; Trewby, M. L. and Kennedy, M. W. 2002. Carotenoids and egg quality in the lesser black-backed gull *Larus fuscus*: a supplemental feeding study of maternal effects. *Proc. R. Soc. Lond. B.*, **269**: 29-36.
- Boag, P.T. and van Noordwijk, A.J. 1987. Quantitative genetics. In *Avian genetics* (F. Cooke and Buckley, P.A. eds.), pp. 45-78. Academic Press. London.
- Bolton, M. 1991. Determinants of chick survival in the lesser black-backed gull: relative

- contribution of egg size and parental quality. *J. Anim. Ecol.*, **60**: 949-960.
- Clutton-Brock, T.H. 1991. *The evolution of parental care*. Princeton University Press.
- Christe, P.; Møller, A. P. and de Lope, F. 1998. Immunocompetence and nestling survival in the house martin: the tasty chick hypothesis. *Oikos*, **83**: 175-179.
- Christians, J.K. 2002. Avian egg size: variation within species and inflexibility within individuals. *Biol. Rev. Camb. Philos. Soc.*, **77**: 1-26.
- Croxal, J. P.; Rothery, P. and Crisp, A. 1992. The effect of maternal age and experience on egg size and hatchling success in Wandering albatrosses *Diomedea exulanus*. *Ibis*, **134**: 219-228.
- Deeming, D.C. 2001. *Avian incubation. Behaviour, environment, and evolution*. Oxford Ornithology Series. Oxford.
- Einum, S. and Fleming, I. A. 2000. Highly fecund mothers sacrifice offspring survival to maximize fitness. *Nature*, **405**: 565-567.
- Fair, J. M.; Hanse, E. S. and Ricklefs, R. E. 1999. Growth, developmental stability and immune response in juvenile Japanese quails (*Coturnix coturnix japonica*). *Proc. R. Soc. Lond. B.*, **266**: 1735-1742.
- Fargallo, J. A.; Laaksonen, T.; Pöyri, V. and Korpimäki, E. 2002. Inter-sexual differences in the immune response of Eurasian kestrel under food shortage. *Ecol. Let.*, **5**: 95 - 101.
- Fargallo, J. A.; Blanco, G.; Potti, J. and Viñuela, J. 2001. Nestbox provisioning in a rural population of Eurasian Kestrels: breeding performance, nest predation and parasitism. *Bird Study*, **48**: 236-244.
- Fridolfsson, A. K. and Ellegren, H. 1999. A simple and universal method for molecular sexing of non-ratite birds. *J. Avian Biol.*, **30**: 116-121.
- Gasparini, J.; McCoy, K.D.; Tvreea, T. and Boulinier, T. 2002. Related concentrations of specific immunoglobulins against the Lyme disease agent *Borrelia burgdorferi sensu lato* in eggs, young and adults of the kittiwake (*Rissa tridactyla*). *Ecol. Let.*, **5**: 519-524.
- Giron, D. and Casas, J. 2003. Mothers reduce egg provisioning with age. *Ecol. Let.*, **6**: 273-277.
- González, G.; Sorci, G.; Møller, A. P.; Ninni, P.; Haussy, C. and de Lope, F. 1999. Immunocompetence and condition-dependent sexual advertisement in male house sparrow (*Passer domesticus*). *J. Anim. Ecol.*, **68**: 1225-1234.
- Hailman, J.P. 1986. The heritability concept applied to wild birds. *Current Ornithology*, **4**: 71-95.
- Hipfner, J.M., Gaston, A.J., Herzberg, G.R., Brosnan, J.T. and Storey, A.E. 2003. Egg composition in relation to female age and relaying: constraint on egg production in Thick-billed murre (*Uria lomvia*). *Auk*, **120**: 645-657.
- Hörak, P.; Tegelmann, L.; Ots, I. and Møller, A. P. 1999. Immune function and survival of great tit nestling in relation to growth conditions. *Oecologia*, **121**: 316-322.
- Johnsen, A.; Andersen, V.; Sunding, C. and Lifjeld, J. T. 2000. Female bluethroats enhance offspring immunocompetence through extra-pair copulation. *Nature*, **406**: 296-299.
- Klasing, K. 2002. *Comparative avian nutrition*. CABI Publishing. Oxon.
- Littell, R.C.; Milliken, G.A.; Stroup, W.W. and Wolfinger, R.D. 1996. *SAS system for mixed models*. SAS Institute. Cary. New York.
- Magrath, R. D. 1992. The effect of egg mass on the growth and survival of black birds: a field experiment. *J. Zool.*, **227**: 639-653.
- Meijer, T. Masman, D. and Daan, S. 1989. Energetics of reproduction in female kestrels. *Auk*, **106**: 549-559.
- Mousseau, T. A. and Fox, C. W. 1998. *Maternal effects as adaptations*. Oxford University Press, New York.
- Nisbet, I.T.C. 1978. Dependence of fledgling success on egg size, parental performance and egg composition among Common and Roseate Terns, *Sterna Hirundo* and *S. dougallii*. *Ibis*, **120**: 207-215.
- Parsons, J. 1970. Relationship between egg size and post-hatching chick mortality in the Herring



- Gull (*Larus argentatus*). *Nature*, **228**: 1221-1222.
- Petrie, M., H. Schawbl, N. Brande-Lavridsen, and T. Burke. 2001. Sex differences in avian yolk hormone levels. *Nature*, **412**: 498.
- Potti, J. 1999. Maternal effects and the pervasive impact of nestling history on egg size in a passerine bird. *Evolution*, **53**: 279-285.
- Potti, J. 1993. Environmental, ontogenic, and genetic variation in egg size of Pied Flycatchers. *Canadian J. Zool.*, **71**: 1534-1542.
- Reynolds, S.J., Schoech, S.J. and Bowman, R. 2003. Nutritional quality of prebreeding diet influences breeding performance of the Florida scrub-jay. *Oecologia*, **134**: 308-316.
- Roff, D. A. 2002. *Life History Evolution*. Sinauer Associates Inc., Sunderland.
- Saino, N.; Ferrari, R. P.; Martinelli, R.; Romano, M.; Ruboloni, D. and Møller, A. P. 2002. Early maternal effects mediated by immunity depend on sexual ornamentation of the male partner. *Proc. R. Soc. Lond. B.*, **269**: 1005-1009.
- Saino, N.; Calza, S. and Møller, A. P. 1998. Effects of a dipteran ectoparasites on immune response and growth trade-offs in barn swallow, *Hirundo rustica*, nestlings. *Oikos*, **81**: 217-228.
- Smith, H. G. and Bruun, M. 1998. The effect of egg size and habitat on starling nestling growth and survival. *Oecologia*, **115**: 59-63.
- Smits, J. E.; Bortolotti, G. R. and Tella, J. L. Simplifying the phytohaematoglutinin skin-testing technique in studies of avian immunocompetence. *Funct. Ecol.*, **13**: 567-572.
- Sinervo, B. and Huey, R.B. 1990. Allometric engineering: an experimental test of the causes of interpopulation differences in performance. *Science*, **248**: 1106-1109.
- Sockman, K.W. and Schwabl, H. 2000. Yolk androgens reduce offspring survival. *Proc. R. Soc. Lond. B.*, **267**: 1451-1456.
- Soler, M.; Martín-Vivaldi, M.; Marín, J. M. and Møller, A. P. 1999. Weight lifting and health status in the black wheatear. *Behav. Ecol*, **10**: 281-286.
- Soler, J.J.; Moreno, S. and Potti, J. 2003. Environmental, genetic and maternal components of immunocompetence of nestling pied flycatchers from a cross-fostering study. *Evol. Ecol. Res.*, **5**: 259-272.
- Sorci, S.; Soler, M and Møller, A. P. 1997. Reduced immunocompetence of nestlings in replacement clutches of the European magpies (*Pica pica*). *Behav. Ecol.*, **10**: 281-286.
- Stoleson, S.H. and Beisinger, S.R. 1995. Hatching asynchrony and the onset of incubation in birds, revisited. When is the critical period?. *Current Ornithology*, **12**: 191-270.
- Starck, J. M. and Ricklefs, R. E.. 1998. *Avian growth and development. Evolution within the Altricial-precocial spectrum*. Oxford University Press, New York.
- Sterns, S. C. 1992. *The evolution of life histories*. Oxford University Press.
- Tella, J. L.; Bortolotti, G.; Dawson, R. D. and Forero, M. G. 2000a. The T-cell-mediated immune response and return rate of fledgling American kestrel are positively correlated with parental clutch size. *Proc. R. Soc. Lond. B.*, **267**: 891-895.
- Tella, J. L.; Bortolotti, G.; Forero, M. G. and Dawson, R. D. 2000b. Environmental and genetic variation in T-cell-mediated immune response of fledgling American kestrels. *Oecologia*, **123**: 453-459.
- Tella, J. L.; Blanco, G.; Forero, M. G.; Gajón, A.; Donazar, J. A. and Hiraldo, F. 1998. Habitat, world geographic range, and embryonic development of hosts explain the prevalence of avian haematzoa at small spatial and phylogenetic scales. *Proc. Natl. Acad. Sci. USA*, **96**: 1785-1789.
- Tschirren, B., Fitze, P.S. and Richner, H. (2003) Sexual dimorphism in susceptibility to parasites and cell-mediated immunity in great tit nestlings. *J. Anim. Ecol.*, **72**: 839-845.
- Tullet, S.G. and Burton, F.G. 1982. Factors affecting the weight and water status of the chick at hatch. *Brit. Poultry. Sci.*, **23**: 361-369.
- Viñuela, J. 1997. Adaptation vs. constraint: intraclutch egg-mass variation in birds. *J. Anim. Ecol.*, **66**: 781-792.
- Wiebe K.L.; Wiehn J. and Korpimäki E. 1998. The onset of incubation in birds: can females

control hatching patterns?. *Anim. Behav.* **55**:  
1043-1052.

Williams, T. D. 1994. Intraspecific variation in  
egg size and egg composition in birds: effects on  
offspring fitness. *Biol. Rev.*, **68**: 35-59.

## CAPÍTULO IV

### **Los parásitos intestinales no están relacionados con los parámetros reproductivos ni con la respuesta inmune de los pollos en una población silvestre de Cernícalo vulgar (*Falco tinnunculus*)**

Este capítulo reproduce íntegramente el texto del siguiente manuscrito:

**Jesús Martínez-Padilla and Javier Millán.** *En revisión.* Intestinal parasites is not related with reproductive parameters and nestling immune response in wild Eurasian Kestrels *Falco tinnunculus*. *Ibis*.

#### **Resumen del Capítulo**

Las asociaciones parásito-hospedador son una de las relaciones ecológicas más comunes en la naturaleza. Diferentes estudios de aves han estudiado estas relaciones teniendo en cuenta ectoparásitos y hematozoos de los que se infiere un efecto negativo sobre el crecimiento, supervivencia o éxito reproductivo. Sin embargo, pocos estudios en poblaciones silvestres se han llevado a cabo que consideren parásitos intestinales, especialmente en aves rapaces donde se ha visto que este tipo de parásitos tiene importantes efectos en individuos en cautividad. Por ello, en este capítulo hemos realizado análisis coprológicos de 71 Cernícalo vulgar (*Falco tinnunculus*) de una población silvestre reproductora en el Sistema central. Analizamos las relaciones entre presencia y ausencia, junto al efecto de la abundancia de propágulos de este tipo de parásitos y diferentes parámetros reproductivos y medidas de la calidad de los pollos. Además, estudiamos cómo se relaciona la parasitación de los volantones con la respuesta inmune celular mediada por linfocitos T, medida como la reacción frente a la inyección de fitohematoglutina. Detectamos que el 59.2% de los pollos excretaron ooquistes de coccidios del género *Caryospora* sp. La prevalencia estuvo relacionada con el tamaño de nidada y la intensidad de infección varió entre años. Sugerimos que el topillo campesino (*Microtus arvalis*) podría jugar un importante papel en la transmisión de este parásito, por que éstos han sido descritos como hospedadores paraténicos de *Caryospora* sp. Paralelamente, no encontramos relación significativa entre la infección por *Caryospora* y la respuesta inmune celular mediada por linfocitos T, lo que sugiere un escaso efecto sobre la condición del volantón, aunque se sugiere no se descartar posibles efectos tras la emancipación del nido. Estos resultados sugieren una alta prevalencia de *Caryospora* sp. en la población de estudio, por lo que sugerimos que sería conveniente diseñar experimentos para conocer los efectos que este parásito pueda tener el fitness de los volantones.



# Intestinal parasitism is not related with reproductive parameters and nestling immune response in wild Eurasian kestrels *Falco tinnunculus*.

JESÚS MARTÍNEZ-PADILLA AND JAVIER MILLÁN. *Instituto de Investigación en Recursos Cinegéticos (IREC, CSIC - UCLM - JCCLM), PO Box 535, E-13080 Ciudad Real, Spain.*

## ABSTRACT

Host-parasite associations are among the most common ecological relationships in nature. Most avian studies have focussed on ectoparasites and haematzoa suggesting a negative effect on growth, survival or breeding success. However, studies of wild populations have not taken into account intestinal parasites. We have conducted a coprological survey of samples from 71 wild Eurasian kestrel (*Falco tinnunculus*) nestlings in central Spain and analysed the relationships between presence and abundance of parasite propagules and different reproductive parameters and measures of nestling quality. We have also studied whether parasitization was related to nestling T-cell mediated immune response, measured by the phytohaemagglutinin injection assay. We found that 59.2% of chicks excreted *Caryospora* sp. oocysts. Prevalence was related positively to brood size (as expected in direct life-cycle parasites) and the intensity of infection differed between years. We suggest that *Microtus* voles could play an important role in the transmission of this parasite to nestlings, because they have been described as paratenic host of *Caryospora* sp. We did not find a relationship with T-cell-mediated immunity, suggesting weak effects of the parasite on nestling condition. Our findings show a high prevalence of *Caryospora* sp. in our population, so we suggest that more studies are needed in order to know the effects of this parasite on fledgling fitness.

Key words: *Falco tinnunculus*, fledglings, *Caryospora* spp., immune response, brood size, year.

## INTRODUCTION

---

Interactions between parasites and their hosts are among the most common ecological relationships in nature. By definition, parasitism represents a reduction of some component of host fitness (Rigby & Moret, 2000). Several studies conducted in last two decades have detected in this order relationships between parasites and sexual selection (Hamilton & Zuk, 1982), reproductive output (Merino & Potti, 1995), behaviour (Hart, 1997), physiology (Folstad & Karter, 1992), or survival (Millán et al. 2001) meaning an increased understanding about parasite influence on different

reproductive traits, both on parents and their descendants. Most studies on host-parasite relationships in birds have been conducted on blood parasites or ectoparasites (e.g. Merino & Potti 1995, Merino et al. 2000). Intestinal parasites have received less attention, although they have been suggested to cause important fitness reductions for their hosts. For example, helminths probably are a key factor in the regulation of red grouse (*Lagopus lagopus scoticus*) populations (Hudson et al. 1998). In addition, microparasites have been also cited affecting on carotenoids absorption with consequences for plumage pigmentation of house finches (*Carpodacus*

*mexicanus*, Brawner, et al. 2000). However, little is known about their effects on wild raptors populations, despite they having been cited as showing high infections by intestinal parasites, especially coccidia, in captive raptors conditions (Forbes & Simpson, 1997; Upton, 1990). Thus, the distribution and significance of *Caryospora* in free-living birds of prey remains unclear (Krone, 2002).

In this study, we measured the parasite propagule excretion in a wild population of the Eurasian kestrel (*Falco tinnunculus*, kestrel hereafter), a medium size raptor. We focussed on the potential effect that this parasite may have on fledgling physiology because it should affect their growth, development and future survival (see Clayton & Moore, 1997). In addition, because parasites affect negatively the condition and immune response of their hosts in early life (Møller, 1997) and linked to the high virulence of some intestinal parasites that could cause death in wild birds (Krone, 2002), we could expect that intestinal parasite infection influences fledgling immunity. Therefore, the aims of this study are: (1) estimate the prevalence and abundance of parasite propagule excretion in wild Eurasian kestrel fledglings, (2) explore the association of this excretion with different reproductive traits, and (3) study the relation between such excretion and fledgling immunity, where we hypothesise that kestrel immunity should decrease in parasitized birds.

## **MATERIAL AND METHODS.**

---

### **Field procedures.**

The study was carried out in the breeding seasons of 2001 and 2002 in the Campo Azálvaro region, a mountain

grassland in central Spain, with kestrels nestlings. In the study area, kestrels breed in nest-boxes provided by us since 1994 (see Fargallo, et al. 2001 for more details). Breeding kestrels have a wide diet, although it preys mainly on common voles (*Microtus arvalis*) in the study area (unpublished data).

Kestrel pairs were monitored in order to know different reproductive traits such as clutch size, laying date and number of fledglings. We visited all nests every two days before laying, so laying order of eggs was known. When eggs were laid we measured them with callipers to the nearest 0.01mm and weighed them to the nearest 0.25g. We controlled the hatching process, so we accurately knew hatching date, hatching order and number of hatchlings. When nestlings were 25 – 26 days (counted after the first egg hatched), we measured their wing and tarsus lengths to the nearest mm and weighed them to the nearest g. Fledgling size was defined as wing length and fledgling size differences was defined as the differences in wing length between each chick and their largest sibling. We considered fledgling growth as the difference between chick mass at hatching and fledgling (25 d). Nestling sex was determined by molecular procedures (Fridolfsson & Ellegren 1999) using DNA extracted from a drop of blood obtained by brachial venipuncture. We collected faecal samples of chicks when they were 25 – 26 d. Each chick was individually placed on a clean one-use cotton piece, allowing us to identify each faecal sample produced while their siblings were measured and weighed. Faecal samples were kept in a field refrigerator and analysed as soon as possible by the zinc sulfate flotation technique and counted in a MacMaster chamber. Measurements are expressed as oocysts/g of

faecal mass. Propagules were identified following Melhorn et al. (1992). Abundances and prevalences were calculated following Rózsa et al. (2000). The parasitological terms used are based on Bush *et al.* (1997).

### **Immune response.**

Evaluation of immune function has been recognized as a valuable tool in avian ecological studies. Twenty four days after hatching, kestrel chicks were injected intradermally in the left wing web with 0.1ml of a solution of Phytohaematogglutinin PHA (3mg of PHA in 1ml of PBS) after measuring its thickness at the point of injection. 24h after injection we measured again wing web thickness and we considered the difference between initial and final measurement as immune response (Smits, *et al.* 1999). Three measures were taken with a digital spessimeter (Mitutoyo Absolute ID-112B) to the nearest 0.01mm before and after injection. The repeatabilities of initial ( $F_{69,140} = 180.86$ ,  $p < 0.0001$ ) and final ( $F_{69,140} = 117.32$ ,  $p < 0.0001$ ) measurements were high, so we used mean values of it. All measurements were taken by Martínez-Padilla with the same spessimeter each year.

### **Statistical procedures.**

Logistic regression was performed to study the relationships between reproductive parameters of nests and presence-absence of infection. We considered clutch size, sex ratio (proportion of males in a brood), number of chicks that died, laying date, average fledgling mass, mass hierarchy, year and brood size as independent variables and nest infection as dependent variable (1, nest with at least one chick infected; 0, no

chicks infected in the brood). In addition, two models were conducted to assess the effects of parasite infection. (1) To explore individual traits of fledglings, we performed a generalised model (GENMOD procedure in SAS) with nest as factor and chick infection as dependent variable (1, infected chick; 0, uninfected chick) with binomial negative error and log link function. We consider fledgling size, fledgling size differences, fledgling growth and fledgling mass as independent variables and fledgling sex as factor. Fledgling sex in relation to parasite infection was explored with Chi-square tests. (2) In addition, a mixed model (GENMOD procedure in SAS) was performed to explore the relationships between immune response and parasite infection. Because immune response showed a normal distribution, we used normal distribution of errors and an identity link function (GLIMMIX macro of SAS, Littell et al. 1996). Immune response was introduced as dependent variable and *Caryospora* infection and prevalence as covariate and factor respectively in mixed models where nest was considered as random term, allowing us to consider values of each chick as sample unit and avoiding pseudoreplication. Because we used data for two different years where the same nest was occupied, nest was nested in year as random variable to control for the particular characteristics of each nest to avoid pseudoreplication.

## **RESULTS**

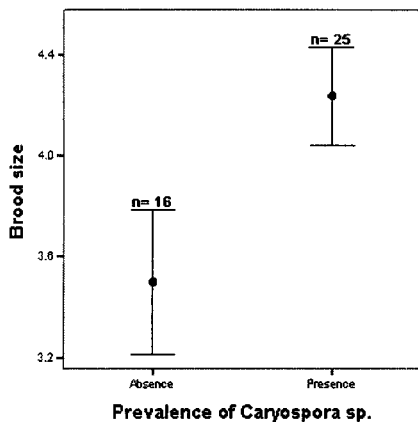
---

### **Intestinal parasites**

We analysed 71 kestrel chicks ( $n = 24$ , 2001;  $n = 47$ , 2002) from 41 different nests ( $n = 15$ ;  $n = 26$ , in 2001 and 2002 respectively), where 42 (59.2%) were

Variable	B	SE	Wald	Df	p
Clutch size	-0.0847	0.6349	0.0178	1	0.8939
Sex ratio	-0.5215	1.4354	0.1320	1	0.7163
Chicks died	-0.9219	0.8020	1.3214	1	0.2503
Laying date	-0.0420	0.0462	0.8269	1	0.3632
Fledgling mass	0.0265	0.0232	1.3046	1	0.2534
Mass hierarchy	-0.0297	0.0184	2.6024	1	0.1067
Year	1.4422	0.8017	3.2359	1	0.0720
<b>Brood size</b>	<b>0.6925</b>	<b>0.3442</b>	<b>4.0464</b>	<b>1</b>	<b>0.0443</b>

**Table 1.** Results of logistic regression on presence-absence of infection by *Caryospora* sp. in kestrel nests. The model assumed 65.85% of the overall variance.



**Figure 1.** Mean  $\pm$  SE of number of fledglings in broods according to infection by *Caryospora* sp. Numbers above bars are sample sizes.

infected by *Caryospora* sp. and two fledglings were infected by *Eimeria* sp. Mean number of oocysts of *Caryospora* sp./g of faeces was  $2991.36 \pm 5838.28$ . Considering infection as presence-absence in nests (see methods), logistic regression was performed in order to know the influence of *Caryospora* infection on different reproductive traits related to the nest. The model showed that the number of fledglings in a brood was the only variable that was retained, assuming 65.85% of the overall variance. Infected nests had a higher number of fledglings than uninfected ones (Table 1; Figure 1). Neither clutch size,

laying date, sex-ratio, mass hierarchy, mean fledgling body mass, chicks died nor year was related to *Caryospora* infection (Table 1). However, considering the abundance of *Caryospora* oocyst excretion, the GLM suggests that year was only the variable related to the intensity of parasite infection. Mean abundance was  $455.6 \pm 849.1$  in 2001 and  $4286.2 \pm 6806.9$  in 2002 (Figure 2. Table 2).

A GLIMMIX model suggests that immune response was not related to abundance of oocyst excretion ( $F_{1,27} = 0.02$ ,  $p = 0.893$ , fledgling mass as covariate in the model  $F_{1,27} = 5.01$ ,  $p = 0.034$ ) or prevalence of *Caryospora* infection ( $F_{1,27} = 0.02$ ,  $p = 0.894$ , controlling for the effect of fledgling mass  $F_{1,27} = 5.09$ ,  $p = 0.032$ ). Other fledgling variables such as fledgling mass, fledgling growth, fledgling size or size differences were not related to parasite infection (all  $p > 0.394$ , where random effect was controlled, all  $p > 0.006$ ). We did not find differences between males and females in parasite infection ( $\chi^2 = 0.876$ ).

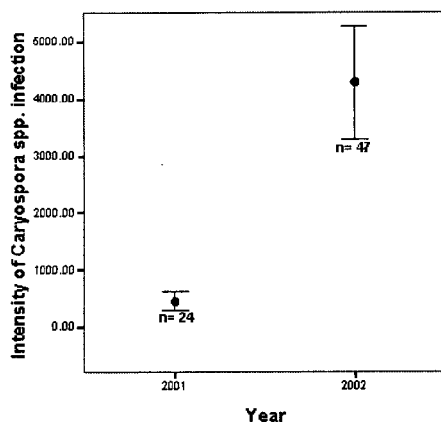
## DISCUSSION

To the best of our knowledge, this is the first study that explores intestinal parasite infestation in wild Eurasian kestrel nestlings. The analysis of parasite propagules in excreta is of special interest



Response term	Explanatory term	Rejected term	F	Df.	P
Abundance of <i>Caryospora</i> infection					
	Year		5.24	1,29	<b>0.0296</b>
		Laying date	1.51	1,27	0.2299
		Size differences	1.36	1,26	0.2537
		Fledgling mass	0.64	1,24	0.4283
		Sex	0.38	1,23	0.5421
		Fledgling mass gain	0.17	1,21	0.6853
		Laying order	0.90	2,19	0.4251
		Fledgling mass	0.03	1,18	0.8616
		Hatchling order	0.12	2,15	0.8916

**Table 2.** GLMM with negative binomial error and log link function of abundance of *Caryospora* infection in Eurasian Kestrel nestlings. Hatching order and laying order, were encoded as first, middle and last laid/hatched eggs. Other potentially influencing variables and the remaining interactions between the variables were not significant ( $P > 0.345$ ).



**Figure 2.** Mean  $\pm$  SE of intensity of *Caryospora* spp. infection in Eurasian kestrel fledglings related to years of study. Note that the graph results from univariate analysis, for multivariate analysis see methods and results. Numbers under bars are sample sizes.

because it enables non-destructive monitoring of wild animal health (Watve & Sukumar 1995). Propagules from faeces have been useful for evaluating interactions and heterogeneity in levels of infection among hosts at a small scale (e.g. Müller-Graf et al 1996; Millán et al. 2002).

*Caryospora* is the third largest coccidian genus from the family Eimeriidae. It is especially present in raptors and snakes, which are suggested as facultatively in development (Upton, 1990). Previous studies on effects of *Caryospora* sp. have been aimed at describing new species of parasites in this genus (Upton, 1990), physiological effects on their hosts (Krone, 2002; Vítovec, et al. 1997; Koudela, et al. 2000) or at describing its life cycle (Volf, et al. 2001), always in captive birds, snakes or mammals (see for instance Vítovec, et al. 1997; Upton & Barnard, 1988), although neither study related *Caryospora* oocysts excretion to reproductive parameters. Our results suggest that brood size at fledging is positively correlated with prevalence of excretion. *Caryospora* are monoxenous parasites. As observed by Simberloff and Moore (1997), host density influences disease transmission, with directly transmitted parasites being more sensitive to host density. For example, in bobwhite quail (*Colinus virginianus*), directly transmitted helminths with short life cycle were related to social group size (Moore, et al. 1988).

However, although little is known about the life-cycle of *Caryospora* sp. in wild conditions, recent studies suggest that common voles (*Microtus arvalis*) could act as a paratenic host of these parasites (Volf, et al. 2001). Other species could be hosts for *Caryospora* sp., mainly reptiles (Koudela, 1993; Matuschka, 1984; Modrý & Koudela, 1994; Telford, 1997; Doszak & Stanley, 2001), that are common prey in our kestrel population. However, the second important prey in our study area were lizards (*Lacerta lepida*), but *Caryospora* sp. were not found in their faecal samples (manuscript in prep.).

Therefore, common voles could represent a key factor to understand the variation about intensity of infection because this vole is one of the most important prey in our kestrel population, representing about 30% of the total prey biomass (unpublished data). Dijkstra et al. (1990) suggest that parental effort increases with brood size in kestrels, and Wiehn & Korpomäki (1997) suggest the same trend for females Finish kestrels under food shortage. Therefore, a higher hunting effort could imply a higher probability of capturing infected voles and consequently transmitting the parasite to their offspring. Parent kestrels with larger broods need high quality prey items such as common voles to provision their nestlings. In addition, a higher number of siblings in a brood could improve the temperature and humidity conditions in the nest for growth and development of parasites. Moreover, a higher number of nestlings in a brood could increase competition for resources, and thereby stress. Nestlings could be more vulnerable to parasite infection in stressful situations due to immunosuppression. The association of infection with large broods may imply a trade-off between nestling

quantity and quality as envisaged by Lack (1954).

The prevalence of excretion differed between years. In relation with other studies, we estimated abundances of *Microtus* voles, lizards and birds, and no variation was found in their abundance between study years (unpublished data). So we suggest that weather could be a factor that modulates populations of intestinal parasites in the wild (Merino & Potti, 1996).

Apparently, *Caryospora* sp. infection was not related to the T-cell mediated immune response against a novel challenge, at least in early life. The response to PHA has been correlated with condition in many studies (Alonso-Álvarez & Tella, 2001). The absence of detectable effects of *Caryospora* on immune response suggests that effects of the parasite on nestling physiology and general condition are weak, although other components of the immune system could reflect the effect of parasites in fledgling fitness, as recent study suggest (Buchanan, et al. 2003). However, effects of parasite infection could be increased in postfledgling period, because that is the moment in which *Caryospora* infection seems to be more virulent (Krone, 2002). Therefore, the effect of *Caryospora* infection in kestrel fitness could emerge after fledgling.

In conclusion, we did not find effects of *Caryospora* infection in reproductive parameters of wild kestrels at least in nestling stage. Therefore, more experimental studies are needed in order to generate better information about the effect of a parasitization with *Caryospora* or other gastrointestinal parasites, in relation with (1) determining physiological effects in our kestrel populations, and (2) establishing

their effects in kestrels populations where *Microtus* voles suffer cyclic periods of abundance.

## ACKNOWLEDGEMENTS

---

We thank the Finat family for kindly allowing us to conduct the study in their property, and J. A. Fargallo, C. Marqués and J. San Teodoro for their collaboration in fieldwork. We thank J. Viñuela, G. Blanco and J. Moreno for their support to conduct the study, for their valuable comments on early draft and for financed the study. J. Moreno financed partially the study through the project BOS 2001-0587-MCyT.

## REFERENCES

---

- Alonso-Álvarez, C. & Tella, J.L. 2001. Effects of experimental food restriction and body-mass changes on the avian T-cell-mediated immune response. *Canadian Journal of Zoology*, **79**: 101 – 105.
- Bush, A.O.; Lafferty, K.D.; Lotz J.M., & Shostak, A.W. 1997. Parasitology meets ecology on its own terms: Margolis *et al.* revisited. *J. Parasitol.* **83**: 575-583.
- Brawner III, W. R.; Hill, G. E. & Sunderman, C. A. 2000. Effects of coccidial and mycoplasmal infections on carotenoids-based plumage pigmentation in male house finches. *The Auk*, **117**: 952-963.
- Buchanan, K.L., Evans, M.R. & Goldsmith, A.R. In press. Testosterone, dominance signalling and immunosuppression in the house sparrow, *Passer domesticus*. *Behavioral Ecology and Sociobiology*.
- Clayton, D. & Moore, J (eds). 1997. Host-parasite evolution: general principles and avian models. Oxford University Press. Oxford.
- Dijkstra, C.; Bult, A.; Bijlsma, S.; Daan, S.; Meijer, T. and Zijlstra, M. 1990. Brood size manipulation in the Kestrel (*Falco tinnunculus*): effects on offspring and parent survival. *Journal of Animal Ecology*, **59**: 269-285.
- Doszak, P. & Satanley, J. B. 2001. A description of two new species of coccidian (Apicomplexa: Eimeriidae) from African reptiles with nomenclatural corrections for two *Caryospora* and one *Eimeria* species from snakes. *Folia Parasitologica*, **48** (1): 1-6.
- Fargallo, J. A., G. Blanco, J. Potti, J. & Viñuela, J. 2001. Nestbox provisioning in a rural population of Eurasian Kestrels: breeding performance, nest predation and parasitism. *Bird Study*, **48**: 236-244.
- Folstad, I. & Karter, A. J. 1992. Parasites, bright males and the immunocompetence handicap. *American Naturalist*, **139**: 603 – 622.
- Forbes, N. A. & Simpson, G. N. 1997. *Caryospora neofalconis*: an emerging threat to captive-bred raptors in the United of Kingdom. *J. Avian Med. Surg.*, **11**: 110 – 114.
- Fridolfsson, A. K. & Ellegren, H. 1999. A simple and universal method for molecular sexing of non-ratite birds. *Journal of Avian Biology*, **30**: 116-121.
- Hamilton, W. D. & Zuk, M. 1982. Heritable true fitness and bright birds: A role for parasites?. *Science*, **218**: 384 – 387.
- Hart, B. J. 1997. Behavioral defence. In D. H. Calyton and J. Moore (Eds.): *Host-parasite evolution. General principles and avian models*. Pp: 59 – 77. Oxford University Press. Oxford.
- Hudson, P.J., Dobson, A.P. & Newborn, D. 1998. Prevention of population cycles by parasite removal. *Science*, **282**: 2256-2258.
- Koudela, B.; Modrý, D.; Volf, J. & Šlapeta, J. R. 2000. SCID mice as tool for evaluation of heteroxenous life cycle pattern of *Caryospora* (Apicomplexa, Eimeriidae) species. *Veterinary Parasitology*, **92**: 191-198.
- Koudela, B. 1993. Experimental transmission of *Caryospora simplex* Watcha et Christiansen, 1982 (Apicomplexa: Eimeriidae) from rattlesnake, *Crotalus atrox*, to rodents and pigs. *Folia parasitologica*, **40**: 81-84.
- Krone, O. 2002. Fatal *Caryospora* infection in a free-living juvenile Eurasian kestrel (*Falco tinnunculus*). *Journal of Raptor Research*, **36**(1): 84-86.

- Lack, D. (1954) The natural regulation of animal numbers. Oxford University Press.
- Littell, R.C.; Milliken, G.A.; Stroup, W.W. & Wolfinger, R.D. 1996. *SAS system for mixed models*. SAS Institute. Cary, New York.
- Matuschka, F. R. 1984. Description of *Caryospora colubris*, sp. n. (Apicomplexa: Eimeriidae) from western whip snake, *Coluber viridiflavus* (Serpentes: Colubridae). *Can. J. Zool.*, **62**: 1525-1527.
- Melhorn, H.; Düwell, D. & Raether, W. 1992. *Atlas de Parasitología Veterinaria*. GRASS Editions, Spain.
- Merino, S., Moreno, J., Sanz, J.J. & Arriero, E. 2000. Are avian blood parasites pathogenic in the wild? A medication experiment in blue tits (*Parus caeruleus*). *Proceedings of the Royal Society of London B.*, **267**: 2507-2510.
- Merino, S. & Potti, J. 1996. Weather dependent effects of nest ectoparasites on their bird hosts. *Ecography*, **19**: 107-113.
- Merino, S. & Potti, J. 1995. Mites and blowflies decrease growth and survival in nestling pied flycatchers. *Oikos*, **73**: 95-103.
- Millán, J., Gortázar, C., Tizzani, P., & Buenestado, F.J. 2002. Do helminths increase the vulnerability of released pheasants to fox predation?. *Journal of Helminthology*, **76**: 225-229.
- Modrý, D. & Koudela, B. 1994. *Caryospora ahaetullae* sp. n. (Apicomplexa: Eimeriidae) from the long nosed vine snake, *Ahaetulla nasuta* (Serpentes: Colubridae). *Folia Parasitol.*, **41**: 233 – 235.
- Møller, A. P. 1997. Parasitism and the evolution of host life history. In: Clayton DH, Moore, J. (eds) *Host-parasite evolution: general principles and avian models*. Oxford University Press, Oxford, pp: 105 – 127.
- Moore, J., Simberloff, D., and Freehling, M. (1988) Relationships between bobwhite quail social group size and intestinal helminth parasitism. *American Naturalist*, **131**: 22-32.
- Müller-Graf, C.D.M., D.A. Collins, M.E.J. Woolhouse 1996. Intestinal parasite burden in five troops of olive baboons (*Papio cynocephalus anubis*) in Gombe Stream National Park, Tanzania. *Parasitology*, **112**: 489-497.
- Rigby, M. C. & Moret, Y. 2000. Life-history trade-offs with immune defenses. In *Evolutionary Biology of Host-Parasite: Theory Meets Reality*. P. Roulin, S. Morand, and A. Skorping (Eds.). Elsevier Science B. V.
- Rózsa, L.; Reiczigel, J. & Majoros, G. 2000. Quantifying parasites in samples of hosts. *J. Parasitol.* **86**: 228-232.
- Simberloff, D., Moore, J. (1997): Community ecology of parasites and free-living animals. In Clayton D.H. and Moore J. (Eds): *Host-parasite evolution: general principles and avian models*, Oxford University Press, Oxford, UK, pp. 174-197.
- Smits, J. E.; Bortolotti, G. R. & Tella, J. L. Simplifying the phytohaematoglutinin skin-testing technique in studies of avian immunocompetence. *Functional ecology*, **13**: 567-572.
- Telford, S. R. 1997. Coccidian parasites (Apicomplexa: Eimeriidae) of endemic Florida snake *Tantilla relicta* Telford (Serpentes: Colubridae). *Systematic Parasitology*, **36** (1): 17-25.
- Upton, S. J.; Campbell, M.; Weigel, M. & McKnown, R. D. 1990. The Eimeriidae (Apicomplexa) of raptors: review of the literature and description of new species of the genera *Caryospora* and *Eimeria*. *Can. J. Zool.*, **68**: 1256 – 1265.
- Upton, S. J. & Barnard, S. M. 1988. Development of *Caryospora bigenica* (Apicomplexa: Eimeriidae) in experimentally infected mice. *Int. J. Parasitol.*, **18**: 15 -20.
- Vitovec, J.; Koudela, B. and Modrý, D. 1997. Pathogenicity of experimental caryosporosis. *International Journal for Parasitology*, **27**(7): 819 – 824.
- Volf, J.; Modrý, D. & Koudela, B. 2001. Experimental transmission of *Caryospora kutzery* (Apicomplexa: Eimeriidae) by rodent hosts. *Folia parasitologica*, **48**: 11- 14.
- Watve, M.G. & R. Sukumar. 1995. Parasite abundance and diversity in mammals: Correlates

with host ecology. *Proc. Natl. Acad. Sci. USA.*,  
**92**: 8945-8949.

Wiehn, J. & Korpimäki, E. 1997. Food  
limitation on brood size: experimental evidence  
in the Eurasian kestrel. *Ecology*, **78**: 2043-2050.



## CAPÍTULO V.

### **Jerarquías de tamaño en la nidada, sexo y parásitos determinan el estrés en volantones de Cernícalo vulgar (*Falco tinnunculus*): un estudio con proteínas de estrés térmico.**

Este capítulo reproduce íntegramente el texto del siguiente manuscrito:

**Martínez-Padilla, J., Martínez, J., Dávila, J. A., Merino, S., Moreno, J. and Millán, J.** *En revisión*. Brood hierarchies, sex and parasites determine stress in Eurasian kestrel (*Falco tinnunculus*) nestlings: a study of heat-shock proteins. *Functional Ecology*.

#### **Resumen del Capítulo**

Las jerarquías de tamaño establecidas a través de la asincronía de eclosión se supone que son costosas para los últimos pollos eclosionados debido a la desventaja que se genera en la eclosión en relación al tamaño y la supervivencia. Un coste adicional que no a sido explorado en la actualidad es el estrés impuesto por la competencia entre los pollos por los recursos alimenticios en el nido. En este capítulo son estudiadas nidadas reproductoras de Cernícalo vulgar (*Falco tinnunculus*) en España. Para ello hemos empleado los niveles de proteínas de estrés térmico (Heat Shock Proteins, HSP's) o proteínas de estrés de los tipos HSP60 y HSP70, así como la relación heterófilos/linfocitos para detectar estrés en pollos justo antes de su emancipación, a la edad de 24 días. Dadas las implicaciones del dimorfismo sexual invertido en rapaces en general y en el Cernícalo en particular, por la competencia en el nido frente a sus hermanos y conocidas el efecto estresante de los parásitos en otras especies, tanto el sexo de los pollos (identificado a través de sexado molecular) y la infección por parásitos intestinales del género *Caryospora sp.*, ambos factores fueron considerados en los análisis. En primer lugar, obtuvimos que aquellos pollos con mayores diferencias en tamaño con respecto a su hermano mayor (estimadas como diferencias en el tamaño del ala), mostraron unos niveles mayores de proteínas de estrés y este efecto fue mayor para los pollos hembra como sugiere la interacción estadísticamente significativa entre la diferencia en tamaño y el sexo de los pollos. Además, aquellas nidadas en las que el pollo mayor fue una hembra mostraron mayores niveles de HSP60 que aquellas en las que el pollo mayor fue macho. Por último, el modelo detectó que los pollos infectados por *Caryospora sp.* También tuvieron mayores niveles de estrés, así como los pollos de puestas tardías. No se observaron, sin embargo y contrariamente a recientes estudios, relación entre las proteínas de estrés y la relación heterófilo/linfocito, lo que podría sugerir que éste último es un peor indicador de estrés. Por tanto, el estrés en volantones de cernícalo está fuertemente determinado por su posición jerárquica dentro de la nidada, y hembras en desventaja en la jerarquía sufren están más estresadas que los machos previsiblemente por sus mayores requerimientos energéticos para crecer. Las proteínas de estrés y especialmente las HSP60, se revelan como unos indicadores adecuados estrés, especialmente aquellos mediados por la competencia entre

hermanos por los recursos así como estimador de estrés por la infección parasitaria en aves en poblaciones naturales. Se sugiere, por tanto, que este tipo de estimador de estrés debería ser incluido en futuros análisis de las repercusiones en el fitness de los pollos debido a la competencia mediada por la jerarquía de tamaños en nidadas de aves.



# Brood hierarchies, sex and parasites determine stress in Eurasian kestrel (*Falco tinnunculus*) nestlings: a study of heat-shock proteins.

JESÚS MARTÍNEZ-PADILLA<sup>1</sup>, JAVIER MARTÍNEZ<sup>2,3</sup>, JOSÉ A. DÁVILA<sup>1</sup>, SANTIAGO MERINO<sup>3</sup>, JUAN MORENO<sup>3</sup> AND JAVIER MILLÁN<sup>1</sup>. <sup>1</sup>*Instituto de Investigación en Recursos Cinegéticos (IREC, CSIC - UCLM - JCCLM), PO Box 535, E-13080 Ciudad Real, Spain.* <sup>2</sup>*Departamento de Microbiología y Parasitología, Facultad de Farmacia, Universidad de Alcalá E-28871 Alcalá de Henares, Spain.* <sup>3</sup>*Museo Nacional de Ciencias Naturales, CSIC, C/ José Gutiérrez Abascal, 2 E-28006 Madrid, Spain.*

## ABSTRACT

Brood hierarchies established through hatching asynchrony are supposed to be costly for small chicks due to impaired growth and survival. An additional cost that has remained unexplored is the stress imposed by competition for resources in the nest. In the present study of broods of Eurasian kestrels, we have used the level of heat-shock proteins like HSP60 and HSP70 in peripheral blood as well as the heterophil/lymphocyte ratio to detect stress in nestlings. The sex of nestlings and their *Caryospora* sp. oocyst excretion were included in analyses. Nestlings showing a large size difference with respect to their largest sibling had higher levels of both stress proteins, and this effect was stronger for female chicks as indicated by a significant interaction sex-size difference presumably due to their higher food requirements for growth. Nestlings for which the largest sibling was a female had higher levels of HSP60 than when it was a male. The H/L index was a much poorer predictor of competitive stress. Stress proteins, are effective estimators of competitive, nutritional and parasite-mediated stress in nestlings in the wild. The cost of sustained stress has to be included in future analyses of the fitness repercussions of dominance hierarchies in avian broods.

*Key words:* *Caryospora* sp., Eurasian kestrel, HSP60, sibling competition, hatching asynchrony.

## INTRODUCTION

Hatching asynchrony is a common trait in birds although its functional basis is still poorly understood (Stoleson & Beissinger, 1995). However, there is no doubt that the first consequence of hatching asynchrony for broods is the creation of a hierarchy in age and size at hatching. Thus, a competitive disadvantage is imposed by hatching asynchrony on the last hatched chicks. This handicap could be compensated through a more competitive disposition in last hatched chicks, enabling them to catch up with their larger siblings (Saino, *et al.* 2001). Competition in the nest may be mediated hormonally through a higher

hormone activity in small siblings (Nuñez-de la Mora, Drummond & Wingfield 1996). It has been suggested that females may use differential allocation of testosterone in last laid eggs in order to improve their capacity for sibling competition at hatching (Schwabl, 1993, but see Ellis, Borst & Thompson 2001). Growth compensation through aggressiveness in the nest may incur risks due to the stress effected by a higher competitive disposition. Although the costs of growth compensation are beginning to receive their due share of attention by ecologists (Metcalf & Monaghan 2001), little attention has yet been paid to the costs of maintaining high levels of competition towards siblings, although size hierarchies

imposed by hatching asynchrony have been proposed to be, at least in some species, including raptors, a mechanism to reduce sibling competition (Hahn, 1981; Wiebe & Bortolotti, 1994; Viñuela 1999).

The bird sex is one of the factors which may affect competitive disposition in nestling birds (Drummond *et al.*, 1991; Bortolotti, 1986). Additionally, differential mortality between male and female fledglings during the period of parental care has been found to favour survival of the smaller sex (Clutton-Brock, 1991). In reversed sexually dimorphic birds such as raptors, males may suffer in conditions of food shortage due to size-dependent capacity to compete for resources in the nest (Bortolotti, 1986; Anderson, *et al.* 1993; Arroyo, 2002; Fargallo *et al.* 2002; Fargallo *et al.* 2003). However, in another species with reversed sex dimorphism, last hatched females suffered a greater increase in mortality than last hatched males due to their greater food demands (Torres & Drummond, 1997). Thus, it is not obvious which sex should suffer more from stress given the implications of size both for competitive capacity and for food requirements. In any case, the relationship between within-brood size differences and fledgling sex may be reflected in stress conditions. To our knowledge, HSP has still not been employed as stress measure to describe such relationship. Another consequence of sexual size dimorphism in the nest may be that dominance hierarchies could depend on the sex of the larger siblings, with situations when the oldest sibling belongs to the larger sex being more stressful for its smaller broodmates.

Aggressive competition within broods may result in stress as expressed by higher

levels of circulating corticosterone (Nuñez-de la Mora, *et al.* 1996; Tarlow, Wikelski, & Anderson 2001; although see Sockman & Schwabl, 2001). Although corticosterone may represent a good stress indicator, its measurement in the wild is fraught with problems due to the rapid reactions of the endocrine system to capture and handling (Romero & Romero, 2002). Other non-destructive stress indicators that may not fluctuate as rapidly following handling of wild birds are the heterophil/lymphocyte ratio (H/L) and heat-shock protein levels in peripheral blood. The H/L is widely accepted as a reliable and accurate physiological indicator of stress in domestic birds (Maxwell 1993, Maxwell & Robertson 1998). Its relevance for studies of nutritional stress in wild avian populations has recently been explored (Hörak *et al.* 1999; Moreno *et al.* 2002). Stress proteins, traditionally called heat-shock proteins or HSPs, are evolutionarily highly conserved molecules that help cells in recovering from stress situations by correcting misconfigurations in protein structures (Morimoto 1991). HSP levels do not fluctuate as rapidly as hormone levels (see Burel, *et al.* 1992), and may thus be independent of capture and handling stress. Moreno *et al.* (2002) have observed that HSP levels are related to H/L and to nestling growth in a wild bird population. They detected a positive association between mean HSP level in broods and the within-brood variance in size traits, and suggested that HSP levels reflected the stress imposed by asymmetric sibling competition. In addition, parasitism has been revealed as an important source of stress (Møller, 1997), as shown by recent studies of HSP levels in relation to ectoparasite load and blood parasites (Merino, *et al.* 1998; Merino, *et al.* 2002).

However, in wild raptors little is known about the impact of intestinal parasites in different aspects of their ecology. Therefore, we studied ectoparasites, blood parasites and intestinal parasites in relation to stress proteins.

Here we have tried to explore four related questions in a wild population of Eurasian kestrels *Falco tinnunculus*. The first issue is the influence of size differences among siblings on stress due to competition for food. The second question is whether there are sexual differences in stress in a species where females are larger than males, and whether this difference interacts with the size differences imposed by hatching asynchrony. Merino, Møller, & de Lope (2002) have suggested that there are sexual differences in adult barn swallow HSP levels, while nothing is known about differences in nestlings. In addition, the third issue is the influence of sex of the largest sibling in the brood on stress imposed by its larger size or its stronger competitive disposition (Anderson, *et al.* 1993; Fargallo, *et al.* 2003). Finally, the fourth issue was related to explore the effects of the different types of parasites of nestling kestrels in our study population.

## **MATERIAL AND METHODS**

---

### **Field procedures**

We monitored breeding in Eurasian kestrels, a small and sexually dimorphic falcon, for which females are 20 % heavier than males (Village 1990). The study was conducted during the breeding season of 2002 in the Campo Azávaro region, a montane (1300m.a.s.l.) grassland area in central Spain (for more details see Fargallo *et al.* 2001). All licences that allowed us to

work in the field work and to manipulate fledglings were issued by Junta de Comunidades de Castilla y León.

All broods used in our study were located in nest boxes that were monitored every two days during laying and eggs were marked according to laying order (one egg is laid every two days, see Aparicio 1994; Wiebe, Wiehn & Korpimäki 1998). To record hatching order, we visited nests every day from 25 days after the end of laying, and twice every day from the day when we detected the first evidence of hatching until all eggs of the clutch hatched. We estimated hatching asynchrony as the number of hours elapsed between the estimated hatching time for the first and last hatched chicks in a brood (Viñuela, 2000). Hatchlings were identified by marking them with indelible and harmless ink on the hatching tooth during the hatching process, and later with ink on the head until banding. Chicks were weighed with a Pesola spring balance to the nearest 0.25 g and wing length was measured with calliper to the nearest 0.01mm just after hatching. When they were 24 days old were weighed to the nearest g with Pesola spring balance and their wing length measured with calliper to the nearest mm. Additionally, 250 µl of blood were extracted by brachial venipuncture. A drop of blood was used to determine nestling sex by molecular procedures (Fridolfsson & Ellegren 1999), and another drop for leukocyte counts and blood parasite screening in blood smears.

### **Haematology and parasites**

Blood smears were immediately air dried and later fixed with absolute ethanol and stained with Giemsa (1/10 v/v) for 45 min. Half of the symmetrical smear was

scanned at x200 magnification in search of large blood parasites, whereas small intra-erythrocytic parasites were searched at x1000 magnification (Merino & Potti 1995a; Merino, Potti & Fargallo 1997). Smears were scanned at 1000 × magnification and at least 100 white blood cells were counted to obtain the differential leukocyte count. We counted leukocytes in a part of the smear where cells are separated in a monolayer and we scanned the smear along its short axis to minimize differences in the thickness of the blood layer. This method has been reported to be highly repeatable, (Moreno *et al.* 1998; Saino, Møller, & Bolzern 1995). Heterophils, eosinophils, basophils, lymphocytes and monocytes were differentiated and counted following recommendations by Dein (1986) and Hawkey & Dennett (1989).

When fledglings were 24 days we collected faecal samples of chicks during manipulation just after defecation. Every chick was kept on a separate clean one-use paper towel, so we know the origin of each faecal sample. Chicks were maintained on individual towels while their sibs were measured and weighed. This method only allowed the collection of 44 samples, that were analysed by zinc sulfate flotation and counting in a MacMaster chamber. Both quantitative and qualitative analyses were carried out. Propagules were identified following Melhorn, Düwell & Raether (1992). The parasitological terms used are based on Bush *et al.* (1992). In addition, *Carnus* spp. flies were taken into account in our study. To estimate *Carnus* sp., chicks were taken from the nest and immediately were introduced in a bag blowing them and counting overall flies that were collected in the bag.

### Measurement of heat-shock proteins (HSPs)

All procedures to obtain soluble proteins were carried out at 4°C to prevent denaturation. Blood cells were homogenised by sonication in approximately 0.4 ml of distilled water to release HSPs. The homogenate was centrifuged (14,000 g, 20 min) and the supernatant collected. The total protein concentration was determined using the Bio-Rad Protein Assay. Samples of soluble proteins obtained from the blood cells (35 µg/well) were separated by SDS-PAGE. The total protein value was within the linear range of the antibody-antigen response for the species and antibodies studied. Electrophoresis was performed in a discontinuous buffer system. Polyacrylamide gels consisted of a stacking gel (4%, pH = 6.8) and a separating gel (10%, pH = 8.8). Protein separation was performed at a constant voltage of 200 v, following the manufacturer's (Bio-Rad) guidelines. Electroblot transfer from the polyacrylamide gels was performed at 150 v for 1.5 h.

The polyvinylidene fluoride (PVDF) blots were washed in PBS containing 0.05% Tween-20 (PBS-Tw), and incubated with 5% non-fat powdered milk in PBS-Tw for 1 h to block all additional binding capacity of the PVDF membranes. After incubation, blots were tested with antiserum. The primary monoclonal antibodies (Sigma) were anti-HSP60 (clone LK2) and anti-HSP70 (clone BRM22) diluted 1/1500 and 1/5000 in PBS-Tw. These antibodies react specifically with HSP60 and HSP70 respectively, and have been used to recognize these HSPs in a large variety of vertebrates, including birds.

A peroxidase-conjugated secondary antibody (Sigma) was used at 1/6000 dilution. This dilution was chosen because it allows clear detection of HSPs without unspecific binding. Positive bands were detected using 50mM Tris buffer containing 0.03% diaminobenzidine and 0.001% hydrogen peroxide. Primary and secondary antibodies were incubated overnight at 4°C and for 2 h at room temperature respectively. Three washes with PBS-Tw were performed after each step. Finally, protein bands were quantified using image analysis software for Windows (Scion Corporation). Immunoreactivities (arbitrary units) were obtained using the following formula:

$$\text{Immunoreactivity} = \text{area} \times \text{mean intensity of the band}$$

### Statistical procedures

Since eggs, hatchlings and fledglings from the same nest share parents and environment we used the chick as the unit and the nest as a random factor in general linear mixed models (GLMM) using a normal distribution of errors and an identity link function (Littell *et al.* 1996) in SAS statistical software (SAS 1989-96 Institute Inc., Cary, NC, USA). Additionally, by introducing the nests as a random term we ensured that the effects of the fixed variables were not influenced by characteristics of the parents or the nest ( $Z = 2.59$ ,  $P = 0.0048$ ), allowing the use of chick as sample unit and avoiding pseudoreplication. In addition, HSP levels were analysed in three different days where random variability in measurements were found. Thus, 'analysis day' was introduced into the model as random factor in mixed models ( $Z = 6.03$ ,  $p < 0.0001$ ), allowing us to control this

variation. Residuals of the mixed model with HSP60 as dependent variable controlling by random effects of 'nest' and 'analysis day' were employed as 'y' axis to give graphic results.

We performed a mixed model to evaluate the effect of the nest when we explored variables related to each different nestling in each nest. The effect of position in the within-brood size hierarchy was expressed as size differences of each nestling with respect to its largest sibling. These differences were included as a covariate in the mixed model like laying date and clutch size. The mass and wing length differences of each individual chick with their largest sibling at 24 days of age were considered as mass differences and size differences, respectively. Differences between hatchling and fledgling (24 days of age) mass and wing length were considered as mass gain and wing growth. Other variables such as laying order and hatching order were also considered as fixed factors. Laying order and hatching order were encoded as first, middle or last hatched/laid eggs, allowing us to compare laying and hatching sequences among different clutch sizes. *Caryospora* infection as found in our kestrel population was treated as a factor (0: uninfected fledgling; 1: infected fledgling) and as a covariate (abundance of infection). Some of the explanatory variables could covary, so we fitted their effects to the observed data following a forward stepwise procedure, testing the significance of each variable and adding only the variable that resulted in a better fit of the model. The significance of the remaining variables was tested again until no additional variable or interaction reached significance. All tests are two-tailed.

## RESULTS

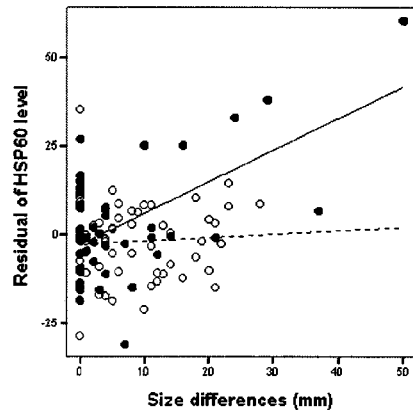
### Hatching asynchrony

A logistic regression showed that fledgling size hierarchy was only related to hatching asynchrony of chicks by assuming 84% of overall variance (Wald statistic = 18.97,  $p < 0.0001$ ), suggesting that within-brood size differences were imposed by hatching asynchrony. Hatching asynchrony increased with laying order ( $F_{2,106} = 11.91$ ,  $p < 0.0001$ ), indicating that last hatched chicks hatched from last laid eggs and consequently showed larger size differences in relation to their siblings ( $F_{2,109} = 6.33$ ,  $p = 0.003$ ).

### HSP levels in relation to size hierarchies within broods and sex

The mean levels of HSP60 and HSP70, expressed as immunoreactivities, of 112 Eurasian kestrel fledglings were  $188.0 \pm 22.3$  and  $118.9 \pm 26.0$  respectively. Of these, 61 were females and 51 were males. The HSP60 level was related negatively to fledgling wing length ( $F_{1,78} = 26.09$ ,  $p < 0.0001$ ), wing growth ( $F_{1,75} = 26.15$ ,  $p < 0.0001$ ), mass ( $F_{1,78} = 9.33$ ,  $p = 0.0031$ ), and mass gain ( $F_{1,75} = 9.21$ ,  $p = 0.0033$ ) and positively to size difference ( $F_{1,78} = 30.65$ ,  $p < 0.0001$ ) and mass difference ( $F_{1,78} = 14.01$ ,  $p = 0.0003$ ).

GLIMMIX only found the positive relationship between HSP60 level and size differences, measured as wing length differences with the largest sibling (see methods) when all variables were considered in the model (Table 1), suggesting that size differences was the most adequate variable related to HSP60 levels. This indicates that undersized nestlings with respect to the largest sibling in the brood showed higher levels of stress



**Figure 1.** Effects of the interaction between sex and size difference with respect to largest sibling, on the levels of HSP60 from peripheral blood of Eurasian kestrel fledglings. Continuous line represents females and broken line represents males. The 'y' axis represents the residuals from the GLIMMIX model controlling by the random effects of 'nest' and 'analysis day' (see methods for more details).

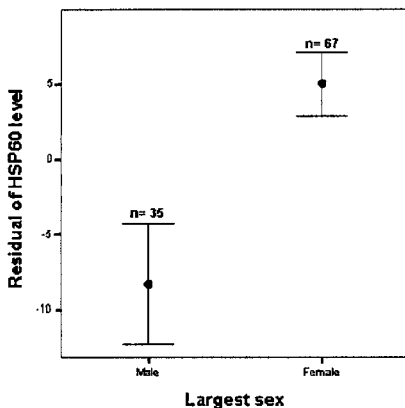
(Figure 1). In addition, fledgling sex was related to HSP60 level ( $F_{1,78} = 12.39$ ,  $p = 0.0007$ ), although this relationship disappeared when the interaction between sex and size hierarchy was considered (Table 1). The significant interaction indicates that females are more stressed than males for a similar level of size differences with the largest sibling in the brood (Figure 1).

In addition, sex of the largest sibling was related to HSP60 level of its broodmates (Table 1) and with mean HSP60 level in broods, with those broods where the largest chick was a female showing a higher HSP60 level (Figure 2). Laying date was also positively related to HSP60 levels (Table 1), suggesting that stress increases during the breeding season (Figure 3). HSP70 levels were related to size differences with the largest sibling ( $F_{1,77} = 20.55$ ,  $p < 0.0001$ ) and fledgling sex ( $F_{1,77} = 12.42$ ,  $p = 0.0007$ ), where undersized

Response term	Explanatory term	Rejected term	F	df.	P
HSP60	Size difference		14.66	1,69	<b>0.0003</b>
	Fledgling sex		0.51	1,69	0.4768
	Sex*Size difference		8.73	1,69	<b>0.0043</b>
	Sex of largest sibling		6.23	1,69	<b>0.0150</b>
	Laying date		4.29	1,69	<b>0.0420</b>
	Mass difference		2.02	1,69	0.1596
	Clutch size		1.38	1,68	0.2444
	Fledgling mass		0.94	1,67	0.3361
	Wing growth		0.58	1,63	0.4501
	Wing length		0.75	1,63	0.3898
Laying order		0.89	2,61	0.4155	
Hatching order		0.43	2,59	0.6514	
Mass gain		0.00	1,58	0.9679	

**Table 1.** GLMM with normal error and identity link function on intensity of HSP60 levels of Eurasian Kestrel fledglings. The model retained the variance of nest introduced into the model as a random term ( $Z = 2.59$ ,  $P = 0.0048$ ). Other potentially influencing variables and the remaining interactions between the variables were not significant ( $P > 0.07$ ). In the final model 'fledgling sex' was maintained to explore interaction. Statistically significant p-values in bold type.

nestlings were more stressed and females were more stressed than males ( $193.6 \pm 22.8$  and  $183.3 \pm 20.9$ , respectively) without any significant interaction (all  $p > 0.3491$ ).



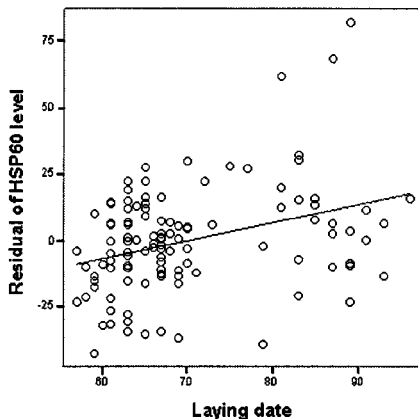
**Figure 2.** Mean ( $\pm$  SE) HSP60 level according to sex of the largest sibling in a brood. Numbers above bars are sample sizes. The 'y' axis represents the residuals from the GLIMMIX model between random effect of the nest and HSP60 level.

### Leukocyte counts in relation to size differences within broods and sex

We collect 88 blood samples from all nestlings in order to explore leukocyte counts and blood parasites. No relationships were found between numbers of different types of leukocytes and HSP60 (all  $p > 0.139$ ) or HSP70 levels (all  $p > 0.217$ ). There was also no relationship between H/L and HSP60 and HSP70 levels ( $F_{1,54} = 1.76$ ,  $p = 0.1899$ ;  $F_{1,55} = 0.02$ ,  $p = 0.8856$ , respectively). However, H/L was positively correlated to fledgling size differences ( $F_{1,59} = 4.19$ ,  $p = 0.0451$ ), wing length ( $F_{1,59} = 5.00$ ,  $p = 0.0291$ ) and wing growth ( $F_{1,56} = 4.50$ ,  $p = 0.0384$ ) in independent analyses, but only the second association remained significant when all variables were included in a GLIMMIX analysis (Table 2). Neither sex nor fledgling size differences were related to H/L and no significant interactions were found (all  $p > 0.53$ ).

Response term	Explanatory term	Rejected term	F	df.	p
H/L index	Wing length		5.00	1,59	<b>0.0291</b>
		Wing growth	1.98	1,55	0.1654
		Hatching order	1.41	2,53	0.2530
		Mass gain	1.01	1,51	0.3187
		Fledgling mass	0.82	1,52	0.3683
		Laying date	0.66	1,50	0.4209
		Sex	0.34	1,49	0.5628
		Size difference	0.23	1,49	0.6359
		Laying order	0.10	2,47	0.9054

**Table 2.** GLMM with normal error and identity link function on H/L of Eurasian Kestrel fledglings. The model retained the variance of nest, introduced in to the model as random term ( $Z = 1.73$ ,  $P = 0.0420$ ). Other potentially influencing variables, and the remaining interactions between the variables were not significant ( $P > 0.21$ ).



**Figure 3.** Correlation between laying date (0 is March 1) and HSP60 level (see Table 1 for statistics). The 'y' axis represents the residuals from the GLIMMIX model of the association between random effect of the nest and HSP60 level.

### Parasites and stress

No ectoparasites were found on fledglings of 24 days. The screen of blood smears of 86 fledglings for haematozoa was also negative. We could collect 44 faecal samples from fledglings for which we could measure HSPs levels, where the coprological of the faeces revealed the presence of *Caryospora sp.* in 29 (65.9%) of

the samples excreted *Caryospora sp.* oocysts. Mean oocyst excretion was  $4379.4 \pm 6851.8$  oocysts / gr of faeces. No other parasite propagules were detected. No relationships were found between infection and laying date, fledgling mass gain, fledgling growth, wing length or size differences (U-Man Whitney tests, all  $p > 0.333$ ). In addition, H/L index was not correlated to parasite infection (U-Man Whitney test, all  $p > 0.511$ ). However, we introduced *Caryospora* infection status as a factor in the model explaining HSP60 and HSP70 levels, finding that *Caryospora* infection increased HSP60 levels (Table 3), but was not related to HSP70 levels ( $F_{1,15} = 0.03$ ,  $p = 0.8584$ ). The intensity of infection was not related to HSP60 and HSP70 levels (both  $p > 0.3101$ ).

## DISCUSSION

### The effect of position in the size hierarchy

The relationship found between HSPs and size differences clearly show that levels of these proteins in kestrel nestlings increase in relation to size differences with respect to their largest sibling and are thus dependent on position in the within-brood size hierarchy for which size differences are



Response term	Explanatory term	Rejected term	<i>F</i>	df.	<i>p</i>
HSP60	Size difference		9.439	1,15	<b>0.008</b>
	Fledgling sex		1.690	1,15	0.210
	Sex*Size difference		6.647	1,15	<b>0.021</b>
	Caryospora infection		11.419	1,15	<b>0.004</b>
		Laying date	1.390	1,14	0.259
		Sex of largest sib	0.100	1,14	0.762

**Table 3.** GLMM with normal error and identity link function on HSP60 levels of Eurasian Kestrel fledglings. The model retained the variance of nest, introduced in to the model as random term ( $Z = 2.51$ ,  $P = 0.0060$ ). Other potentially influencing variables and the remaining interactions between variables were not significant ( $P > 0.35$ ). In the final model 'fledgling sex' was maintained to explore interaction.

imposed by the within-brood hatching pattern. Both stress proteins show a similar trend, reinforcing the evidence for hierarchy-related stress. Competition-related stress could be mediated by hormonal levels, given that smaller chicks increase corticosterone levels under food deprivation (Nuñez-de la Mora, *et al.* 1996) and that testosterone levels increase with laying order in order to compensate for age- and size-related disadvantages in posthatching development (Schwabl, 1993). Earlier studies have shown that HSP60 levels were related to within-brood size variation (Merino, *et al.* 1998; Moreno, *et al.* 2002). These authors suggested that HSP60 could be expressing the greater stress induced through competition in broods with large size differences. However, these studies did not report that the smallest siblings had higher levels of stress proteins, as they worked with brood means. Our results clearly show that the smaller nestlings in each brood are more stressed than their larger siblings, as has been shown in other species under food shortage using stress hormones (Nuñez-de la Mora, *et al.* 1996; Kitaysky, Wingfield & Piatt 2001). We propose that HSP levels, especially for HSP60, may be a good index of sustained competition-related stress in avian broods. It is possible that begging signals and adopting

strategic positions in the nest during parental visits could be stressful (Brzek & Konarzewski, 2001), especially in species like the kestrel where nest position is an important factor determining success in obtaining food under food shortage (Fargallo *et al.* 2003).

Stress protein levels and H/L have shown a positive association in a recent study (Moreno *et al.* 2002), a result which could not be confirmed for nestling kestrels. Also, H/L did not relate to size hierarchies within broods, confirming the suggestion by Moreno *et al.* (2002) that it is a poorer index of the stress due to competition in the nest. There was a positive association of H/L with wing length, a variable that has been shown as a good estimate of nutritional condition in nestling birds (Nowicki, *et al.* 2000). This indicates that as found for broilers food restriction do not affect H/L (Maxwell 1993) and is not a good index of nutritional stress in kestrels.

We also found that HSP60 levels increase during the course of the breeding season. This trend could be related to a seasonal decline in food abundance or to thermal stress imposed by an increase in ambient temperature. Several studies have described that nest parasites increase with breeding season (Merino & Potti, 1995b;

although see Dawson & Bortolotti 1997), suggesting costs for growth and immune response (Merino, *et al.* 2000; although see Roulin, *et al.* 2003 for Eurasian kestrels). However, our results suggest that laying date is not a relevant factor that increases parasitism infection and stress in kestrel chicks.

It is widely assumed that resources decrease with date in most temperate areas (Price, 1996). In our study population there is a seasonal decline in fledgling size and in reproductive success (Fargallo *et al.* 2001) which can be due to low food allocation by parents to their fledglings, because late poorly breeding kestrels are relegated to poor territories. It is thus reasonable to suppose that competition for food will increase with date and lead to more competition-related stress. Poor nutrition itself could also be driving the seasonal trend in stress (Moreno *et al.* 2002). However, the expected seasonal increase in ambient temperature could be reflected in a thermal increase in nest boxes, so HSP60 levels could reflect a seasonal increment in thermal stress (Krebs & Feder, 1995). These two possibilities are not mutually exclusive, so future research should explore their relative importance.

### **The effect of fledgling sex**

Our results suggest that stress protein levels of small siblings depend on sex when size hierarchies are extreme. Thus, subordinate female fledglings were more stressed than males when the size differences with respect to the largest sibling increased. In raptors, female nestlings are larger than males, suggesting that males are in a competitive disadvantage under conditions of food scarcity (Anderson *et al.* 1993, Fargallo *et al.* 2003), meaning a

higher male mortality (Arroyo, 2002) or lower capacity of mounting an adequate immune response under food shortage (Fargallo *et al.* 2002). Our results are partially in disagreement because fledgling sex is excluded in our model, while our results suggest additionally that female fledgling kestrels are more stressed than males when size differences increase in relation to their siblings. In addition, different studies have shown that the larger sex has higher energetic requirements for growth suggesting an adaptive disadvantage in relation to the smaller sex (Arroyo, 2002; Anderson, *et al.* 1993; Torres & Drummond, 1997; Bortolotti, 1986). This implies that they would have to compete more strongly for food when in a subordinate position in the brood hierarchy. This could lead to a higher stress for small female siblings. Our results suggest that female kestrel chicks are more stressed than males when they lose their competitive size advantage due to the higher food requirements in this sex, suggesting additionally that males may better confront a size-disadvantage situation. The association of competition-related stress to sex-dependent hormone levels in nestlings remains to be studied to clarify the role of testosterone and corticosterone in the determination of stress levels.

Our results show that when the largest chick in a brood is a female, average stress protein levels in a brood are higher than if it is a male. This suggests that females in a dominant position impose a more competitive situation for their siblings, which is expressed in increased HSP levels. Female nestlings in Eurasian (Fargallo, *et al.* 2003) and American kestrels (Anderson *et al.* 1993) compete more successfully than males in conditions of food shortage, possibly due to their larger average size.

In our population, fledgling mass decreased with hatching order in clutches initiated with male eggs, while fledgling mass of chicks hatched in different orders did not differ in nests initiated with female eggs (Blanco *et al.* unpublished manuscript). This result suggests that when females are in a dominant position, subordinate siblings may compensate for their size handicap by competing more effectively for food. However, the results of the present study indicate that this compensation may not be without the costs imposed by higher stress levels. In general, reviews of the costs of growth compensation (Metcalf & Monaghan 2001) have not considered the effects of stress induced by increased sibling competition.

### **Stress and parasites**

Although the quantitative faecal examination is only an approximate indication of the actual intensity of infection, this is the only non-invasive technique suitable for the study of intestinal parasites in wild bird nestlings (Watve & Sukumar 1995). Our results suggest that infection by *Caryospora sp.* increase individual stress, at least as measured by stress proteins. The effect of parasites is independent of position in the hierarchy and sex. Precedent findings suggest that last chick in the laying sequence could be the target of parasites (Christie *et al.* 1998). Our results do not suggest this finding although they are not mutually exclusives because different patterns of fledgling *Caryospora* infection could be described more related to random effects than with adaptive consequences by knowing in addition, that hatching order or size hierarchy do not influence on immune response (unpublished data: Martínez-Padilla, J & Dávila, J.). On

this order, chicks could be infected to be in contact to faeces of their sibs or directly to eat a infected prey, so we suggest that intestinal parasite infection could be modulates by other patterns suggested by Christie, Møller, & de Lope (1998) although more researches are needed to elucidate this question. Very little is known about coccidian infection in wild populations of raptors, although it has been described that severe coccidiosis could cause death in free living kestrels (Krone, 2002). Protozoan parasites, are large enough to avoid phagocytic uptake, although inflammatory cells could act directly by adhering to them and releasing a variety of potent mediators creating a hostile microenvironment (Wakelin & Apanius, 1997). In kestrel nestlings, *Caryospora* infection could induce fever and an inflammatory response which in turn could raise stress protein levels (Macario, 1995). However, in vitro studies have shown that stress proteins could be stimulated by the parasite itself (Garbe, 1992), especially during the first stage of infection. Krone (2002) observed that a *Caryospora*-infected kestrel are more severe 40 - 45 days after infection, so infection-related stress may increase further after fledging, suggesting that parasite infection in wild fledgling kestrels could be reflected as a stress factor in the nest stage.

To conclude, we have shown the usefulness of stress proteins from peripheral blood for detecting the effects of different types of stressors for nestling birds. A low position in the brood hierarchy increases stress, and this effect is more marked for female chicks in species where females are larger and require more food in the nest. Parasites and breeding date also affect stress. When the dominant chicks belong to the larger sex, their siblings are more

stressed. Trying to compensate for a bad start in life may be stressful, and this stress may have long-term consequences for fitness. The link between stress protein levels and future survival and reproduction in the wild remains to be elucidated to confirm its utility for ecological studies.

## ACKNOWLEDGEMENTS

We thank the Finat family for kindly allowing us to conduct the study in their property, and C. Marqués and J. San Teodoro for their collaboration in field work. JMP did not receive any grant or scholarship during the course of this study. We thank J. Viñuela for providing comments on early drafts. J. A. Fargallo improved the original manuscript with his comments and helped me occasionally in the field work. Project BOS2001-0587 to J. Moreno, J. Viñuela and G. Blanco partly supported the field work, while project BOS 2000-1125 to S. Merino and J. Martínez financed blood smear and HSP analyses. J. Redondo, Sáez, A. and Pérez, R. could repair JMP's computer, thus saving data and allowing JMP to continue his research.

## REFERENCES

- Anderson, D.J. Budde, C. Apanius, V. Martínez-Gómez, J.E. Bird, D.M. & Weathers, W.W. (1993) Prey size influences competitive dominance in nestling American kestrels (*Falco sparverius*). *Ecology* **74**, 367-376.
- Aparicio, J.M. (1994) The effect of variation in the laying interval on proximate determination of clutch size in the European Kestrel. *Journal of Avian Biology* **25**, 275-280.
- Arroyo, B. (2002) Sex-biased nestling mortality in the Montagu's harrier *Circus pygargus*. *Journal of Avian Biology* **33**, 455-460.
- Bortolotti, G. (1986) Influence of sibling competition on nestling sex ratios of sexually dimorphic birds. *American Naturalist* **127**, 495-597.
- Brzek, P. & Konarzewski, M. (2001) Effect of food shortage on the physiology and competitive abilities of sand martin (*Riparia riparia*) nestlings. *Journal of Experimental Biology* **204**, 3065-3074.
- Burel, C. Mezger, V. Pinto, M. Rallu, M. Trigon, S. & Morange, M. (1992) Mammalian heat shock protein families. Expression and functions. *Experientia* **48**, 629-634.
- Bush, A.O. Lafferty, K.D. Lotz, J.M. & Shostak, A.W. (1997) Parasitology meets ecology on its own terms: Margolis *et al.* revisited. *Journal of Parasitology* **83**, 575-583.
- Christe, P. Møller, A.P. & de Lope, F. (1998) Immunocompetence and nestling survival in the house martin: the tasty chick hypothesis. *Oikos* **83**, 175-179.
- Clutton-Brock, T.H. (1991) *The evolution of parental care*. Princeton University Press. Princeton. New Jersey.
- Dawson, R. & Bortolotti, G. (1997) Ecology of parasitism of nestling American kestrels by *Carnus hemapterus* (Diptera: Carnidae). *Canadian Journal of Zoology* **75**, 2021-2026.
- Dein, F.J. 1986. Hematology. *Clinical Avian Medicine and Surgery* (eds B. G. H. Harrison & L. R. Harrison), pp. 174-191. Philadelphia.
- Drummond, H. Osorno, J.L. Torres, R. García-Chavelas, C. & Merchant-Larios, H. (1991) Sexual size dimorphism and sibling competition: implications for avian sex ratios. *American Naturalist* **138**, 623-641.
- Ellis, L. A. Borst, D. W. & Thompson, C. F. (2001). Hatching asynchrony and maternal androgens in egg yolks of House Wrens. *Journal of Avian Biology* **32**, 26-30.
- Fargallo, J.A. Blanco, G. Potti, J. & Viñuela, J. (2001) Nestbox provisioning in a rural population of Eurasian Kestrels: breeding performance, nest predation and parasitism. *Bird Study* **48**, 236-244.
- Fargallo, J.A. Laaksonen, T. Poyri, V. & Korpimäki, E. (2002) Inter-sexual differences in the immune response of Eurasian kestrel

- nestlings under food shortage. *Ecology Letters* **5**, 95-101.
- Fargallo, J.A. Laaksonen, T. Korpimäki, E. Pöyri, V. Griffith, S.C. & Valkama, J. (2003) Size-mediated dominance and begging behaviour in Eurasian kestrel broods. *Evolutionary Ecology Research* **5**, 549-558.
- Fridolfsson, A.K. & Ellegren, H. (1999) A simple and universal method for molecular sexing of non-ratite birds. *Journal of Avian Biology* **30**, 116-121.
- Garbe, T.R. (1992) Heat shock proteins and infection: Interactions of pathogen and host. *Experientia* **48**, 635-639.
- Hahn, D.C. (1981) Asynchronous hatching in the laughing gull: cutting losses and reducing rivalry. *Animal Behaviour* **29**, 421-427.
- Hawkey, C.M. & Dennet, T.B. (1989) *Atlas de Hematología Veterinaria Comparada*. Grass Editions, London.
- Hörak, P. Tegelman, L. Ots, I. & Møller, A.P. (1999) Immune function and survival of great tit nestlings in relation to growth conditions. *Oecologia* **121**, 316-322.
- Kitaysky, A.S. Wingfield, J.C. & Piatt, J.F. 2001. Corticosterone facilitates begging and affects resource allocation in the black-legged kittiwake. *Behavioral Ecology* **12**, 619-625.
- Krebs, R.A. & Feder, M.E. (1995) Natural variation in the expression of the heat-shock protein HSP70 in a population of *Drosophila melanogaster* and its correlation with tolerance of ecologically relevant thermal stress. *Evolution* **51**, 173-179.
- Krone, O. (2002) Fatal Caryospora infection in a free-living juvenile Eurasian kestrel (*Falco tinnunculus*). *Journal of Raptor Research* **36**, 84-86.
- Littell, R.C. Milliken, G.A. Stroup, W.W. & Wolfinger, R. D. (1996) *SAS system for mixed models*. SAS Institute. Cary. New York.
- Macario, A.J.L. (1995) Heat-shock proteins and molecular chaperones: Implications for pathogenesis, diagnostics, and therapeutics. *International Journal of Clinical & Laboratory Research* **25**, 59-70.
- Maxwell, M.H. (1993) Avian blood leukocyte responses to stress. *World Poultry Science Journal* **49**, 34-43.
- Maxwell, M.H. & Robertson, G.W. (1998) The avian heterophil leucocyte: A review. *World Poultry Science Journal* **54**, 155-178.
- Melhorn, H. Düwell, D. & Raether, W. (1992) *Atlas de Parasitología Veterinaria*. GRASS Editions. Spain.
- Merino, S. Martínez, J. Barbosa, A. Møller, A.P. de Lope, F. Pérez, J. & Rodríguez-Cabeiro, F. (1998) Increase in a heat-shock protein from blood cells in response of nestling house martins (*Delichon urbica*) to parasitism: an experimental approach. *Oecologia* **116**, 343-347.
- Merino, S. Martínez, J. Møller, A. P. Barbosa, A. de Lope, F. & Rodríguez-Cabeiro, F. (2002) Blood stress protein levels in relation to sex and parasitism of barn swallows (*Hirundo rustica*). *Ecoscience* **9**, 300-305.
- Merino, S. Møller, A.P. & de Lope, F. (2000) Seasonal changes in cell-mediated immunocompetence and mass gain in nestling barn swallows: a parasite-mediated effect?. *Oikos* **90**, 327-332.
- Merino, S. Potti, J. & Fargallo, J.A. (1997) Blood parasites of some passerine birds from central Spain. *Journal of Wildlife Diseases* **33**, 638-641.
- Merino, S. & Potti, J. (1995a) High prevalence of hematozoa in nestlings of a passerine species, the Pied flycatcher (*Ficedula hypoleuca*). *Auk* **112**, 1041-1043.
- Merino, S. & Potti, J. (1995b) Mites and blowflies decrease growth and survival in nestling pied flycatcher. *Oikos* **73**, 95-103.
- Metcalf, N.B. & Monaghan, P. (2001) Compensation for a bad start: grow now, pay later?. *Trends in Ecology and Evolution* **16**, 254-260.
- Møller, A.P. (1997) Parasitism and the evolution of host life history. *Host-parasite evolution: general principles and avian models* (eds D. H. Clayton & J. Moore), pp: 105-127. Oxford University Press, Oxford.

- Moreno, J. Merino, S. Martínez, J. Sanz, J.J. & Arriero, E. (2002) Heterophil/lymphocyte ratios and heat-shock protein levels are related to growth in nestling birds. *Ecoscience* **9**, 434-439.
- Moreno, J. de León, A. Fargallo, J.A. & Moreno, E. (1998a) Breeding time, health and immune response in the chinstrap penguin *Pygoscelis antarctica*. *Oecologia* **115**, 312-315.
- Morimoto, R.I. (1991) Heat shock: the role of transient inducible responses in cell damage, transformation, and differentiation. *Cancer Cells* **3**, 295-268.
- Núñez-de la Mora, A. Drummond, H. & Wingfield, J.C. (1996) Hormonal correlates of dominance and starvation-induced aggression in chicks of the Blue-footed booby. *Ethology* **102**, 748-761.
- Nowicki, S. Hasselquist, D. Bensch, S. & Peters, S. (2000) Nestling growth and song repertoire size in great reed warblers: Evidence for song learning as an indicator mechanism in mate choice. *Proceedings of the Royal Society of London Series B* **267**, 2419-2424.
- Price, T. 1996. Maternal and paternal effects in birds. Effects on offspring fitness. *Maternal effects as adaptation* (eds A. M. Timothy & W. Charles), pp 202-226. Oxford University Press, Oxford.
- Romero, L.M. & Romero, R.C. (2002). Corticosterone responses in wild birds: the importance of rapid initial sampling. *Condor* **104**, 129-135.
- Roulin, A. Brinkhof, M.W. G. Bize, P. Richner, H. Jungi, T.W. Bavoux, C. Boileau, N. & Burnealeau, G. (2003) Which chick is tasty to parasites? The importance of host immunology vs. parasite life history. *Journal of Animal Ecology* **72**, 75-81.
- Saino, N. Møller, A.P. & Bolzern, A.M. (1995) Testosterone effects on the immune system and parasite infestations in the barn swallow (*Hirundo rustica*): an experimental test of the immunocompetence hypothesis. *Behavioral Ecology* **6**, 397-404.
- Saino, N. Incagli, M. Martinelli, R. Ambrosini, R. & Møller, A.P. (2001) Immunity, growth and begging behaviour of nestling Barn Swallows *Hirundo rustica* in relation to hatching order. *Journal of Avian Biology* **32**, 263-270.
- Schwabl, H. (1993) Yolk is a source of maternal testosterone for developing birds. *Proceedings of the National Academy of Sciences of the USA* **90**, 11446-11450.
- Sockman, K.W. & Schwabl, H. (2001) Plasma corticosterone in nestling american kestrels: effects of age, handling stress, yolk androgens, and body condition. *General and Comparative Endocrinology* **122**, 205-212.
- Stoleson, S.H. & Beisinger, S.R. (1995) Hatching asynchrony and the onset of incubation in birds, revisited. When is the critical period?. *Current Ornithology* **12**, 191-270.
- Tarlow, E. Wikelski, M. & Anderson, D.J. (2001) Hormonal correlates of siblicide in Galapagos Nazca boobies. *Hormones and Behaviour* **40**, 14-20.
- Torres, R. & Drummond, H. (1997) Female-biased mortality in nestling of a bird with size dimorphism. *Journal of Animal Ecology* **66**, 859-865.
- Village, A. (1990) *The Kestrel*. T. & A. D. Poyser. London.
- Viñuela, J. (1999) Sibling aggression, hatching asynchrony and nestling mortality in the black kite (*Milvus migrans*). *Behavioral Ecology Sociobiology* **45**, 33-45.
- Viñuela, J. (2000) Opposing selective pressures on hatching asynchrony: egg viability, brood reduction and nestling growth. *Behavioral Ecology Sociobiology* **48**, 333-343.
- Wakelin, D. & Apanius, V. (1997) Immune defence: genetic control. Host-parasite evolution: general principles and avian models (eds D. H. Clayton & J. Moore). Oxford University Press. Oxford.
- Watve, M.G. & Sukumar, R. (1995) Parasite abundance and diversity in mammals. Correlates with host ecology. *Proceedings of the National Academy of Sciences of the USA* **92**, 8945-8949.
- Wiebe, K.L. & Bortolotti, G. (1994) Energetic efficiency of reproduction: the benefits of asynchronous hatching for American kestrels. *Journal of Animal Ecology* **63**, 551-560.

Wiebe, K.L. Wiehn, J. & Korpimäki, E. (1998)  
The onset of incubation in birds: can females

control hatching patterns?. *Animal Behaviour* **55**,  
1043-1052.





## CAPÍTULO VI

### **Influencia de la asincronía de eclosión, sexo y reducción de nidada en la inmunidad de volantones de Cernícalo vulgar.**

Este capítulo reproduce íntegramente el texto del siguiente manuscrito:

**Jesús Martínez-Padilla and José A. Dávila.** *En revisión.* Influence of hatching asynchrony, sex and brood reduction on immunity of Eurasian kestrel nestlings. *Journal of Animal Ecology*.

#### **Resumen del Capítulo**

El inicio de la incubación en aves antes del final de la puesta es común en aves altriciales. Este patrón implica una obligada asincronía en la eclosión y por tanto una jerarquía posterior de los pollos en la nidada, lo que ha sido sugerido como una estrategia parental para mejorar la calidad de los volantones y/o su supervivencia. Sin embargo, son escasas las evidencias que sugieran algún tipo de correlación entre estos dos índices en casos de mortalidad debida a la asincronía de eclosión. En este capítulo, se estudia la relación entre la respuesta inmune celular mediada por linfocitos-T como una medida de calidad fenotípica y la asincronía de eclosión de volantones de Cernícalo vulgar (*Falco tinnunculus*) en España durante tres temporadas de estudio. Paralelamente, el sexo de los pollos es considerado ya se ha comprobado que éste puede afectar a la inmunidad de los pollos. Los resultados sugieren que (1) la relación entre la respuesta inmune y la asincronía de eclosión, está mediada por la mortalidad en el nido, donde las puestas de eclosión asincrónica que sufrieron reducción de nidada, mostraron los niveles más altos de respuesta inmune celular. Por otro lado, (2) los pollos macho, aunque no las hembras y contrariamente a resultados precedentes, incrementaron su respuesta inmune celular en puestas que sufrieron reducción de nidada. Además, (3) el año es una fuente importante de variación en la respuesta inmune, lo que sugiere que debería tenerse en cuenta en cuanto a la hora de interpretar futuros estudios en relación a la respuesta inmune y las historias vitales. Nuestros resultados estarían de acuerdo con la idea de la reducción de nidada postulada por Lack por que la mortalidad de algún pollos en puestas de eclosión asincrónica mejoraron la calidad de los volantones.



# Influence of hatching asynchrony, sex and brood reduction on immunity of Eurasian kestrel nestlings.

JESÚS MARTÍNEZ-PADILLA AND JOSÉ A. DÁVILA. <sup>1</sup>*Instituto de Investigación en Recursos Cinegéticos (C.S.I.C.-U.C.L.M.), Ronda de Toledo s/n, 13005 Ciudad Real, Spain,*

## ABSTRACT

The onset of incubation in birds before the end of laying is common in altricial birds. This pattern implies an obligate asynchrony in hatching and therefore a size hierarchy in the brood that has been suggested as a parental strategy to improve fledgling quality and survival. However, little is known about the relationship between these two traits in cases of mortality due to hatching asynchrony. In the present study, we investigate the relationship between T-cell mediated immune response as a measure of phenotypic quality and hatching asynchrony of Eurasian kestrel (*Falco tinnunculus*) fledglings in central Spain during three breeding seasons. In addition, fledgling sex is considered because it may affect immunity. Our results suggest that (1) the relationship between immune response and hatching asynchrony was different depending on the occurrence of nestling mortality, where asynchronously hatched broods suffering reduction showed the highest immunocompetence, (2) male chicks, but not females, increase their immune response in broods suffering reduction than in other broods, and (3) year is an important source of variation in immune response, suggesting that it must be taken into account in future studies of immunity and life history traits. Our results are in agreement with Lack's brood reduction hypothesis because mortality in asynchronously hatching broods leads to improvements in the quality of offspring.

Key words: *Falco tinnunculus*, sexual differences, T-cell mediated immunity, nestling mortality.

## INTRODUCTION

---

Life-history theory predicts that parents should maximise lifetime reproductive success by adjusting reproductive effort according to prevailing environmental and body conditions (Clutton-Brock 1991). Under different environmental or physiological conditions, parents should adopt different adaptive strategies with respect to the development of costly physiological functions such as the immune response (Martin II et al. 2002; Whitaker & Fair 2002). Recent studies have focused on

studying how the immune function may be affected by different factors such as sex (Moreno et al. 2001; Barbosa & Moreno, 2002; Fargallo et al. 2002; Tschirren, Fitze & Richner, 2003), clutch size (Moreno, Sanz & Arriero 1999; Tella et al. 2000a), brood size (Sorci, Soler & Møller, 1997; Hōrak et al. 1999), secondary sexual traits (González et al. 1999; Velando, Lessells & Márquez 2001), paternity (Johnsen et al. 2000), fluctuating asymmetry (Fair, Hansen & Ricklefs 1999; Whitaker & Fair 2002) or intensity of parasitism (Saino, Calza & Møller 1998; Christe, Møller and de Lope

1998), as well as on performing comparative interspecific analyses (Tella, Scheuerlein & Ricklefs 2002; Martin et al. 2001).

Hatching asynchrony is a common trait in birds that may individually affect growth and survival of chicks depending on their position within the hatching sequence (Stoleson & Beissinger 1995). Multiple hypotheses have been advanced to explain the existence of hatching asynchrony in birds (Stoleson & Beissinger 1995), most of them considering this trait as an adaptation (although see Nilsson 1993). These adaptive hypotheses suggest that evolutionary pressures could be acting on different breeding stages or actors involved on: 1) chick survival and growth during the incubation, 2) nestling or fledgling periods or 3) parental survival and condition (reviewed by Stoleson and Beissinger 1995). Thus, different evolutionary pressures could be acting depending on the species (Viñuela and Carrascal 2000) or within a species (Viñuela 1999; Wiebe, Korpimäki & Wiehn 1998).

Recent experimental studies suggest that different opposing selective pressures could be regulating hatching asynchrony patterns, and that parent birds could choose among different hatching patterns (by regulating the onset of incubation), depending on prevailing conditions (Wiebe et al. 1998; Viñuela 2000). The basic idea behind adaptive hypotheses to explain hatching asynchrony is that the observed pattern of hatching asynchrony should maximise lifetime reproductive output (Dawson & Bortolotti 2002). In this respect, available data support that hatching asynchrony may depend on maternal prelaying conditions (Hanssen, Engebretsen & Erikstad 2002), that it may affect chick survival, growth rate or fledgling mass (e.g. Viñuela 2000;

Massemin et al. 2002), that may be adjusted to minimise predation rate (Clark & Wilson 1981) or to maximise egg viability (Arnold, Rohwer & Armstrong 1987), or be affected by a combination of these (Viñuela 2000). However, most studies on hatching asynchrony in birds have estimated reproductive performance using classical variables, such as growth rate, fledging mass or nestling survival (Stoleson & Beisinger 1995), while studies assessing the effects of hatching asynchrony on aspects of the physiological condition of fledglings such as immunity are scarce (Christe et al. 1998).

In addition, has been suggested that parasites could modulate life-histories strategies (Hamilton & Zuk 1982), mainly on fledgling stage because they could not escape from parasites being immunity their main defence. Recent studies suggest that mount a good immunitary system could be more important than mass gain (Merino, Møller & de Lope 2000) and that immune response is a good predictor of survival (Soler et al. 1999; Hórak et al. 1999) at least in same cases or species (Tella et al. 2000b). Therefore, physiological condition measured as immune response could be a important trait to evaluate than classical variables described in hatching asynchrony studies.

Brood reduction is particularly common in raptors (Newton 1979). Several studies in Eurasian kestrels *Falco tinnunculus* have shown that an increased food supply reduces nestling mortality (Korpimäki, 1989; Dijkstra et al. 1990; Wiehn & Korpimäki 1997). Therefore, when food abundance is not sufficient to adequately raise all siblings, changes in physiological condition of nestlings, such as a lower immune response, are expected (Saino et al. 1998; Hórak et al.

1999, Merino et al. 2000). Although these studies did not take into account hatching asynchrony, they apparently contradict the classical brood reduction hypothesis that suggest that hatching asynchrony improve the quality and/or quantity of chicks under food shortage eliminating the last hatched chicks (Lack 1954). Under brood reduction idea, hatching asynchrony should act promoting a quick death of last hatched chicks in the brood when food is scarce, thus saving resources for their early-hatched siblings. Consequently not showing any negative effects of food scarcity on their immune system. In this respect, some studies in American *Falco sparverius* and Eurasian kestrels have reported that experimentally increased food supply does not improve fledgling mass and/or physical condition (Wiehn & Korpimäki 1997; Dawson & Bortolotti 2002), but no information is available about how the interaction between hatching asynchrony and food supply affects other fitness components, such as the development of the immune system. In this paper we explore the relationships among hatching asynchrony, sex, mortality and immunity in fledgling Eurasian kestrels.

We take in to account fledgling sex because recent studies demonstrate that sexual differences in immune response are expected in nestling stage in Eurasian kestrels under food shortage (Fargallo et al. 2002) and in great tits nestlings under different experimental parasitism conditions (Tschirren et al. 2003).

In addition, breeding success and fledgling immune response is measured in different years in the same population to study the possible effect of interannual variation on the variables we measured.

## **MATERIAL AND METHODS**

---

### **Laying order and hatching pattern**

The study was conducted between 2000 to 2002 in an Eurasian kestrel (kestrel hereafter) population in Campo Azálvaro region (40°40'N, 4°20'W, 1300m.a.s.l.), an homogenous mountain grassland area in central Spain, where 55 nest boxes were provided (see Fargallo et al. 2001 for more details).

We monitored nest boxes every 1 – 2 days during the prelaying and laying periods to estimate laying date. Eggs were marked with felt pens as they were laid in order to know the laying sequence. Four to-five days before the expected date of hatching (20 days after laying of the last egg) we started to monitor nest-boxes daily until we detected the first clues of hatching (chick sounds inside the unbroken eggshell), and 2 or 3 times (as required) every day until the end of hatching.

Hatching span was estimated as the number of hours elapsed between the first and last hatched egg in a brood (Viñuela 2000). Hatching asynchrony was considered as “asynchronous” broods when the time elapsed between the first and last hatched eggs was equal or longer than 24 h, while “synchronous” broods where those in which all eggs in the brood hatched in less than 24 h (Stoleson & Beissinger 1995). In addition, we considered “asynchronous chicks” as those hatched at least 24h after their immediately older sibling, and “synchronous chicks” as those hatched within 24 h (Stoleson & Beissinger 1995). In order to assign each egg to its corresponding nestling, hatchlings were identified by marking them with indelible ink on the hatching tooth when eggs were

found during the hatching process, and subsequently on the head until banding. Chicks were weighed with a Pesola balance to the nearest  $\pm 0.25$  g, and measured as to wing length to the nearest 0.01 mm just after the egg hatched. When they were 24 days they were weighed to the nearest g and measured as to wing length to the nearest mm.

Mass differences of each chick with respect to its largest sibling, were measured when the oldest chick in the brood was 25 days old, and considered as size differences. Mass gain was defined as the difference in body mass between hatching and fledging (25 d). Nestling sex was determined by molecular procedures (Fridolfsson & Ellegren 1999) using DNA extracted from a drop of blood obtained by brachial venipuncture when fledglings were 24 days old. Adult females were captured between a week after the end of laying and a week before the onset of hatching.

### **Immune response**

To evaluate the condition of the immune system, a mitogen was injected to generate a local proliferation of circulating T lymphocytes. Twenty four days after hatching, kestrel chicks were injected intradermally in the left wing web with 0.1ml of a solution of Phytohaematogglutinin PHA (3mg of PHA in 1ml of PBS) after measuring its thickness at the point of injection. Twenty four hours after injection we measured wing web thickness again and we considered the difference between initial and final measurement as the cell-mediated immune response (Smits, Bortolotti & Tella 1999). Three measures were taken with a digital spessimeter (Mitutoyo Absolute ID-112B) to the nearest 0.01mm before and after

injection. The repeatability of initial ( $F_{338,678} = 130.275$ ,  $p < 0.0001$ ) and final ( $F_{338,678} = 205.384$ ,  $p < 0.0001$ ) measurements was high, so we used the mean values of the three measurements in the analyses. The same spessimeter was employed in all measurements.

### **Statistical procedures**

Since hatchlings and fledglings from the same nest had probably more similar mass than those from different nests due to the influence of sharing common parents and rearing environments, they were not considered as independent samples. Therefore, we performed generalized linear mixed models (GLIMMIX) using a normal distribution of errors and an identity link function (GLIMMIX macro of SAS, Littell et al. 1996) and nest as random factor in order to avoid pseudoreplication when employing chick as sample unit. However, we considered additionally year as random factor in which nest were nested in order to avoid pseudoreplication by the use of the same nest as sample unit throughout years. With this procedure we could consider each nest as independent sample in spite of sometimes the same nest was employed for breeding in different years.

In order to avoid the confounding effects of clutch size on hatching asynchrony and following Wiebe & Bortolotti (1994), we only considered modal clutches (4 and 5 five eggs,  $n = 74$ , 78.7% of all broods studied,  $n = 94$ ) because these clutches could hatch synchronously or asynchronously. Clutches of 3 eggs ( $n = 4$ ) always hatched synchronously and clutches of 6 ( $n = 14$ ) and 7 eggs ( $n = 2$ ) always hatched asynchronously. In addition, we removed randomly from analyses those females that bred in different years in order

to avoid pseudoreplication. Some of the explanatory variables could covary, so we fitted their effects to the observed data following a forward stepwise procedure, testing the significance of each variable and adding only the variable that resulted in a better fit of the model. The significance of the remaining variables was tested again until no additional variable or interaction reached significance. All tests are two-tailed.

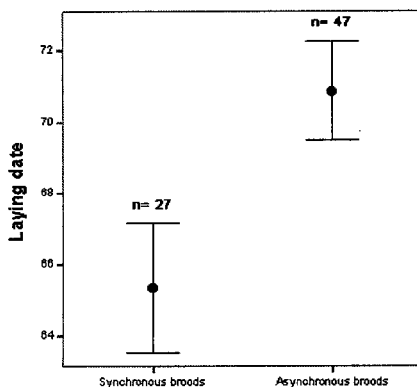
To analyse the variation in hatching asynchrony and CMI response, we performed a (general) mixed model in which the effect of nest was removed by including it as a random factor in the model being immune response as dependent variable. Hatching asynchrony (synchronous vs. asynchronous broods), brood reduction (broods with mortality vs. broods without mortality) and fledgling sex were included as fixed factors. Because in our analyses we included only clutches with 4 and 5 eggs, we considered clutch size as factor. Other variables such as chicks hatched, size differences, hatchling mass,

brood size, laying date and hatching span were also considered as covariates. Given that we could not determine for each nestling the egg that they were hatched, we only considered in models those chicks that we could assign the egg which each nestling hatched. Laying and hatching order were encoded as first-, last- and middle- (in between) laid/hatched eggs, allowing us to compare laying and hatching sequences across different clutch sizes. The same model was repeated considering fledgling body mass and fledgling wing length as dependent variable where nest as random variable and brood size as covariate was the only variables retained by the model (both  $p < 0.0015$ ).

## RESULTS

### Hatching asynchrony and nestling mortality

We could determine hatching span of 86 broods, being the time elapsed between the early and last hatched egg  $13.24 \pm 9.65$  h for synchronous ( $n = 26$ ) and  $48.08 \pm 19.76$  h for asynchronous ( $n = 60$ ) broods. Considering only modal clutches that we could determine hatching span and therefore encoded broods as synchronous and asynchronous broods ( $n = 74$ , 4 and 5 eggs), a logistic regression showed that hatching asynchrony was affected by laying date (Wald = 5.2876,  $p = 0.022$ ) but not by clutch size ( $p = 0.084$ ). Asynchronous broods were laid later than synchronous broods (Figure 1). GLIMMIX models showed that fledgling body condition, fledgling mass, egg mass, hatchling mass and size were not significantly different between synchronous and asynchronous broods (all  $p > 0.127$  and always considering year as factor). Fledgling body



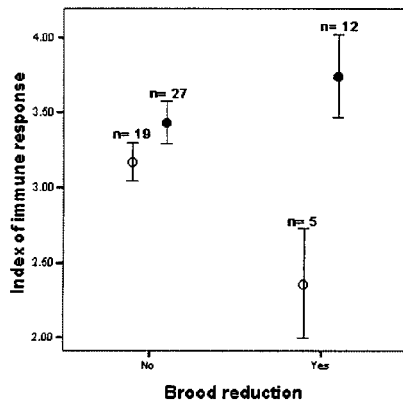
**Figure 1.** Mean  $\pm$  SE of laying date according to hatching asynchrony of broods. Numbers on bars are sample sizes. Note that graphic results are from univariate analysis, for multivariate analyses see results.

Variable	B	Wald	p
Egg mass	-0.0927	0.0681	0.7941
Sex ratio	0.5390	0.2220	0.6376
Hatchling mass	0.2218	0.5545	0.4565
Laying date	0.0388	0.5916	0.4418
Year	0.5355	1.3746	0.2410
Clutch size	-1.2461	1.8347	0.1756
Size difference	-0.0372	2.7358	0.0981
<b>Hatching span</b>	<b>0.0006</b>	<b>4.8143</b>	<b>0.0282</b>

**Table 1.** Logistic regression with brood reduction as dependent variable (1: nest where brood reduction occurred; 0: nest with no brood reduction; n = 74). Sex ratio is expressed as proportion of males within the brood. Other variables are defined in methods.

condition was not affected by brood reduction, hatching asynchrony or the interaction of both ( $p > 0.149$ ).

From the overall number of eggs laid in the three years of the study ( $n = 481$ ), 83.6 % chicks hatched ( $n = 402$ ), and from them 9.7 % ( $n = 37$ ) died after hatch. A logistic regression showed that hatching span was the only variable explaining nest mortality



**Figure 2.** Mean  $\pm$  SE relative immune response according to hatching asynchrony and mortality. Full symbols represent asynchronous broods and open symbols represent synchronous broods. Numbers on bars are broods analysed. Note that graphic results are from univariate analyses, for multivariate analyses see results.

( $n = 245$ , Wald = 16.182,  $p < 0.0001$ ), which assumed 77.3 % of the overall variance of the model (Table 1) suggesting that last hatched chicks in kestrels broods had a higher probability to die. However, no differences in the number of dead fledglings were found between synchronous and asynchronous broods ( $F_{1,71} = 2.656$ ,  $p = 0.108$ ), laying date and clutch size did not affect significantly (both  $p > 0.481$ ). When brood reduction occurred, more chicks died in asynchronous broods ( $\chi^2 = 4.263$ ,  $p = 0.039$ ).

### Immune response and body condition

We evaluated the cell-mediated immune (CMI) response of 217 chicks from 63 different broods over the 3 years of study for clutches with 4 and 5 eggs. The average immune response was  $3.31 \pm 1.11$  mm (2000:  $3.81 \pm 1.20$ ,  $n = 51$ ; 2001:  $2.87 \pm 1.08$ ,  $n = 76$ ; 2002:  $3.41 \pm 0.75$ ,  $n = 90$ ), after controlling for the significant effects of year ( $F_{2,156} = 7.48$ ,  $p = 0.0008$ ) and fledgling mass ( $F_{1,156} = 35.58$ ,  $p < 0.0001$ ), GLIMMIX showed that the relationship between immune response and hatching asynchrony was different depending on the occurrence of nestling mortality (Figure 2). In the same model, no significant relationship was found between immune response and hatching or laying order (Table 2). In addition, females had a better immune response than males (males:  $3.12 \pm 1.04$ ,  $n = 92$ ; females:  $3.48 \pm 1.048$ ,  $n = 100$ ), but sex did not affect immune response once the effect of body mass was included in the model (Table 2). However, the significant effect of the interaction between mortality and sex suggests that differences in immune response were different in relation to nest mortality (Table 2 and Figure 3).



Response term	Explanatory term	Rejected term	Estimate	F	Df.	P
Immune Response	FINAL MODEL	Fledgling sex <sup>†</sup>	-0.1635	1.04	1,133	0.3096
		Nest mortality <sup>†</sup>	-0.1799	2.73	1,133	0.1010
		Fledgling mass	0.0160	20.07	1,133	< 0.0001
		Hatching asynchrony of brood <sup>†</sup>	-0.3197	8.28	1,133	0.1554
		Fledgling sex * Nest mortality	0.6361	5.36	1,133	0.0221
		Hatching asynchrony * Nest mortality	-1.5216	3.93	1,133	0.0494
		Size difference	0.0101	3.27	1,131	0.0730
		Chicks hatched	-0.2073	2.80	1,131	0.0967
		Laying order	-0.0770	1.89	2,128	0.1153
		Hatchling mass	-0.0364	0.71	1,116	0.4015
		Chick asynchrony <sup>†</sup>	-0.0623	0.09	1,114	0.7637
		Hatching order <sup>†</sup>	0.1926	0.51	2,112	0.6004
		Laying date	0.0028	0.06	1,111	0.8044
		Clutch size	-0.0690	0.06	1,111	0.8038
Brood size	0.9527	0.00	1,111	0.9527		
<i>Scaled deviance</i>			148.4423			

**Table 2.** GLMM with normal error and identity link function on intensity of immune response of Eurasian Kestrel fledglings. The model explained 22.3 % of original variance, without considering the deviance explained by parental and nest characteristics which were controlled for by including the nest as a random term nested in year (see methods,  $Z = 3.15$ ,  $P = 0.0008$ ). Other potentially influencing variables and the remaining interactions between the variables were not significant ( $P > 0.07$ ). Parameter estimates and *SE* for the levels of fixed factor (\*) were calculated considering a reference value of zero. In the final model 'hatching asynchrony', 'nest mortality' and 'fledgling sex' variables were maintained to explore interactions.

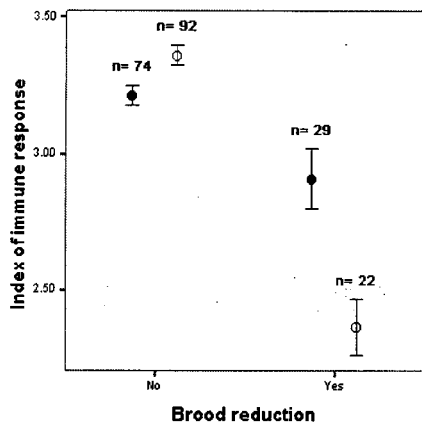
Fledgling body mass was affected by fledgling sex although was not influenced by brood reduction ( $F_{1,156} = 0.00$ ,  $p = 0.951$ ). Separately, males and females did not vary their body mass when brood reduction occurred (males:  $F_{1,58} = 0.85$ ,  $p = 0.360$ ; females:  $F_{1,61} = 0.02$ ,  $p = 0.893$ ). In addition, wing length was affected by brood reduction decreasing when brood reduction occurred ( $F_{1,154} = 6.71$ ,  $p = 0.011$ ) and fledgling sex where females were bigger than males ( $F_{1,154} = 23.68$ ,  $p < 0.0001$ ). However, this variation affected differentially to males and females where wing length of females was not influenced by brood reduction ( $F_{1,59} =$

2.58,  $p = 0.114$ ) while in males decrease significantly ( $F_{1,59} = 5.89$ ,  $p = 0.018$ ).

## DISCUSSION

### Hatching pattern and immune response

Brood reduction was relatively rare in this population (9.7 % of nesting attempts), and the degree of hatching asynchrony was also relatively low, affecting mainly the last hatched chicks, that were also those affected by brood reduction. We found a lower immune response of chicks in synchronous nests with brood reduction. This result may indicate that in the absence of a clear



**Figure 3.** Mean  $\pm$  SE relative immune response according to sex and mortality. Full symbols represent males and open symbols represent females. Numbers above bars are sample sizes. Note that graphic results are from univariate analyses, for multivariate analyses see results.

within-brood size hierarchy all nestlings may attain an impaired physiological condition due to food scarcity, or that synchronous broods require more food due to increased sibling competition (Wiebe & Bortolotti, 1994, Viñuela 1999). This result is in agreement with the brood reduction hypothesis (Lack, 1954), that predicts a better quality or quantity of nestlings in asynchronous broods under food shortage. It means that the early elimination of the last hatched chick could improve the quality of the surviving siblings.

Our results show that hatching asynchrony did not increase fledgling success and that fledgling body condition was not affected by hatching pattern studied, meaning that the benefit of brood reduction could be expressed in other terms than fledgling success or size. Similar results were obtained by Wiehn et al. (2000), who found that brood reduction did not increase the body mass of the surviving fledglings. Therefore, nestling mortality related to

hatching asynchrony could be advantageous only for the physiological condition of chicks, because no effects of brood reduction associated to hatching asynchrony were detected for fledgling mass gain, fledgling final mass or intra-brood size hierarchy. This suggests that the benefit of nestling mortality in relation to energy intake for each nestling could be assigned to maintain and generate a competitive immune response. Our findings suggest the existence of a trade-off between rising a large number of chicks or a reduced brood with a better immune response, because the loss of fitness induced by reduced brood size could be compensated by an improved immune system, that may be a good indicator of survival (Hörak et al. 1999; Soler et al. 1999; Tella et al. 2000b).

In this context, the question remains of why not all the broods in this population are asynchronous, because a relatively high percentage of pairs (35.1 %) hatched their eggs synchronously. Perhaps synchronous broods could be favoured in terms of better offspring survival supported by several studies in different animal groups that suggest that early breeders produce more offspring (Daan et al. 1989; Schultz 1993; Olson & Shine 1997) which have a higher chance of survival and reproduction than those produced by late breeders (Harris et al. 1992.; Ydenberg et al. 1995; Brinkhof et al. 1997).

We did not find differences in immune response between early and last hatched chicks, once the significant effect of fledgling mass was taken into account. This result does not support previous findings (Christie et al. 1998; Saino et al. 2001), and suggests that differences induced by hatching order may be explained by condition of nestlings. However, these

contrasting results among studies could also reflect the existence of different reproductive strategies in relation to hatching order, because in the Eurasian kestrel, the relatively small size hierarchy among sibs imposed by hatching asynchrony could decrease the variance of immune response between early and last hatched chicks. In addition, our results show that nestling mortality is correlated with immune response of a brood only through the interaction with hatching asynchrony, in contrast with previous studies finding an effect of nestling mortality “per se” (Merino et al. 2000; Hörak et al. 1999). Given that in those studies the authors did not consider the effect of hatching asynchrony, it is difficult to decide if those contrasting results are due to differences in ecological strategies followed by each species in relation to hatching patterns, or explained by methodological differences. Overall, these contrasting results indicate that more research is needed to understand the effects of brood reduction on the immune system in different species.

Also, we found that year is a very important factor affecting the immune response that could be related to environmental conditions in different years. To our knowledge, this is the first time that a field study about immunity, measured as PHA response, has been carried out in more than a single year. This result suggests that year-to-year variability should be taken into account in descriptive studies of immune response.

### **Sex and immune response**

Female nestlings tended to show a better immune response than males, but this difference was statistically significant only

in nests where brood reduction did not occur, because male kestrel nestlings in broods with mortality showed a better immune response than in those without mortality. This result partially supports previous findings in captive (Grossman 1985; Olsen & Kovacs 1996) and wild populations of birds (Moreno et al. 2001; Fargallo et al. 2002; Barbosa & Moreno 2002). Body mass had a positive effect on immune response, supporting that fledglings in good condition were able to develop stronger immune responses. However, the effect of sex can not be explained by possible differences in physical condition between sexes, since the significant effect of body mass was taken into account in the models. Thus, our results should be explained by other physiological differences between males and females.

We found that when brood reduction occur, fledglings grow less than in nests where all chicks fledged but not in fledgling mass according to previous findings with Eurasian kestrels (Whien & Korpimäki 1997), suggesting that brood reduction is reflect of food scarcity. However, food scarcity was not reflected in the similar way on males than females because while females fledglings did not vary their body mass or wing length, males decrease significantly their wing length when brood reduction occur. Linked to previous findings that suggest a trade-off between growth and immune response (Merino et al. 2000), our results support partially this idea because only males could be adjust their growth to their immune response, suggesting that under food shortage males, not females, could traded-off growth and immune response. Therefore, it suggests that males under food scarcity conditions could adjust better their growth and were resources

assigned to immune response could be maintained under food scarcity, while females maintain their growth (structural and weight) independently of the environmental conditions. Thus, the high cost of the female high growth rate could be increased under food shortage in terms of the immune defence, suggesting two different strategies for males and females in this food scarcity conditions. Therefore, we suggest that the largest sex (female) in the kestrel nestlings could pay a higher cost in fitness under food shortage by their high growth rate, as precedent results suggest (Drummond 1991; Velando 2002).

Given that we found a more variable immune response in males depending on the rearing environment (occurrence of brood reduction), this result suggests that males could have a more flexible system to assign resources to immunity with respect to other competing traits. In this context, female fledglings are more vulnerable to stress when they are in size disadvantage imposed by hatching asynchrony (unpublished data), supporting a higher physiological flexibility in males. Males developed a stronger immune response when brood reduction occurred, apparently assigning more resources to immune function when food availability is poor (brood reduction occurred). This change in the assignment of resources could be a “security system” to face the effects of parasites or pathogens when food is scarce, or simply reflect that parasites and pathogens are more common in nests suffering from food scarcity, thus inducing higher immune response in those nests. In any case, males in nests in which brood reduction did not occur, apparently removed resources from immune function to other unidentified trait, as could be production of sexual characters (Gil et al.

1999; Verhulst et al. 1999), or more intense activity, perhaps induced by differences in hormonal levels between males and females (Petrie et al. 2001). This finding, however, is partially contrary to the results suggested by Fargallo et al. (2002) in relation to food scarcity in kestrel nestlings in Finland. They found inter-sexual differences in immunity in control and in those broods that were enlarged. However, we suggest that clutch enlargement and brood reduction could generate different responses in siblings.

In our study, brood reduction is a response to food scarcity, may be an adaptive pattern that could improve the immune response of the siblings (see above), while in a Fargallo’s experiment food scarcity is forced experimentally where parents could be constrained in order to improve the manipulation. Therefore, we suggest that although food scarcity conditions is described in both studies, different adaptive responses could be adopted by parents in laying period that is the stage that the female could favour brood reduction, controlling the hatching sequence by the onset of incubation, because the main cause of mortality in our population is the time elapsed between hatchlings as our results suggest.

To conclude, we have shown that brood reduction induced by hatching asynchrony may benefit surviving nestlings by improving their immune response, a trait not considered in earlier studies. This benefit is only apparent for male nestlings, given their comparatively low immune response when compared to females. Tests of Lack’s original hypothesis may be enriched by considerations of crucial aspects of fitness like immunity and by analysing separately effects on male and female nestlings.

## ACKNOWLEDGMENTS

---

We thank J. Viñuela, J. Moreno, and G. Blanco that partially financed field work and helped us with essential ideas for the study. In addition, J. A. Fargallo helped us with valuable comments on the manuscript and fieldwork. Finat family allowing us to conduct the study in their property, and C. Marqués, L. Bolonio and J. San Teodoro for their collaboration in fieldwork. J. Redondo, A. Sáez and R. Pérez were able to repair JMP's computer, thereby saving data and allowing JMP to continue his research. JMP was not supported by any grant during the course of the study.

## REFERENCES

---

- Arnold, T.W., Rohwer, F.C. & Armstrong, T. (1987) Egg viability, nest predation, and the adaptive significance of clutch size in prairie ducks. *American Naturalist*, **130**, 643 – 653.
- Barbosa, A. & Moreno, E. (2002) Sex differences in the T-cell-mediated immune response in wintering great tits *Parus major*. *Avian Science*, **2**, 99 – 102.
- Brinkhof, M.W.G., Cave, A.J. & Perdeck, A.C. (1997) The seasonal decline in the first year survival of juvenile coots, an experimental approach. *Journal of Animal Ecology*, **66**, 73 – 82.
- Clark, A.B. & Wilson, D.S. (1981) Avian breeding adaptations, hatching asynchrony, brood reduction, and nestling failure. *Quarterly Review of Biology*, **56**, 253 – 277.
- Clutton-Brock, T.H. 1991. *The evolution of parental care*. Princeton University Press, Princeton.
- Christe, P., Møller, A.P. & de Lope, F. (1998) Immunocompetence & nestling survival in the house martin, the tasty chick hypothesis. *Oikos*, **83**, 175-179.
- Daan, S., Dijkstra, C., Drent, R. & Meijer, T. (1989) Food supply and the annual timing of avian reproduction. *Acta International Ornithological Congress*, **19**, 392 – 407.
- Dawson, R.D. & Bortolotti, G.R. (2002) Experimental evidence for food limitation and sex-specific strategies of American kestrels (*Falco sparverius*) provisioning offspring. *Behavioral Ecology and Sociobiology*, **52**, 43 – 52.
- Dijkstra, C., Bult, A., Bijlsma, S., Daan, S., Meijer, T. & Zijlstra, M. (1990) Brood size manipulation in the kestrel (*Falco tinnunculus*), effects on offspring and parent survival. *Journal of Animal Ecology*, **59**, 269 – 285.
- Drummond, H., Osorno, J.L., Torres, R., García Chavelas, C. & Larson, H.M. (1991) Sexual size dimorphism & sibling competition, implications for avian sex ratios. *American Naturalist*, **138**, 623 – 641.
- Fair, J.M., Hansen, E.S. & Ricklefs, R.E. (1999) Growth, developmental stability & immune response in juvenile Japanese quails (*Coturnix coturnix japonica*). *Proceedings of the Royal Society of London B*, **266**, 1735 – 1742.
- Fargallo, J.A., Blanco, G., Potti, J. & Viñuela, J. (2001) Nestbox provisioning in a rural population of Eurasian Kestrels, breeding performance, nest predation & parasitism. *Bird Study*, **48**, 236-244.
- Fargallo, J. A., Laaksonen, T., Pöyri, V. & Korpimäki, E. (2002) Inter-sexual differences in the immune response of Eurasian kestrel under food shortage. *Ecology Letters*, **5**, 95 – 101.
- Fridolfsson, A.K. & Ellegren, H. (1999) A simple & universal method for molecular sexing of non-ratite birds. *Journal of Avian Biology*, **30**, 116-121.
- Gil, D., Graves, J., Hazon, N. & Wells, A. (1999) Male attractiveness & differential testosterone investment in zebra finch eggs. *Science*, **286**, 126-128.
- González, G., Sorci, G., Møller, A.P., Ninni, P., Haussy, C. & de Lope, F. (1999) Immunocompetence and condition-dependent sexual advertisement in male house sparrow (*Passer domesticus*). *Journal of Animal Ecology*, **68**, 1225 – 1234.
- Grossman, 1985. Interaction between the gonadal steroids and the immune system. *Science*, **227**, 257 – 261.

- Hamilton, W.D. & Zuk, M. (1982) Heritable true fitness and bright birds, a role for parasites?. *Science*, **218**, 384-387.
- Hanssen, S.A., Engebretsen, H. & Erikstad, K.E. (2002) Incubation start and egg size in relation to body reserves in common eider. *Behavioural Ecology and Sociobiology*, **52**, 282-288.
- Harris, M.P., Halley, D.J. & Wanless, S. (1992) The postfledging survival of young guillemots *Uria aalga* in relation to hatching date and growth. *Ibis*, **134**, 335 - 339.
- Hörak, P., Tegelmann, L., Ots, I. & Møller, A.P. (1999) Immune function & survival of great tit nestling in relation to growth conditions. *Oecologia*, **121**, 316-322.
- Johnsen, A., Andersen, V., Sunding, C. & Lifjeld, J.T. (2000) Female bluethroats enhance offspring immunocompetence through extra-pair copulation. *Nature*, **406**, 296 - 299.
- Korpimäki, E. (1989) Breeding performance of Tengmalm's owl *Aegolius funereus*, effects of supplementary feeding in a peak vole year. *Ibis*, **131**, 51 - 56.
- Lack, D. (1954) *The natural regulation of animal numbers*. Oxford University Press.
- Littell, R.C., Milliken, G.A., Stroup, W.W. & Wolfinger, R.D. (1996) *SAS system for mixed models*. SAS Institute. Cary. New York.
- Martin, T.E., Møller, A.P., Merino, S. & Clobert, J. (2001) Does clutch size evolve in response to parasites & immunocompetence?. *Proceedings of the National Academy of Sciences of the USA*, **98**, 2071 - 2076.
- Martin II, L.B., Scheuerlein, A. & Wikelski, M. 2003. Immune activity elevates energy expenditure of house sparrows, a link between direct & indirect costs?. *Proc. R. Soc. Lond. B.*, **270**, 153 - 158.
- Massemin, S., Korpimäki, E., Pöyri, V. & Zorn, T. (2002) Influence of hatching order on growth rate and resting metabolism of kestrel nestlings. *Journal of Avian Biology*, **33**, 235 - 244.
- Merino, S., Møller, A.P. & de Lope, F. 2000. Seasonal changes in cell-mediated immunocompetence & mass gain in nestling barn swallows, a parasite-mediated effect?. *Oikos*, **90**, 327-332.
- Moreno, J., Potti, J., Yorio, P. & García, P. (2001) Sex differences in cell-mediated immunity in the Magellanic penguin *Spheniscus magellanicus*. *Annales of Zool. Fenn.*, **38**, 111 - 116.
- Moreno, J., Sanz, J.J. & Arriero, E. (1999) Reproductive effort & T-lymphocyte cell-mediated immunocompetence in female pied flycatcher *Ficedula hypoleuca*. *Proceedings of the Royal Society of London B.*, **266**, 1105 - 1109.
- Newton, I. (1979) *Population ecology of raptors*. T&AD Poyser.
- Nilsson, J.Å. 1993. Energetic constraints on hatching asynchrony. *American Naturalist*, **142**, 712 - 717.
- Olsen, N.J. & Kovacs, W.J. (1996) Gonadal steroids and immunity. *Endocrinology Review*, **17**, 369 - 384.
- Olson, M. & Shine, R. (1997) The seasonal timing of oviposition in sand lizards (*Lacerta agilis*), why early clutches are better. *Journal of Evolutionary Biology*, **10**, 369 - 381.
- Petrie, M., Schwabl, H., Brande-Lavridsen, N & Burke, T. (2001) Sex differences in avian hormone levels. *Nature*, **412**, 498-499.
- Saino, N., Calza, S. & Møller, A.P. (1998) Effects of a dipteran ectoparasites on immune response and growth trade-offs in barn swallow, *Hirundo rustica*, nestlings. *Oikos*, **81**, 217 - 228.
- Saino, N., Incagli, M., Martinelli, R., Ambrosini, R. & Møller, A.P. (2001) Immunity, growth & begging behaviour of nestling Barn Swallows *Hirundo rustica* in relation to hatching order. *Journal of Avian Biology*, **32**, 263 - 270.
- Schultz, E.T. (1993) The effect of birth date on fitness of female dwarf perch, *Micronotrus minimus* (Perciformes, Embiotocidae). *Evolution*, **47**, 520 - 539.
- Smits, J.E., Bortolotti, G.R. & Tella, J.L. (1999) Simplifying the phytohaematoglutinin skin-testing technique in studies of avian immunocompetence. *Functional ecology*, **13**, 567-572.
- Soler, M., Martín-Vivaldi, M., Marín, J.M., Møller, A.P. (1999) Weight lifting and health

- status in the black wheatear. *Behavioral Ecology*, **10**, 281 – 286.
- Sorci, S., Soler, M & Møller, A.P. (1997) Reduced immunocompetence of nestlings in replacement clutches of the European magpies (*Pica pica*). *Behavioral Ecology*, **10**, 281 – 286.
- Stoleson, S.H. & Beisinger, S.R. (1995) Hatching asynchrony and the onset of incubation in birds, revisited. When is the critical period?. *Current Ornithology*, **12**, 191-270.
- Tella, J.L., Scheuerlein, A. & Ricklefs, R.E. (2002) Is cell-mediated immunity related to the evolution of life-history strategies in birds?. *Proceedings of the Royal Society of London B*, **269**, 1059 – 1066.
- Tella, J.L., Bortolotti, G., Dawson, R.D. & Forero, M. G. (2000a) The T-cell-mediated immune response and return rate of fledgling American kestrel are positively correlated with parental clutch size. *Proceedings of the Royal Society of London B*, **267**, 891 – 895.
- Tella, J.L., Bortolotti, G., Forero, M.G. & Dawson, R.D. (2000b) Environmental and genetic variation in T-cell-mediated immune response of fledgling American kestrels. *Oecologia*, **123**, 453- 459.
- Tschirren, B., Fitze, P.S. & Richner, H. (2003) Sexual dimorphism in susceptibility to parasites & cell-mediated immunity in great tit nestlings. *Journal of Animal Ecology*, **72**, 839 – 845.
- Velando, A. 2002. Experimental manipulation of maternal effort produces differential effects in son and daughters, implications for adaptive sex ratios in the blue-footed boobies. *Behavioral Ecology*, **13**, 443 – 449.
- Velando, A., Lessells, C.M. & Márquez, J.C. (2001) The function of female and male ornaments in the Inca tern, evidence for link between ornament expression and both adult condition and reproductive performance. *Journal of Avian Biology*, **32**, 311 – 318.
- Verhulst, S., Dieleman, S.J. & Parmentier, H.K. (1999) A tradeoff between immunocompetence and sexual ornamentation in domestic fowl. *Proceedings of the National Academy of Sciences of the USA*, **96**, 4478 – 4481.
- Viñuela, J. (2000) Opposing selective pressures on hatching asynchrony, egg viability, brood reduction & nestling growth. *Behavioral Ecology and Sociobiology*, **48**, 333-343.
- Viñuela, J. (1999) Sibling aggression, hatching asynchrony, & nestling mortality in the black kite (*Milvus migrans*). *Behavioral Ecology and Sociobiology*, **45**, 33-45.
- Viñuela, J. & Carrascal, L.M. (1999) Hatching patterns in nonprecocial birds, a preliminary comparative analysis (eds N.J. Adams, & R.H. Slotow). *Proceedings of the 22 International Ornithological Congress*, 584-599.
- Whitaker, S. & Fair, J. (2002) The cost of immunological challenge to developing mountain chickadees, *Poecile gambeli*, in the wild. *Oikos*, **99**, 161 – 165.
- Wiebe, K.L. & Bortolotti, G. (1994) Energetic efficiency of reproduction, the benefits of asynchronous hatching for American kestrels. *Journal of Animal Ecology*, **63**, 551 – 560.
- Wiebe, K.L., Korpimäki, E. & Wiehn, J. (1998) Hatching asynchrony in Eurasian kestrels in relation to the abundance and predictability of cyclic prey. *Journal of Animal Ecology*, **67**(6), 908 – 917.
- Wiehn, J., Petteri, I., Korpimäki, E., Pahkala, M. & Wiebe, K. (2000) Hatching asynchrony in the Eurasian kestrel *Falco tinnunculus*, an experimental test of the brood reduction hypothesis. *Journal of Animal Ecology*, **69**, 85-95.
- Wiehn, J. & Korpimäki, E. (1997) Food limitation on brood size, experimental evidence in the Eurasian kestrel. *Ecology*, **78**, 2043 – 2050.
- Ydenberg, R.C., Clark, C.W. & Harfenist, A. (1995) Intraspecific fledging mass variation in Alcidae, with special reference to the seasonal fledging mass decline. *American Naturalist*, **145**, 412 – 433.



UNIVERSIDAD DE ALCALÁ  
SERVICIO DE POSTGRADO

DILIGENCIA PARA HACER CONSTAR QUE EL  
PRESENTE EJEMPLAR DE LA TESIS PRESENTADA  
POR D. Jesús Martínez Padilla  
CONSTA DE 128 PAGINAS Y HA SIDO ENTREGADA  
CON FECHA 29 de octubre de 2001  
A EFECTOS DE DEPOSITO DE TESIS.

EL FUNCIONARIO.



Reunido el Tribunal que suscribe en  
el día de la fecha acordó otorgar  
a la presente Tesis Doctoral la  
calificación de .....

SOBRESALIENTE " CUM LAUDE "

POR UNANIMIDAD

Alcalá de Henares, 15 de MARZO de 2001

Roberto Bar Ramos.

José Miguel Apomero

José Luis Telle

Alberto Velasco

Juan José Soler