



TESIS DOCTORAL

# Aproximación múltiple a las estrategias vitales en passeriformes

---

Estudios en competencia espermática, muda, parasitismo y coloración de plumas

**Ignacio García Hermosell**

Departamento de Anatomía, Biología Celular y Zoología

2015



TESIS DOCTORAL 2015

Departamento de Anatomía, Biología Celular y Zoología

# **Aproximación múltiple a las estrategias vitales en paseriformes**

---

Estudios en competencia espermática, muda, parasitismo y coloración de plumas

Ignacio García Hermosell

Conformidad de los directores:

Fdo:

Fdo:

Alfonso Marzal Reynolds

Javier Balbontín Arenas



DOCTORAL THESIS 2015

# **Multi-focus approach to life histories in passerines**

---

Studies on sperm competition, moult, parasitism and feather coloration

Ignacio García Hermosell

A mi padres, mis hermanas  
y a Guada



CONTENTS

<b>AGRADECIMIENTOS - AKNOWLEDGMENTS.....</b>	<b>7</b>
<b>INTRODUCTION .....</b>	<b>12</b>
ECOLOGY, LIFE HISTORIES AND EVOLUTION .....	12
IMPROVING METHODOLOGIES: MULTIVARIATE STATISTICS TO SEX BIRDS .....	12
FEATHERS AND THEIR ROLE IN LIFE HISTORY TRAITS.....	14
SPERM COMPETITION .....	16
OBJECTIVES.....	19
REFERENCES.....	20
<b>CAPÍTULO 1 .....</b>	<b>27</b>
INTRODUCTION .....	29
MATERIAL AND METHODS .....	30
RESULTS .....	32
DISCUSSION .....	33
ACKNOWLEDGMENTS .....	33
REFERENCES.....	34
<b>CAPÍTULO 2 .....</b>	<b>41</b>
INTRODUCTION .....	43
MATERIALS AND METHODS .....	45
RESULTS .....	51
DISCUSSION .....	53
ACKNOWLEDGEMENTS .....	58
REFERENCES.....	58
<b>CAPÍTULO 3 .....</b>	<b>77</b>
INTRODUCTION .....	79
MATERIALS AND METHODS .....	81

RESULTS .....	84
DISCUSSION.....	86
REFERENCES.....	91
<b>CAPÍTULO 4.....</b>	<b>105</b>
INTRODUCTION .....	107
MATERIAL AND METHODS .....	109
RESULTS .....	112
DISCUSSION.....	113
REFERENCES.....	117
<b>CAPÍTULO 5.....</b>	<b>127</b>
INTRODUCTION .....	129
MATERIAL AND METHODS .....	130
RESULTS .....	131
DISCUSSION.....	132
REFERENCES.....	133
<b>GENERAL RESULTS AND DISCUSSION.....</b>	<b>139</b>
REFERENCES.....	145
<b>CONCLUSIONS.....</b>	<b>150</b>
<b>PAPERS .....</b>	<b>151</b>

## **AGRADECIMIENTOS - AKNOWLEDGMENTS**

Siempre me llamó la atención este apartado de agradecimientos. Y siempre me parecieron exagerados. Pero cuando me he visto aquí, con la tesis terminada, y he vuelto la vista atrás me he dado cuenta del porqué de todos esos vastos reconocimientos. Y es que es la tesis no es sólo un largo trabajo de investigación. Son muchas las personas que conoces durante los años en los que la estás desarrollando. Personas que influyen de forma más o menos directa en ella, pero que te pueden marcar durante este periodo tan importante. Por eso me temo que ésta va a ser otra de esas largas listas de agradecimientos...

Mi tesis ha tenido dos grandes etapas: la extremeña y la asturiana, así que iré por orden cronológico. En Badajoz tengo que nombrar por supuesto a mi familia, y en especial a mis padres y mis hermanas. Mis padres siempre apoyaron mis decisiones, estuviesen más o menos de acuerdo con ellas. Siempre estuvieron ahí de forma incondicional. Sin vosotros jamás habría llegado tan lejos. Os debo mucho más de lo que podría describir en este pequeño espacio. ¡Ahora al fin podremos descorchar esa botella que tanto tiempo lleva reservada!

Mis amigos, que aunque nunca tuvieron muy claro que en qué trabajaba más allá de “pajaritos” y “tapar nidos”, siempre han estado en los buenos y los malos momentos cada vez que los he necesitado. La lista de todos ellos es (afortunadamente) muy larga, y variada y lamento no poder nombrarlos a todos. Desde aquellos que conozco desde que tengo uso de razón, la vieja guardia, (Marti, Juanma, Bree, Luisete), los que se fueron sumando en el camino (Toni, Lines, Soraya y Tomás, Suizo y Ana, Beto, Chema y Marisa, Jhoani) y los que volvieron del pasado y hasta del extranjero para quedarse (Mario, Rebeca, Montse). De entre todos ellos les tengo que dedicar un aparte a Raquel (son muchos años ya) y a Jordi (que una época muy baja me ayudó a volver a estar a tope). Y por supuesto a Nando, con quien comparto neurona, nave nodriza y un largo historial de épicas aventuras, viajes y cursos.

Pero también he de extender mi agradecimiento a sus padres y su hermana, que durante muchos años fueron (y siguen siendo) mi segunda familia. Muchas gracias!!

Tras la carrera siguieron llegando nuevos amigos (Alf, que no me aguantas una party, Jorge, Wen...) y una de mis mejores etapas. Gracias a El Dragón Verde y los Hijos del Dragón mi vida se llenó de grandes momentos y aún mejores personas. Experiencias inolvidables, como aquellos REV, esas tardes de viernes en la Concejalía de Juventud o aquellas cenas multitudinarias en el wok. Conservo muchos recuerdos y muy buenos amigos de entonces. La lista sería más larga que la genealogía de los Grandes Reyes de antaño, así que no me extenderé mucho. Pero sabed que me acuerdo de todos y cada uno de vosotros. A pesar de ello, de este vasto grupo tengo que destacar a algunos, mis jinetes de sangre que siempre han estado ahí, aún ahora en la distancia y que sobrevivieron a la caída de Númenor y al cierre de la tienda y la asociación. Walki, Mon, Zel, Moli, Marta, Diego, Omar, Geep, Kite, Magister, la alocada Sara, Azzacov, Keldorn, Elibella, (compañera “salmantina” de viajes), y Astaroth, que ya es también un biólogo de pleno derecho. Sé que sabréis disculpar que algunos os mente por el nick en vez de por vuestro verdadero nombre, pero tienen un sabor nostálgico que no me resisto a paladear. Y también tengo que dar las gracias a Desi, que me apoyó y me acompañó durante casi toda esta etapa extremeña de mi tesis. ¡Mil gracias a todos por haber estado ahí!

Después ya empecé a currar en la universidad con aquella FPI, iniciando esta gran andadura que ahora culmina. Gracias a Carlos Navarro, porque con él me metí en este mundillo. Gracias a ti aprendí a manipular y medir gorriones en el antiguo aviario . Gracias a mis compañeros de despacho por tantas, tantísimas horas de paciente trabajo de campo, por los innumerables aviones y golondrinas que pasaron por nuestras manos y las incontables celdas de excel a nuestras espaldas: Carmen, Yolanda, Maribel, Sergio, Luz (joven padawan convertida ya en toda una doctora)... ¡sois los mejores!. Y por supuesto al resto de gente que poblaba el tercer piso. Entre todos hacíais que trabajar allí fuese un placer. Y me es imposible

hablar de la universidad sin tener un recuerdo especial para mis entrañables compañeros de carrera: el inefable Fernando Mayo, Mario (ya todo un papá), Paconan (otra padrazo también), Paco Ferri y Marga (que pese a todo lo pasado, nunca habría llegado a biólogo sin sus apuntes y su amistad). Por todas esas piedras levantadas en busca de anfibios y reptiles, por tantas cañas departamentales, por todo el tiempo que pasamos juntos. Gracias por todo.

Pero mi tesis tiene también una vertiente asturiana, si no en la investigación en sí, al menos en el tiempo y el espacio. Una época de cambios muy importantes, con receso en la tesis incluido, que me marcó profundamente. Mi más hondo agradecimiento a Sergi, compañero de andanzas lobunas en Portugal y responsable directo de que acabase en tierras astures. Y por supuesto a la encantadora Marian. Ambos me acogieron nada más llegar a la tierrina y me hicieron sentirme como en casa. ¡Sois muy grandes! Tampoco puedo evitar acordarme de esa gran familia de Biosfera, que me recibieron como uno más desde el primer día y con la que tantos kilómetros, tantos jaggermeister y tantos chuletones he compartido. Gracias a todos los que pasaron por allí, a los que estaban cuando llegué, a los que siguieron cuando me fui, a los que se fueron, a los que llegaron, a los de prácticas. Hicisteis que Asturias fuese mucho mejor de lo que ya era.

Y de Asturias también tengo que hablar de mi grupo de amigos, de esas noches frikis visitando los Sietes Reinos. De esas noches de billares y dardos y de esas cervecitas en el Bartolomeo. Cuestiones de espacio me impiden nombraros a todos como os merecéis, pero estoy seguro de que sabéis que mi agradecimiento va por vosotros. Sin embargo no me puedo ir sin una mención especial a Nuria, Eva, Carla y Adriana. Vosotras habéis hecho que Asturias se convierta en mi segundo hogar. Ahora que dejé Asturias sois lo que más echo de menos. Gracias por haber estado ahí. Gracias por el apoyo y gracias por haber sido parte de mi día a día.

Pero la vuelta a Badajoz me trajo algo maravilloso. Y su nombre es Guada. Ella se ha convertido en el motor que me dio fuerzas para seguir con esto. Ella da color, da luz a todo lo

que me rodea y gracias a ella me siento capaz de cualquier cosa. ¡Hasta de acabar esta tesis! Gracias por confiar en mí. Gracias por seguir a mi lado a pesar de todo. No hay espacio suficiente ni palabras que te hagan justicia. Son tantas las cosas que podría decirte y tan poco el espacio del que dispongo que tendré que resumirlo todo en un enorme y sincero ¡GRACIAS!

También quiero agradecer a aquellos que me acogieron durante las estancias breves (y no tan breves). A Juan José Soler, en Almería, y a todos los que conocí allí. No sabéis cuánto disfruté de El Chumbo. Thanks to Anders Moller for his wise advise while I was in Paris and for that great dinner. And for all those comments that improved the papers so much (specially the last one). And thanks to Jan and his team for all those times he invited me to Oslo. And special thanks to Terje too, who collaborated very closely with me in two chapters of this thesis. I have learnt very very much with all and each one of you.

Y no, no me he olvidado de alguien. Pero les reservo un lugar especial. Y al igual que en los papers el último lugar en la lista de autores es un puesto de honor, les he reservado también el último puesto en esta (interminable) lista de agradecimientos. Son las tres personas que más me han influido en mi vida académica e investigadora y, por supuesto, en esta tesis. Muchas gracias a Tinín, maestro y mentor, que recibió a un bisoño estudiante de biología en su despacho y le hizo un hueco en su equipo. Todo lo que he conseguido hasta ahora como biólogo te lo debo a ti. Se lo debo a tus consejos, a tu guía. Siempre has sido un modelo para mí tanto en lo personal como en lo profesional. Ojalá y puede estar a la altura de todas las oportunidades que me has brindado. Estoy eternamente en deuda contigo. Gracias también a mis directores. Alfonso, con quien he ido aprendiendo a navegar por el proceloso mar de la investigación y quien ha sido el responsable de que ahora pueda estar escribiendo estas líneas. Cuánto he aprendido anillando en la RUCAB (y en tu campo, y en el instituto de Botoa, y en Potosí), y qué bien sabes alternar esas sesiones camperas con esas barbacoas. Gracias por fin a Javier, que estoicamente soportó mis interminables dudas estadísticas (que

Multi-focus approach to life histories in passerines

no fueron pocas). Siempre has estado dispuesto a echarme una mano armado de una paciencia infinita para tratar de hacerme comprensible lo ininteligible. ¡Eres un crack! Ha sido un placer y un privilegio poder trabajar con vosotros tres. Gracias de corazón. Vosotros sois mi familia en el ámbito de la investigación. Sin vosotros esta tesis nunca habría existido y yo, probablemente, no sería el mismo.

¡Gracias a todos!

## **Introduction**

### **Ecology, life histories and evolution**

Haeckel (1866) was the first using the word “Ecology” (from the Greek oikos, meaning “home”, and from logos, meaning “study”). From this simple definition Ecology has been growing and developing in many different branches, always focusing on the conspecific and interspecific relationships between living beings and their environments where they carry on their activities. Nowadays most of the purely biological research is made from an ecological approach.

Evolutionary Ecology is a proxy to Ecology that tackles relationships among living beings and their abiotic environment, focusing on how those relationships and behaviors have originated and evolved. It deals with understanding of how biological lineages change through the time, split and ultimately go extinct (Mayhew 2006). Evolutionary Ecology is a complex research field whose methodology takes part of other scientist branches as Mathematics (Grecian et al. 2003), Chemistry (Wakamatsu and Ito 2002), Physics (Fleishman et al. 2006) or Genetics (Harvey et al. 2006). All these different approaches are continuously evolving.

Life histories are one of the main subjects of study in current Ecology research. They are a group of traits associated with variation in fecundity and survival along the life of one individual, being responsible for maximizing individual fitness. Some major traits are age of first reproduction, aging, life span or senescence. All these life histories are shaped by natural selection by mean of interactions with other living beings and with a dynamical environment like the current climatic change.

### **Improving methodologies: multivariate statistics to sex birds**

Some techniques have been usually employed in Ecology research for decades. But the recent advances in all scientific areas have made them to evolve rapidly, thus a continuous update and innovation in such methodologies is required. Research on improving accuracy in a wide range of techniques is a previous step before carry on further basic research. For

instance, in Evolutionary Ecology, and especially when birds are the model species, many experimental studies require the knowing of the sex of individuals at their first capture. Observations of breeding behavior in the field have been traditionally used to find out the sex of non-dimorphic birds (Delestrade 2001). However, this technique has the disadvantage of that the most diagnostic behavior is usually during courtship, which occurs after a long time of the arrival of individuals to the breeding quarters.

The recent development of Genetics has provided a whole set of new molecular techniques to precisely identify the sex of individuals from a small tissue sample (Griffiths et al. 1998). But these methodologies are expensive and require resources and devices that might be not available for many researchers. Moreover, these techniques can't be immediately applied on the field and consequently, all the biological material must be processed and analyzed in a laboratory, which might be quite time consuming to get a big number of individuals correctly sexed. Computing has also undergone a rapid development in the last decades, thus favoring the blossoming of multivariate statistical methods to sex size dimorphic species. For instance, morphological data have been used to build discriminant functions to sex several bird species (Balbontín et al. 2001; Sarasola and Negro 2004; Figuerola et al. 2006). These techniques have the advantages that, once a model is obtained, it can be applied directly in the field, using just a common calculator. Therefore, this methodology is an inexpensive, fast and very accurate way to sex individuals just after their arrival.

The barn swallow *Hirundo rustica* is a model species in many avian studies that require knowledge of the sex of individuals with accuracy (de Lope and Møller 1993; Garamszegi et al. 2005; Cuervo and Møller 2006). In many of these investigations individuals have been sexed after observation of presence (females) or absence (males) of a brood patch (Saino and Møller 1995; Møller 2002). However, many experimental studies require knowing the sex of individuals at first capture after arrival at the breeding grounds (de Lope and Møller 1993; Cuervo et al. 1996, 2003). So it would be of special interest for researchers using this

small passerine bird as a model species to have a reliable field method to discriminate sexes with precision.

With this aim, our first objective was to develop a more accurate, inexpensive and faster method to sex barn swallows in the field by using multivariate statistic,

### **Feathers and their role in life history traits**

The importance of plumage color as an indicator of individual quality has long been studied in sexual selection. The presence of colored or highly elongated feathers, bright beaks and skins, and the occurrence of elaborated structures have turned birds into an important taxon for studies of sexual selection and intra-sexual competition (Jawor and Breitwisch 2003). The variety of information displayed by these color signs is wide and rich. Two main pigments are accountable for many of these colorations: melanins and carotenoids. Melanins can be synthesized by animals from the common precursor, L-tyrosine, to produce two different pigments: pheomelanin, which is responsible for the reddish-brown colors, and eumelanin, which is responsible for gray to black color (Prota 1992; Hearing 1993; Ito and Wakamatsu 2006; Simon et al. 2009). In contrast, carotenoids must be obtained directly from the diet by the individual (Goodwin 1984). Therefore, the production of carotenoid-based signals is assumed to be highly condition-dependent and indicators of foraging ability (Møller et al. 2000; Olson and Owens 1998; Peters et al. 2007, 2008). However, there is not scientific consensus that melanin-based coloration reflects the condition of individuals and hence the role in sexual selection.

Here we investigate whether MBC is condition-dependent and how it reflects different components of fitness in barn swallows. Specifically, we study if melanin-based coloration of dorsal feathers in barn swallows covary with body condition and tail length, which is a well-known secondary sexual character in males of this species. We also assessed if melanin-based coloration was correlated with adult survival rate of males and females, arrival or laying date. Finally, we investigate how coloration of back feathers of the two members of the

breeding pair is correlated with the number of fledglings produced during a given year.

Bird feathers are also constantly exposed to abrasion (Butler and Johnson 2004), ultraviolet light (Bergman 1982) or bacterial degradation (Burt and Ichida 1999) that might break the whole or part of the feather. Moulting renews these worn or broken feathers, improving flying performance and thermo-regulation, thus increasing their fitness. Molt is a highly costly process in terms of energy and survival for birds (Swaddle et al. 1999). Also, the moult process involves changes in nutritional and energetic demands of birds (Klaassen 1995; Murphy 1996), and important reallocation of resources among organs and functions (Murphy and Taruscio 1995; Murphy 1996; Nava et al. 2001). Accordingly, both the duration and timing of moult is usually adjusted within the annual cycle of birds to minimise the overlap with other energetically demanding activities such as breeding and migration (Jenni and Winkler 1994; Yuri and Rohwer 1997; de la Hera et al. (2009). For instance, many birds moult their feathers in the winter quarters, prior to migration, so any delay in moult timing would affect to the arrival to the breeding quarters, with the subsequent costs in breeding performance (Kokko 1999; Møller et al. (2009).

Parasitism has turned into one of the most powerful forces modeling life histories through time, including moult patterns. Although avian malaria and related haemosporidian parasites are known to exert negative effects on the life histories traits of their avian hosts (Marzal et al. (2005, 2008; Valkiūnas 2005; Palinauskas et al. ( 2008), whether avian blood parasites affect the growth rate of an individual feather still remains unknown.

With this aim, here we analyse whether the presence of malaria infection during the breeding season is associated with the feather growth rate during moult the preceding winter in Africa. Also, we address the carry-over effects of these two events taking place at the African winter grounds (rectrix growth rate and haemosporidian infection) on the breeding success in Europe

## **Sperm competition**

Sperm competition is the competitive process between spermatozoa of two or more different males to fertilize the same ova (Parker 1970). Sperm competition is an evolutionary pressure on males, and has led to the development of adaptations to increase males' chance of reproductive success. Hence, it should favor those traits that enhance and improve male ability to fertilize females. In this sense, two main powerful selective forces have been suggested to drive and shape sperm morphology and variability: i) the physical, chemical and immunologic obstacles that sperm cells face in female reproductive track (Cohen and McNaughton 1974; Birkhead et al. 1993), and ii) the strong competition between spermatozoa from different males to fertilize promiscuous females (Parker 1998).

Sperm morphology shows a really high variability in Animal kingdom (Cohen 1977). But the difference is specially striking in birds (Laskemoen et al. 2007; Kleven et al. 2008; Lifjeld et al. 2010), even among different populations on the same species (Pitnick et al. 2003; Joly et al. 2004). A typical sperm in birds shows three different and clearly separated parts: the head, a helical body or midpiece, and the tail or flagellum. The range of variation in this taxon reflects not only in morphology but also in relative and absolute length of the different parts. This variability occurs not only at interspecific (Lüpold et al. 2009b), but also at intraespecific level (Laskemoen et al. 2007), or even between different ejaculates within the same individual. Thereby, postcopulatory selection becomes a really important factor that might shape and drive species evolution, pushing towards speciation through geographical variability. However, very little is still known about geographical variation in sperm size.

Recent comparative studies in passerine birds have shown that intraespecific variation in total sperm length and other sperm components are negatively associated with the risk of sperm competition (Calhim et al. 2007; Lifjeld et al. 2010). This can be interpreted as evidence of stronger stabilizing selection on males producing an optimal sperm type when the risk of sperm competition increases. However, this relationship has not been investigated among

populations of the same species. Also, it is well known that levels of extrapair paternity vary among populations (Petrie and Kempenaers 1998).

Therefore, another objective of this research is to investigate if this variation amongst populations in sperm competition could also be reflected in between-male variation in sperm size.

It has also been shown that, under high risk of predation, an elongation of the midpiece favour faster swimming abilities although total sperm length didn't differ among populations (Kleven et al. 2008; Immler et al. 2008; Elgee et al. 2010; Lifjeld et al. 2010).

Furthermore, many external factor might be affecting to sperm performance and therefore to reproductive success. Some of these factors can be female's cryptic choice of sperm (Møller et al 2008b; Gasparini and Pilastro 2011), sperm morphology (Lüpold et al. 2009a), or DNA damage by the attack of reactive oxygen species (ROS) (De Lamirande et al. 1997; Tremellen 2008). Nowadays another factor has gained in importance: radioactive contamination. Radioactivity is naturally presented in environment in low non-toxic doses. But recent catastrophes of Fukushima and Chernobyl have released into the environment huge amount of radioactive isotopes, whose effects on living beings will prevail for hundreds of years because of the long half-life of some of those isotopes. After the accident in the reactor number 4 at Chernobyl Nuclear Power Plant in 1986, the subsequent radioactive clouds extended over most of the Northern Hemisphere (Yablokov et al. 2009). Flora, fauna and human communities were seriously affected. The frequencies of abnormalities and birth defects increased (Feshchenko et al. 2002; UNDP/UNICEF 2002; Scherb and Weigelt 2003; Lazjuk et al. 2003; Chernobyl Forum 2005a, b) revealing an important incidence on sperm development, with changes in their ultra-structure (Fischbein et al. 1997), decreased number of motile spermatozoa (Sakharov et al. 1996), higher presence of aberrants forms (Makeeva et al. 1996; Møller et al. 2005; Møller et al. 2008a) and lower survival prospects (Møller 1993; Møller et al. 2007).

But despite all these studies, the effect of radioactivity on living beings is still being broadly discussed in scientific literature, as well as the susceptibility of different individuals or even species to those deleterious effects. Further studies on the effects of radioactivity over sperm production and morphology and the subsequent influence on fertility are necessary in all taxa.

For this reason, here we examined the effect of radioactivity on the frequency of aberrant spermatozoa in different passerine species along with the relationship between sperm length and its structural stability.

## **Objectives**

- ✓ To find out an accurate, fast and inexpensive methodology to sex barn swallows in the field
- ✓ To investigate the relationship between melanin-based coloration and fitness and survival in the European barn swallow.
- ✓ To study the effect of avian malaria infection on feather growth rate while moulting.
- ✓ To evaluate the possible costs and the hypothetic carry-over effects of delayed moult in the next breeding season
- ✓ To analyze geographic differences in sperm morphology and in sperm competition in different populations of a widespread species of migratory bird.
- ✓ To assess the effect of radioactivity on patterns of sperm damage in different species of Ukrainian passerines.
- ✓ To investigate the nature of these aberrant forms by using Scanning Electron Microscopy.

## References

- Balbontín J, Ferrer M, Casado E (2001) Sex determination in Booted Eagles (*Hieraaetus pennatus*) using molecular procedures and discriminant function analysis. *J Raptor Res* 35:20–23
- Bergman G (1982) Why are the wings of *Larus f. fuscus* so dark? *Ornis Fenn* 59:77–83
- Birkhead TR, Møller AP, Sutherland WJ (1993) Why do females make it so difficult for males to fertilize their eggs? *J Theor Biol* 161:51–60
- Burt E, Ichida JM (1999) Occurrence of feather-degrading bacilli in the plumage of birds. *Auk* 116:364–372
- Butler M, Johnson AS (2004) Are melanized feather barbs stronger? *J Exp Biol* 207:285–293
- Calhim S, Immler S, Birkhead TR (2007) Postcopulatory sexual selection is associated with reduced variation in sperm morphology. *PloS ONE* 2:e413
- Chernobyl Forum (2005a) Chernobyl's legacy: health, environmental and socioeconomic impacts. New York, NY
- Chernobyl Forum (2005b) Chernobyl: the true scale of the accident. 20 years later a UN report provides definitive answers and ways to repair lives.
- Cohen J (1977) *Reproduction*. Butterworths, London
- Cohen J, McNaughton DC (1974) Spermatozoa: The probable selection of a small population by the genital tract of the female rabbit. *J Reprod Fertil* 39:297–310
- Cuervo JJ, de Lope F, Møller AP, Moreno J (1996) Energetic cost of tail streamers in the barn swallow (*Hirundo rustica*). *Oecologia* 108:252-258
- Cuervo JJ, Møller AP (2006) Experimental tail elongation in male Barn Swallows *Hirundo rustica* reduces provisioning of young, but only in second broods. *Ibis* 148:449–458
- Cuervo JJ, Møller AP, de Lope F (2003) Experimental manipulation of tail length in female barn swallows (*Hirundo rustica*) affects their future reproductive success. *Behav*

Ecol 14:451–456

De la Hera I, Díaz JA, Pérez-Tris J, Luis Tellería J (2009) A comparative study of migratory behaviour and body mass as determinants of moult duration in passerines. *J Avian Biol* 40:461–465

De Lamirande E, Jiang H, Zini A, Kodama H, Gagnon C (1997) Reactive oxygen species and sperm physiology. *Rev Reprod* 2:48–54

De Lope F, Møller AP (1993) Female reproductive effort depends on the degree of ornamentation of their mates. *Evolution* 47:1152–1160

Delestrade A (2001) Sexual size dimorphism and positive assortative mating in Alpine Choughs (*Pyrrhocorax graculus*). *Auk* 118:553–556

Elgee KE, Evans JP, Ramnarine IW, Rush SA, Pitcher TE (2010) Geographic variation in sperm traits reflects predation risk and natural rates of multiple paternity in the guppy. *J Evol Biol* 23:1331–1338

Feshchenko SPS, Schröder HC, Müller WEG, Lazjuk GIG, Schroder H, Muller W (2002) Congenital malformations among newborns and developmental abnormalities among human embryos in Belarus after Chernobyl accident. *Cell Mol Biol (Noisy-le-grand)* 48:423–426

Figuerola J, García L, Green AJ, Ibáñez F, Mañez M, Del Valle JL, Garrido H, Arroyo JL, Rodríguez, R (2006) Sex determination in glossy ibis chicks based on morphological characters. *Ardeola* 53:229–235

Fischbein A, Zabludovsky N, Eltes F, Grischenko V, Bartoov B (1997) Ultramorphological sperm characteristics in the risk assessment of health effects after radiation exposure among salvage workers in Chernobyl. *Environ Health Persp* 105:1445–1449

Fleishman LJ, Leal M, Sheehan J (2006) Illumination geometry, detector position and the objective determination of animal signal colours in natural light. *Anim Behav* 71:463–474

- Garamszegi L, Heylen D, Møller AP, Eens M, de Lope F (2005) Age-dependent health status and song characteristics in the barn swallow. *Behav Ecol* 16:580–591
- Gasparini C, Pilastro A (2011) Cryptic female preference for genetically unrelated males is mediated by ovarian fluid in the guppy. *Proc R Soc B* 278:2495-2501
- Goodwin TW (1984) *The Biochemistry of Carotenoids, Vol 2. Animals*. Chapman & Hall, New York
- Grecian VD, Diamond AW, Chardine JW (2003) Sexing razorbills *Alca torda* at Machias Seal Island, New Brunswick, Canada, using discriminant function analysis. *Atl Seabirds* 5:73–80
- Griffiths R, Double MC, Orr K, Dawson, RJG (1998) A DNA test to sex most birds. *Mol Ecol* 7:1071–1075
- Haeckel E (1866) *Generelle Morphologie der Organismen. Allgemeine Grundzüge der organischen Formen- Wissenschaft, mechanisch begründet durch die von Charles Darwin reformirte Descendenz-Theorie*. Berlin, Alemania
- Harvey MG, Bonter DN, Stenzler LM, Lovette IJ (2006) A comparison of plucked feathers versus blood samples as DNA sources for molecular sexing. *J Field Ornithol* 77:136–140
- Hearing VJ (1993) Unraveling the melanocyte. *Am J Hum Genet* 52:1–7
- Immler S, Calhim S, Birkhead TR (2008) Increased postcopulatory sexual selection reduces the intramale variation in sperm design. *Evolution* 62:1538–43
- Ito S, Wakamatsu K (2006) Chemistry of Melanins. In: Nordlund JJ, Boissy VJ, Hearing VJ, et al (eds) *The Pigmentary System. Physiology and Pathophysiology* 2nd edn. Blackwell Publishing, Oxford, UK, pp 282–310
- Jawor JM, Breitwisch R (2003) Melanin ornaments, honesty, and sexual selection. *Auk* 120:249–265
- Jenni L, Winkler R (1994) *Moult and ageing of european passerines*. Academic Press, London

- Joly D, Korol A, Nevo E (2004) Sperm size evolution in *Drosophila*: inter- and intraespecific analysis. *Genetica* 120:233–244
- Klaassen M (1995) Moulting and basal metabolic costs in males of two subspecies of stonechats: the European *Saxicola torquata rubicula* and the east African *S. t. axillaris*. *Oecologia* 104:424–432
- Kleven O, Laskemoen T, Fossøy F, Robertson RJ, Lifjeld JT (2008) Intraspecific variation in sperm length is negatively related to sperm competition in passerine birds. *Evolution* 62:494–499
- Kokko H (1999) Competition for early arrival in migratory birds. *J Anim Ecol* 68:940–950
- Laskemoen T, Kleven O, Fossøy F, Lifjeld JT (2007) Intraspecific variation in sperm length in two passerine species, the Bluethroat *Luscinia svecica* and the Willow Warbler *Phylloscopus trochilus*. *Ornis Fenn* 84:131–139
- Lazjuk G, Verger P, Gagnière B, Kravchuk Z, Zatsepin I, Robert-Gnansia E (2003) The congenital anomalies registry in Belarus: a tool for assessing the public health impact of the Chernobyl accident. *Reprod Toxicol* 17:659–666
- Lifjeld JT, Laskemoen T, Kleven O, Albrecht T, Robertson RJ (2010) Sperm length variation as a predictor of extrapair paternity in passerine birds. *PLoS ONE* 5:1–8
- Lüpold S, Calhim S, Immler S, Birkhead TR (2009a) Sperm morphology and sperm velocity in passerine birds. *Proc R Soc B* 276:1175–81
- Lüpold S, Linz GM, Birkhead TR (2009b) Sperm design and variation in the New World blackbirds (Icteridae). *Behav Ecol Sociobiol* 63:899–909
- Makeeva AP, Belova NV, Emel'yanova NT, Verygin BV, Ryabov IN (1996) Condition of reproductive system in motley silver carp *Aristichthys nobilis* in cooling pond of Chernobyl NPP after the accident. *Probl Ichtiol* 36:239–247
- Marzal A, de Lope F, Navarro C, Møller AP (2005) Malarial parasites decrease reproductive success: an experimental study in a passerine bird. *Oecologia* 142:541–545

- Marzal A, Bensch S, Reviriego M, Balbontin J, de Lope F (2008) Effects of malaria double infection in birds: one plus one is not two. *J Evol Biol* 21:979–987
- Mayhew PJ (2006) *Discovering Evolutionary Ecology: bringing together ecology and evolution*. Oxford University Press, New York
- Møller AP (1993) Morphology and sexual selection in the barn swallow *Hirundo rustica* in Chernobyl, Ukraine. *Proc R Society B* 252:51–57
- Møller AP (2002) Temporal change in mite abundance and its effect on barn swallow reproduction and sexual selection. *J Evol Biol* 15:495–504
- Møller AP, Balbontín J, Cuervo JJ, Hermosell IG, de Lope F (2009) Individual differences in protandry, sexual selection, and fitness. *Behav Ecol* 20:433–440
- Møller AP, Biard C, Blount JD, Houston DC, Ninni P, Saino N, Surai PF (2000) Carotenoid-dependent signals: Indicators of foraging efficiency, immunocompetence or detoxification ability? *Poultry Avian Biol Rev* 11:137-159
- Møller AP, Mousseau TA, de Lope F, Saino N (2007) Elevated frequency of abnormalities in barn swallows from Chernobyl. *Biol Lett* 3:414–7
- Møller AP, Mousseau TA, Lynn C, Ostermiller S, Rudolfson G (2008a) Impaired swimming behaviour and morphology of sperm from barn swallows *Hirundo rustica* in Chernobyl. *Mut Res* 650:210–216
- Møller AP, Mousseau, TA, Rudolfson G (2008b) Females affect sperm swimming performance: a field experiment with barn swallows *Hirundo rustica*. *Behav Ecol* 19:1343–1350
- Møller AP, Surai P, Mousseau TA (2005) Antioxidants, radiation and mutation as revealed by sperm abnormality in barn swallows from Chernobyl. *Proc R Soc B* 272:247–53
- Murphy ME (1996) Energetics and nutrition of molt. In: Carey C (ed) *Avian energetics and nutritional ecology*. Plenum, New York, pp 158–198
- Murphy ME, Taruscio TG (1995) Sparrows increase their rates of tissue and whole-body protein synthesis during the annual molt. *Comp Biochem Physiol A Mol Integr*

Physiol 111:385–396

Nava MP, Veiga JP, Puerta M (2001) White blood cell counts in house sparrows (*Passer domesticus*) before and after moult and after testosterone treatment. *Can J Zool* 79:145–148

Olson VA, Owens IP (1998) Costly sexual signals: are carotenoids rare, risky or required? *Trends Ecol Evol* 13:510–514

Palinauskas V, Valkiunas G, Bensch S, Bolshakov VC. 2008. Effects of *Plasmodium relictum* (lineage P-SGS1) on experimentally infected passerine birds. *Exp Parasitol* 120:372–380

Parker GA (1970) Sperm Competition and its evolutionary consequences in the insects. *Biol Rev* 45:525–567

Parker GA (1998) Sperm Competition and Sexual Selection. Academic, London

Peters A, Delhey K, Andersson S, van Noordwijk H, Förchler MI (2008) Condition-dependence of multiple carotenoid-based plumage traits: an experimental study. *Funct Ecol* 22:831–839

Peters A, Delhey K, Johnsen A, Kempenaers B (2007) The condition-dependent development of carotenoid-based and structural plumage in nestling blue tits: males and females differ. *Am Nat* 169:S122–S136

Petrie M, Kempenaers B (1998) Extra-pair paternity in birds: explaining variation between species and populations. *Trends Ecol Evol* 13:52–58

Pitnick S, Miller GT, Schneider K, Markow TA (2003) Ejaculate-female coevolution in *Drosophila mojavensis*. *Proc R Soc London B* 270:1507–1512

Prota G (1992) Melanins and Melanogenesis. Academic Press, New York

Saino N, Møller AP (1995) Testosterone correlates of mate guarding, singing and aggressive behavior in Male Barn Swallows, *Hirundo rustica*. *Anim Behav* 49:465–472

Sakharov IY, Evdokimov VV, Atochina EA (1996) Sperm angiotensin-converting enzyme

- activity in Chernobyl victims and patients with chronic prostatitis. *Andrologia* 28:259–260
- Sarasola JH, Negro JJ (2004) Gender determination in the Swainson's hawk (*Buteo swainsoni*) using molecular procedures and discriminant function analysis. *J Raptor Res* 38:357–361
- Scherb H, Weigelt E (2003) Congenital malformation and stillbirth in Germany and Europe before and after the Chernobyl nuclear power plant accident. *Environ Sci Pollut Res* 117–125
- Simon JD, Peles D, Wakamatsu K, Ito S (2009) Current challenges in understanding melanogenesis: bridging chemistry, biological control, morphology, and function. *Pigment Cell Melanoma Res* 22:563–79
- Swaddle JP, Williams EV, Rayner JMV (1999) The Effect of simulated flight feather moult on escape take-off performance in starlings. *J Avian Biol* 30:351-358
- Tremellen K (2008) Oxidative stress and male infertility - A clinical perspective. *Hum Reprod Update* 14:243–258
- UNDP/UNICEF (2002) The human consequences of the Chernobyl nuclear accident: a strategy for recovery. New York, NY
- Valkiūnas G. 2005. Avian malaria parasites and other haemosporidia. CRC, Boca Raton
- Wakamatsu K, Ito S (2002) Advanced chemical methods in melanin determination. *Pigment Cell Res* 15:174–183
- Yablokov AV, Nesterenko VB, Nesterenko AV (2009) Chernobyl: consequences of the catastrophe for people and the environment. *Ann N Y Acad Sci* 1181:1-327
- Yuri T, Rohwer S (1997) Molt and migration in the northern rough-winged swallow. *Auk* 114:249–262

## Capítulo 1

### **Determinación del sexo en la Golondrina común *Hirundo rustica* por medio de análisis discriminante en dos poblaciones europeas**

Se investigaron las diferencias morfológicas en adultos de Golondrina común usando medidas de huesos y plumas tomadas de pájaros de una población nortea (danesa) y otra sureña (española). Empleamos el Análisis de Función Discriminante para ayudar a identificar el sexo de los individuos de ambas poblaciones. El estudio se desarrolló en Badajoz (España) Kraghede (Dinamarca). Obtuvimos dos funciones discriminantes, una para cada población europea, usando datos biométricos de individuos adultos. Estas funciones se validaron usando datos externos de una muestra de una población de golondrina común italiana y otra ucraniana. Se retuvieron tres variables morfométricas en el análisis discriminante para cada una de las poblaciones estudiadas: la longitud de las rectrices externas, la longitud de las plumas interiores de la cola y la longitud de la quilla. En general, estas funciones nos permitieron determinar el sexo de los individuos adultos con una precisión del 90.1% y el 91.9% para las poblaciones española y danesa, respectivamente. La validación externa usando una muestra de la población italiana para la validez la función discriminante española y de la población ucraniana para la función discriminante danesa mostró que el 91% de las golondrinas italianas y el 86% de las ucranianas fueron clasificadas correctamente. Por tanto, las dos funciones discriminantes obtenidas podrían ser muy valiosas en futuros trabajos llevados a cabo en esta especie, ya que permitiría a los investigadores determinar el sexo de los individuos nada más llegar a las áreas de cría sin tener que esperar al principio del cortejo o de la incubación para un sexado fiable.

**Sex determination in barn swallows *Hirundo rustica* by means of discriminant analysis in two European populations**

Ignacio García Hermosell, Javier Balbontín, Alfonso Marzar, Maribel Reviriefo and Florentino de Lope.

Differences in morphology in adult barn swallows were investigated using bone and feather measurements taken from birds from a northern (Danish) and a southern (Spanish) European population. Discriminant Function Analysis was used to help in discriminating the sex of individuals of both populations. The study was carried out in Badajoz (Spain) and Kraghede (Denmark). Two discriminant functions were obtained using biometric data from adult individuals, one for each European population. These functions were validated using external data from a sample of an Italian and Ukrainian barn swallow populations. Three morphometric variables: the length of the outermost tail feathers, the length of the inner tail feathers and the length of the keel were retained in the discriminant function in the two populations studied. Overall, these functions allowed us to determine the sex of adult birds with 90.1 % and 91.9 % accuracy for the Spanish and the Danish populations, respectively. External validation using a sample from the Italian population to validate Spanish discriminant function and from the Ukrainian population to validate the Danish discriminant function showed that 91 % of Italian and 86 % of Ukrainian swallows were correctly classified. Therefore, the two discriminant functions obtained would be highly valuable in future work carried out on this species because it would allow researchers to determine the sex of individuals shortly after arrival to the breeding areas without having to wait for the beginning of courtship or incubation for reliable sexing.

## Introduction

Observation of breeding behaviour in the field was formerly used by researchers as a method to identify the sex of non-dimorphic birds (Samuel 1971; Tella and Torre 1993; Delestrade 2001). Later, with development of computer software, multivariate statistical methods were employed to sex size dimorphic species. For instance, morphological data have been used to build discriminant functions to sex several bird species (Bortolotti 1984a, 1984b; Garcelon et al. 1985; Edwards and Kochert 1987; Ferrer and Delecourt 1992; Bosch 1996; Balbontín et al. 2001; Bertellotti et al. 2002; Grecian et al. 2003; Devlin et al. 2004; Sarasola and Negro 2004; Figuerola et al. 2006). Recently, advances in genetics have allowed sexing of individuals using molecular methods (Ellegren 1996; Griffiths et al. 1998). However, these techniques are expensive, require resources unavailable for many researchers and they cannot be used rapidly in the field.

The barn swallow *Hirundo rustica* has been used as a model species in many avian studies that require knowledge of the sex of individuals with accuracy (de Lope and Møller 1993; Møller 1993, 1994a; Cuervo et al. 1996a, 1996b; Kose et al. 1999; Møller 2003, 2004; Garamszegi et al. 2005; Cuervo and Møller 2006; Balbontín et al. 2007). In many of these investigations individuals have been sexed after observation of presence (females) or absence (males) of a brood patch (Saino and Møller 1995; Møller 2002). However, many experimental studies require knowing the sex of individuals at first capture after arrival at the breeding grounds (de Lope and Møller 1993; Cuervo et al. 1996b, 2003).

Despite the difference in tail length between males and females, both sexes overlap in a narrow range that might bias sex assignment by pure observation, especially in southern populations, due to the clinal variation in sexual size dimorphism in length of the outermost tail feathers (de Lope 1985; Møller 1995). The use of secondary features is also used to assign sex in this species: females usually have lighter red-brown throat patch (Ninni and Møller, unpublished data), whiter belly feathers, smaller white patches in the tail feathers (Kose and

Møller 1999) and tips of tail feathers much wider than males (Møller et al. 1995). But only very experienced observers can sex individuals accurately using these traits. So it would be of special interest for researchers using this small passerine bird as a model species to have a reliable field method to discriminate sexes with precision.

## **Material and methods**

### Study area

Our research group has been studying populations of barn swallows in several farms from 1976 to 2006 on the outskirts of Badajoz, south-west Spain (39° N, 7° W). Danish barn swallows data were collected at Kraghede (57° 12' N, 10° 00' E), Denmark, during 1984 - 2006. The areas were mainly agricultural with scattered groups of trees in Spain (de Lope 1983) and plantations, ponds and hedgerows in Denmark (Møller 1994b). Barn swallows breed in rooms in farm houses, getting permanent access through open doors and windows.

### Field procedures

Just after arrival of birds and before the breeding season we captured adults at dawn by using mist nets placed across windows and doors of the breeding rooms. Each bird was identified with a numbered metal ring and a combination of coloured PVC rings, so we could recognize each adult visually. All birds were also provided with an individual combination of colour markings on their belly feathers using stamp ink. Individuals were sexed from the presence (females) or absence (males) of a brood patch and from observation of breeding behaviour during the courtship and incubation period.

We took several morphological measurements for each individual: length of bill's exposed culmen (CULMEN), tarsus (TAR), keel (KEEL), wing (WING), wingspan (WSPAN), length of the outermost tail feathers (TAILOUT), and length of the inner tail feathers (TAILIN). BILL, TAR and KEEL were measured with a digital caliper to the nearest 0.01mm; WING, WSPAN, TAILOUT and TAILIN were measured with a graduated ruler to the nearest mm. All

birds were measured by the same person every year (FdeL in Spain and APM in Denmark).

We also tracked all reproductive events and identified visually each individual in the pairs using binoculars from inside a hide, to avoid disturbance during reproduction. Once females start laying, we did daily observation sessions inside a hide, observing each occupied nest, with the aim of identifying individuals based on colour rings and ink marks. We were able to identify each individual in the pair and their nest for the majority of the occupied nest sites.

Only those individuals with all their morphological data recorded and whose sex was well known by researchers were used in the analysis. Final sample was 806 individuals (391 males and 415 females) in the Spanish population from one of our study farms and 2115 individuals (1030 males and 1085 females) in the Danish one.

#### Statistical analysis

Multivariate analysis of variance (MANOVA) was used to evaluate differences in morphological traits between sexes and locations. Discriminant Function Analysis (DFA) was used to obtain a mathematical model for sexing birds based on morphometric data. We used a leaving-one-out resampling method (jackknife) to test the performance of the discriminant functions.

We also validated the two discriminant functions using external data taken from other Spanish and Danish barn swallows colonies. Specifically, for the Spanish swallows we used the sample from one of our study colonies "Potosi" (280 males and 301 females,  $n = 581$ ) to obtain the Spanish discriminant function validating it using a sample of individuals captured in other colonies ( $n = 223$ ). For the Danish population, we used 918 males and 973 females ( $n = 1891$ ) leaving a separated sample of the same colony to evaluate the Danish discriminant function ( $n = 223$ ).

Additionally, we also validated the discriminant functions of both populations from a

sample of individuals taken from a different geographical area with the aim of knowing if researchers that use this species as a model on their studies could use reliably these discriminant functions to sex their birds. Concretely, we validated the discriminant function obtained from the Spanish population with a sample of an Italian population ( $n = 100$ ) and the discriminant function obtained from the Danish population with a sample of a Ukrainian population ( $n = 100$ ). Statistical analysis was performed using SPSS 13.01 for Windows software (Lachenbruch and Goldstein 1979; Huberty and Lowman 1997).

## Results

Multivariate analysis of variance (MANOVA) showed that there is a difference in size between the Spanish and Danish barn swallows. This analysis showed there are differences in size between males and females and between populations, as it is stated by the significant interaction between location and sex (Table 1). The most dimorphic traits in both populations were the length of the keel, the length of the inner tail and the length of the outermost tail feather. All morphometric data from both populations are summarized in Table 2.

The discriminant function for adult Spanish barn swallow was:

$$D = 0.256 * KEEL + 0.158 * TAILOUT - 0.154 * TAILIN - 13.139$$

Values of  $D > 0$  correspond to males and values  $D < 0$  to females. This function classified correctly 90.2 % of individuals using a jackknife validation procedure. Individuals with discriminant scores between -0.450 and 0.450 should remain unsexed (17 % of total sample), if the total error committed is wanted to be reduced at 5 % level.

The discriminant function for adult Danish barn swallows was:

$$D = 0.406 * KEEL + 0.125 * TAILOUT - 0.192 * TAILIN - 12.581$$

It classified correctly 91.6 % of individuals using a jackknife validation procedure. The percentage of reliability was 91.9 % when other data from the same population were used.

Individuals with scores between -0.300 and 0.300 should remain unsexed (10 % of total sample), if the total error committed is wanted to be reduced at 5 % level.

Reliability of the two discriminant functions was similar when we used a sample of individuals taken either from our own study areas (but not used to obtain the discriminant functions) or from populations located in a different geographical area. The Spanish function classified correctly 91.0 % and 90.1 % of Italian and Spanish individuals and the Danish function classified correctly 86.0 % and 91.9 % of Ukrainian and Danish individuals (Table 3).

## **Discussion**

The use of three morphometric traits was enough for sexing individuals with a great precision and improved current discriminant methods proposed for this species (Calleja et al. 2006). All biometric measurements can easily be taken in the field with great accuracy, because they show low variability when the same morphometric measure is taken by different observers (Møller et al. 2006). Another advantage of the method proposed is that researchers can select the percentage of individuals correctly classified by selecting a narrower range of the discriminant scores, with the cost of leaving out a sample of individuals to be sexed by other methods. Because we proved that the discriminant functions were reliable enough for sexing Barns swallows from very distant geographical area, we encourage the use of the discriminant function obtained in this study by researchers working with this species in a distant geographical area. We proposed that researchers or naturalists working on populations placed at European southern latitudes should use the discriminant function obtained for the Spanish population, whereas studies on northern populations should use the discriminant function obtained for Danish populations.

## **Acknowledgments**

Thanks to all the people who helped to obtain data in the field, especially F. Mateos, C. Navarro, P. Ninni, J. Cuervo, A. Barbosa, J. Moreno, S. Merino, D. Gil, L. Garamszegi and A. N. Dreiss. Special thanks to Nicola Saino and Anders P. Møller for providing us the Italian

data and the Danish and Ukrainian data, respectively. This research was supported by the Spanish Ministry of Education and Science (CGL-2006-01937). AM was supported by post-doctoral (EX-2006-0557) grant from Spanish Ministry of Education and Science.

## References

- Balbontín J, Ferrer J, Casado E (2001) Sex determination in Booted Eagles (*Hieraaetus pennatus*) using molecular procedures and discriminant function analysis. *J Raptor Res* 35:20-23
- Balbontín J, Hermosell IG, Marzal A, Revieriego M, de Lope F, Møller AP (2007) Age-related change in breeding performance in early life is associated with an increase in competence in the migratory barn swallow *Hirundo rustica*. *J Anim Ecol* 76: 915-925
- Bertellotti M, Tella JL, Godoy JA, Blanco G, Forero MG, Donazar JA, Ceballos O (2002) Determining sex of Magellanic Penguins using molecular procedures and discriminant functions. *Waterbirds* 25:479-484
- Bortolotti GR (1984a) Age and sex size variation in Golden Eagles. *J Field Ornithol* 55:54-66.
- Bortolotti GR (1984b) Sexual size dimorphism and age-related size variation in Bald Eagles. *J Wildl Manage* 48: 72-81
- Bosch M (1996) Sexual size dimorphism and determination of sex in Yellow-legged Gulls. *J Field Ornithol* 67:534-541
- Calleja JA, Pone C, Díaz A, Leal A, Cruz A (2006) Biometría de la Golondrina común (*Hirundo rustica*) en la colonia reproductora de la Universidad Autónoma de Madrid. *Revista de Anillamiento* 16-17:59-62
- Cuervo JJ, Møller AP (2006) Experimental tail elongation in male Barn Swallows *Hirundo rustica* reduces provisioning of young, but only in second broods. *Ibis* 148:449-458
- Cuervo JJ, de Lope F, Møller AP (1996a) The function of long tails in female barn swallows (*Hirundo rustica*): An experimental study. *Behav Ecol* 7:132-136

- Cuervo JJ, Møller AP, de Lope F (2003) Experimental manipulation of tail length in female barn swallows (*Hirundo rustica*) affects their future reproductive success. *Behav Ecol* 14:451-456
- Cuervo JJ, de Lope F, Møller AP, Moreno J (1996b) Energetic cost of tail streamers in the barn swallow (*Hirundo rustica*). *Oecologia* 108:252-258
- de Lope F (1983) La avifauna de las Vegas Bajas del Guadiana. *Doñana Acta Vertebrata* 10:91-121
- de Lope F (1985) Pterilosis y dimorfismo sexual de *Hirundo rustica rustica* L en Extremadura (España). *Ardeola* 32:3-8
- de Lope F, Møller AP (1993) Female reproductive effort depends on the degree of ornamentation of their mates. *Evolution* 47:1152-1160
- Delestrade A (2001) Sexual size dimorphism and positive assortative mating in Alpine Choughs (*Pyrrhocorax graculus*). *Auk* 118:553-556
- Devlin CM, Diamond AW, Saunders GW (2004) Sexing Arctic Terns in the field and laboratory. *Waterbirds* 27:314-320
- Edwards TC, Kochert MN (1987) Use of body weight and length of footpad as predictors of sex in Golden Eagles. *J Field Ornithol* 58:144-147
- Ellegren H (1996) First gene on the avian W chromosome (CHD) provides a tag for universal sexing of non-ratite birds. *Proc R Soc B* 263:1635-1641
- Ferrer M, Delecourt C (1992) Sex Identification in the Spanish Imperial Eagle. *J Field Ornithol* 63:359-364
- Figuerola J, Garcia L, Green AJ, Ibañez F, Mañez F, del Valle JL, Garrido H, Arroyo JL, Rodríguez R (2006) Sex identification in Glossy Ibis chicks based on morphological characters. *Ardeola* 53:229-235
- Garamszegi LZ, Heylen D, Møller AP, Eens M, de Lope F (2005) Age-dependent health status and song characteristics in the barn swallow. *Behav Ecol* 16:580-591
- Garcelon DK, Martell MS, Redig PT, Buoen, LC (1985) Morphometric, karyotypic, and

- laparoscopic techniques for determining sex in Bald Eagles. *J Wildl Manage* 49:595-599
- Grecian VD, Diamond AW, Chardine JW (2003) Sexing razorbills *Alca torda* at Machias Seal Island, New Brunswick, Canada, using discriminant function analysis. *Atl Seabirds*. 5:73-80
- Griffiths R, Double MC, Orr K and Dawson, RJG. 1998. A DNA test to sex most birds. *Mol Ecol* 7: 1071-1075
- Huberty CJ, Lowman LL (1997) Discriminant analysis via statistical packages. *Educ Psychol Meas* 57:759-784
- Kose M, Møller AP (1999) Sexual selection, feather breakage and parasites: the importance of white spots in the tail of the barn swallow (*Hirundo rustica*). *Behav Ecol and Sociobiol* 45:430-436
- Kose M, Mänd R, Møller AP (1999) Sexual selection for white tail spots in the barn swallow in relation to habitat choice by feather lice. *Anim Behav* 58:1201-1205
- Lachenbruch PA, Goldstein M (1979) Discriminant analysis. *Biometrics*. 35:69-85
- Møller AP (1993) Female preference for apparently symmetrical male sexual ornaments in the Barn Swallow *Hirundo rustica*. *Behav Ecol and Sociobiol* 32:371-376
- Møller AP (1994a) Symmetrical male sexual ornaments, paternal care, and offspring quality. *Behav Ecol* 5:188-194
- Møller AP (1994b) *Sexual selection and the Barn swallow*. Oxford University Press, Oxford, UK
- Møller AP (1995) Sexual selection in the Barn Swallow (*Hirundo rustica*). 5. Geographic variation in ornament size. *J Evol Biol* 8:3-19.
- Møller AP (2002) Temporal change in mite abundance and its effect on barn swallow reproduction and sexual selection. *J Evol Biol* 15:495-504
- Møller AP (2003) Sexual selection and extinction: why sex matters and why asexual models are insufficient. *Ann Zool Fennici* 40:221-230

Multi-focus approach to life histories in passerines

Møller AP (2004) Protandry, sexual selection and climate change. *Global Change Biol* 10:2028-2035

Møller AP, de Lope F, Saino N (1995) Sexual selection in the barn swallow *Hirundo rustica*. 6. Aerodynamic adaptations. *J Evol Biol* 8: 671-687

Møller AP, Chabi Y, Cuervo JJ, de Lope F, Kilpimaa J, Kose M, Matyjasiak P, Pap PL, Saino N, Sakraoui R, Schifferli L, Hirschheydt J (2006) An analysis of continentwide patterns of sexual selection in a Passerine bird. *Evolution* 60:856-868

Saino, N Møller AP (1995) Testosterone correlates of mate guarding, singing and aggressive behaviour in male Barn Swallows *Hirundo rustica*. *Anim Behav* 49:465-472

Samuel DE (1971) Field methods for determining the sex of Barn Swallows (*Hirundo rustica*). *Ohio J Sci* 71:125-128

Sarasola JH, Negro JJ (2004) Gender determination in the Swainson's hawk (*Buteo swainsoni*) using molecular procedures and discriminant function analysis. *J Raptor Res* 38:357-361

Tella JL, Torre I (1993) Sexual size dimorphism and determination of sex in the Chough (*Pyrrhocorax pyrrhocorax*). *J Ornithol* 134:187-190

TABLES

**Table 1.** Difference in the size of barn swallows between location and gender using Multivariate Analysis of Variance (MANOVA). Shown statistic is the Hotelling's trace (error df = 2911).

	Value	F	Hypthesis df	P
Location	0.687	285.898	7	< 0.001
Sex	1.242	516.618	7	< 0.001
Location * Sex	0.021	8.671	7	< 0.001

**Table 2.** Morphometric measurements for males and females from both populations (mean  $\pm$  SD, sample in brackets).

	SPAIN		DENMARK	
	Males (280)	Females (301)	Males (981)	Females (973)
CULMEN	7.50 $\pm$ 0.53	7.47 $\pm$ 0.54	7.79 $\pm$ 0.49	7.88 $\pm$ 0.52
KEEL	21.17 $\pm$ 0.90	20.50 $\pm$ 0.92	21.46 $\pm$ 0.74	20.74 $\pm$ 0.76
TAR	11.06 $\pm$ 0.48	11.15 $\pm$ 0.49	11.09 $\pm$ 0.56	11.08 $\pm$ 0.58
WING	121.23 $\pm$ 2.96	119.88 $\pm$ 3.08	126.43 $\pm$ 2.90	124.39 $\pm$ 2.82
TAIL_OUT	99.51 $\pm$ 7.33	85.23 $\pm$ 5.02	109.62 $\pm$ 8.92	91.37 $\pm$ 6.05
TAIL_IN	43.23 $\pm$ 2.14	43.57 $\pm$ 1.64	43.97 $\pm$ 1.64	44.51 $\pm$ 1.69
WSPAN	320.53 $\pm$ 9.35	317.21 $\pm$ 9.38	332.35 $\pm$ 11.64	327.91 $\pm$ 6.29

**Table 3.** Validation of the two discriminant functions (Spanish and Danish) using external data from the same studyarea at the Spanish and Danish populations and from two different geographical area on an Italian and Ukrainian barn swallows populations.

Sex	Pronosticated sex					
	SPAIN			DENMARK		
	Males	Females	Total	Males	Females	Total
Males	244	36	280	824	94	918
Females	21	280	301	65	908	973
	ITALY			UKRAINE		
	Males	Females	Total	Males	Females	Total
Males	49	1	50	42	8	50
Females	8	42	50	6	44	50

## Capítulo 2

### **La coloración basada en melanina como indicador fiable del éxito reproductivo en la Golondrina común (*Hirundo rustica*)**

La importancia del color del plumaje como indicador de calidad individual ha sido el foco de atención de los estudios de selección sexual durante mucho tiempo. A pesar de que la coloración basada en melanina es uno de los tipos de ornamentos de plumaje más extendido entre las aves aún hay controversia acerca de la información que dicha coloración aporta. Aquí analizamos la coloración basada en melanina de las plumas dorsales de las golondrinas *Hirundo rustica* para investigar la fiabilidad de este color como indicador de la calidad individual y cómo se relaciona con el desempeño reproductivo y la supervivencia. La coloración basada en melanina no fue dependiente de la condición física, pero predijo la longitud de las rectrices externas en los machos, que es un carácter sexual secundario. Curiosamente, la coloración basada en melanina se correlacionó de manera diferente con la supervivencia de los machos y hembras. Así, mientras que las hembras con una coloración más oscura de las plumas dorsales sobrevivieron mejor al año siguiente que las hembras con una coloración más pálida, ese no fue el caso de los machos. Las hembras con una coloración basada en melanina más saturada llegaron antes a las zonas de cría y comenzaron a reproducirse antes que las hembras con una coloración a base de melanina menos saturada. Finalmente, el número de crías producidas por la pareja reproductora aumentó con la intensidad de la coloración basada en melanina de las hembras, pero no la de los machos. Por lo tanto, la intensidad de la coloración basada en melanina del plumaje dorsal covaría con una serie de importantes elementos de las historias vitales de una manera específica de cada sexo en golondrinas.

**Melanin-based coloration as a reliable indicator of reproductive success and survival in barn swallows (*Hirundo rustica*)**

Ignacio G Hermosell, Javier Balbontín, Florentino de Lope, Anders Pape Møller, Maribel Reviriego, Luz García-Longoria, Carmen Relinque, Alfonso Marzal

The importance of plumage color as an indicator of individual quality has long been the focus of studies of sexual selection. Despite melanin-based coloration being one of the most widespread types of plumage ornamentation in birds, there is still controversy over the information this kind of coloration provides. Here we analyzed melanin-based coloration of the dorsal feathers of barn swallows *Hirundo rustica* in order to investigate the reliability of this color as an indicator of individual quality, and how it relates to reproductive performance and survival. Melanin-based coloration was not condition-dependent, but it predicted the length of the outermost tail feathers in males, which is a secondary sexual character. Interestingly, melanin-based coloration was differently correlated with the survival of males and females. Thus, whereas females with a darker coloration of dorsal feathers survived better to the next year than females with paler coloration, that was not the case for males. Females with a more saturated melanin-based coloration arrived at the breeding grounds earlier and started to reproduce earlier than females with a less saturated melanin-based coloration. Finally, the number of fledglings produced by the breeding pair increased with the intensity of melanin-based coloration of females, but not in males. Therefore, the intensity of melanin-based coloration of the dorsal plumage covaries with a number of important life history traits in a sex-specific manner in barn swallows.

## Introduction

The importance of plumage color as an indicator of individual quality has long been studied in sexual selection. Theory predicts that color traits must impose costs in order to evolve as reliable signals of individual quality (Andersson 1994). Melanins and carotenoids are the two main pigments accountable for much of animal coloration. Melanins can be synthesized by animals from the common precursor, L-tyrosine, to produce two different pigments: pheomelanin, which is responsible for the reddish-brown colors, and eumelanin, which is responsible for gray to black color (Prota 1992; Hearing 1993; Ito and Wakamatsu 2006; Simon et al. 2009). In contrast, carotenoids must be obtained directly from the diet by the individual (Goodwin 1984). Therefore, the production of carotenoid-based signals is assumed to be highly condition-dependent (Møller et al. 2000; Peters et al. 2007, 2008). However, there is not scientific consensus that melanin-based coloration (hereafter: MBC) reflects the condition of individuals and hence the role in sexual selection.

Although melanin color may act as antioxidants, it was traditionally thought that these pigments are used as background body color, for camouflage, flight displays or to increase resistance of feather to abrasion and bacterial degradation (Zink and Remsen 1986; Prota 1992; Bonser 1995; Bokony et al. 2003; Burt and Ichida 2004; Goldstein et al. 2004; McGraw 2006). The possible costs associated with MBC are still poorly understood in birds, and they could be taxon specific (Stoehr 2006). Some studies have found a relationship between different life-history traits and MBC. For instance, previous studies have shown that more relatively eumelanic male barn swallows survive better than paler ones (Saino et al. 2013b), or that darker melanic feral pigeons (*Columba livia*) had lower endoparasite intensity and greater cellular immune response than less eumelanic individuals (Jacquin et al. 2011). MBC also acts as sex-specific signals in some species (Roulin 1999; Roulin et al. 2000, 2001a, b, 2010; Roulin and Altwegg 2007); and it has been suggested that they even signal personality traits (Mateos-González and Senar 2012). Therefore, evidence suggests that MBC might be acting as honest signals in sexual selection at least in some species (Bokony et al. 2003; Parker et

al. 2003; Bokony and Liker 2005; Tarof et al. 2005; Griffith et al. 2006; Kingma et al. 2008; McGraw 2008). If this is true, this color trait should impose costs in order to evolve as a reliable signal (Andersson 1994).

Recently, several studies focused on such possible pleiotropic effects of melanogenesis-related genes on different fitness traits (Ducrest et al. 2008; Roulin and Ducrest 2013; Roulin 2015). Melanogenesis is a very complex process that involves many enzymes and hormones that are also integrated in other physiological and behavioral functions. The melanocortin peptides bind up to five different melanocortin receptor (MC1-5R) located in different tissues and with different physiological and behavioral functions. Melanocortin peptides not only bind to MC1R, the principal melanocortin receptor implicated in melanogenesis, but also to the remaining MCR's, related to stress response (MC2R), anti-inflammatory activity (MC3R), sexual activity (MC4R) or aggressiveness (MC5R), among other functions. There might exist covariation between MBC and up to five categories of traits including sexual behavior and exocrine gland activity, aggressiveness, immune function, stress response and energy homeostasis, among other factors (Ducrest et al. 2008). A literature review including different groups of vertebrates provided evidence that darker, more eumelanic vertebrates are more aggressive, sexually more active and more resistant to stress than lighter individuals (Ducrest et al. 2008). Therefore we might expect that back coloration in barn swallows could provide some insights about individual quality to facilitate female mate choice.

The barn swallow *Hirundo rustica* is a small, 20-g insectivorous, semi-colonial bird. It is a long-distance migrant that breeds in large parts of temperate and subtropical zones of the northern hemisphere. Populations breeding in Europe winter in Africa south of the Sahara desert. In the Iberian Peninsula, barn swallows arrive at the breeding grounds on average 26 March ( $\pm 21$  days SD) calculated in one study considering 812 localities (Gordo et al. 2007). Males arrive on average earlier than females (Møller 1994a). Males and females have similar

morphology with the exception of the length of the outermost tail feathers, which are longer in males due to a female preference for mates with longer tails (Møller 1994a). They are socially monogamous with frequent extra-pair paternity and may have two or even three clutches per year. Ventral MBC have been studied in this species. Males, but not females, with paler, relatively more eumelanic feathers have higher survival rates than rufous, more pheomelanic ones (Saino et al. 2013b). Individual of both sexes with paler ventral feathers have better immune and stress response (Saino et al 2013a). However, the function of this MBC varies when measured from different body region or among geographical populations (Saino et al. 2013c).

Here we investigate whether MBC is condition-dependent and to which extent it reflects different components of fitness in barn swallows. First, we studied if the three color variables that defines MBC (i.e.,  $I\theta I$ ,  $\Phi$  and  $rA$ , see Materials and methods for further details) covary with body condition of barn swallows. Second, we analyzed whether melanin coloration covary with a well-known secondary sexual character (i.e., tail length) in males of this species. If MBC honestly reflects condition in barn swallows, we should expect MBC to covary with body condition of both male and female and /or the length of the tail feathers in males. We also assessed if MBC was related to fitness in this species. We investigated if MBC was correlated with adult survival rate of males and females and investigated if MBC was correlated with arrival and laying date. Finally, we investigated how MBC of back feathers of the two members of the breeding pair was correlated with the number of fledglings produced during a given year.

## **Materials and methods**

### *Field procedures*

The study was carried out in a barn swallow colony located on a farm at the outskirts of Badajoz, southwest Spain (39° N, 7° W) 2004-2007. The area mainly consisted of agricultural fields with scattered groups of trees (de Lope 1983). Early after arrival adult birds were

captured at dawn by using mist nets placed across windows and doors of the breeding rooms. Each individual was identified with a numbered metal ring and a combination of colored PVC rings. In addition, all birds were provided with a unique combination of color markings on their belly feathers using stamp ink to allow identification. Individuals were sexed from the presence (females) or absence (males) of a brood patch and from observation of breeding behavior during the courtship and incubation periods. We took several morphological measurements of each individual. Tarsus length was measured with a digital caliper to the nearest 0.01 mm, tail length was measured to the nearest mm with a graduated ruler, and body mass was measured with a Pesola spring balance to the nearest 0.5 g. The same person measured all birds every year. When each animal was measured, 3-4 black feathers from the back were plucked, placed in a hermetic, sterile bag and stored in darkness for later analysis as recommended by McGraw et al. (2004).

We used body mass and tarsus length to calculate the scaled mass index (Peig and Green 2009) as an estimate of body condition. They defined body condition as the energy capital accumulated in the body as a result of feeding, assumed to be an indicator of an animal's health and quality. This method is an application of Thorpe-Lleonart method to standardize measures of body size with respect to another (Thorpe 1975) by calculating the scaling component by standardized major axis regression of body mass and a length measure. An important advantage is that, unlike ordinary least squares residuals, the scaled mass index can easily be compared between different populations and studies with adequate sample sizes (Peig and Green 2009).

Once females started laying, we performed daily observations to track all reproductive events. We used different phenological and reproductive traits as indicators of reproductive success. Arrival date was the date of first capture (day 1 = 1st January), date of first laying the date when the first egg was laid (day 1 = 1st January) and the number of fledglings was the sum of all fledglings produced during the whole breeding season. We calculated the age and

survival to the next year of barn swallows confidently because this species exhibits high breeding philopatry, and because first recruitment occurs at an age of one year (Møller 1992; Møller et al. 2005; Balbontín et al. 2009). Thus, we assumed unbanded birds to be yearlings at first capture and assuming that disappearance of color-banded breeders from colonies indicated mortality instead of dispersal.

#### *Measuring coloration by spectrophotometry*

Spectrophotometer technology allows accurate measurement of the spectral reflectance of any colored surface under controlled conditions. This sensitive technique can reveal variation in colors unappreciated by the human eye measuring both the visible spectrum range (400 - 700 nm) and the ultraviolet wavelengths (300 - 400 nm) (Hill and Brawnner 1998; McGraw and Hill 2000). Feather reflectance was measured with an Ocean Optics USB2000+ spectrophotometer under laboratory conditions. Each sample was illuminated with both a deuterium bulb and a tungsten-halogen bulb light source in order to record measurements in the ultraviolet and the visible spectrum, respectively. Illumination was made at a 90° angle with respect to the sample. All measurements were made with reference to a WS-1 (Diffuse Reflectance Standard) from Ocean Optics, which has a reflectance exceeding 98% and a dark spectrum to eliminate noise from the spectrometer when no light is reaching the detector. A total of 3-4 feathers were mounted on a neutral surface, as they would be placed in the animal in order to get the most realistic results. Three consecutive measurements were taken from each sample of feathers. Mean measures for hue, tone and brightness were recorded for each individual. The acquisition values for the spectrophotometer were the same throughout all sessions. The same person measured all samples.

All color measurements were processed and transformed before use in the tetrachromatic color space model elaborated by Goldsmith (1990) using the TetraColorSpace program version 1a (Stoddard and Prum 2008). This approach has been successfully applied recently in several studies of birds (Antonov et al. 2010; Saino et al. 2013b, c). It uses not only

the data from the plumage reflectance, but also data on bird cone sensitivity functions, and, therefore, we can describe these as how birds perceive color in a very realistic way. For the analysis we assumed a UVS cone type retina and used the spectral sensitivity of the blue tit (*Cyanistes caeruleus*), because this species is the phylogenetically most closely related species to the barn swallow among the species whose spectral sensitivity is implemented in TetraColorSpace. In this color space model, color is described by the theta component ( $\theta$ ), which represents hue in the red-green-blue, the phi component ( $\Phi$ ), which represents the ultraviolet spectra, and the chroma ( $r$ ), which is a measure of color saturation.  $\theta$  is the horizontal, azimuth angle from the positive X-axis to the color vector, which runs between the m (green) and l (red) vertices. Values of  $\theta$  range from  $-\pi$  to  $+\pi$ . The angle  $\Phi$  is the vertical elevation angle from the X-Y plane to the color vector and it ranges from  $-\pi/2$  to  $+\pi/2$ . The chroma, or saturation, of a color is given by the magnitude of  $r$ , or its distance from the achromatic origin, i.e. the length of the color vector. Because the color space is a tetrahedron and not a sphere, different hues vary in their potential maximum chroma ( $r_{max}$ ). Therefore, the chroma of a color is defined with respect to the maximum possible chroma for his hue, which is called the “achieved chroma” ( $r_A$ ). TetraColorSpace calculates the achieved chroma for each color patch as  $r_A = r / r_{max}$ . A wider, more extensive explanation of this methodology can be found in Stoddard and Prum (2008).

In our case, values of  $\theta$  around zero represent less black individuals (closer to the l vertex), i.e. with higher relative concentration of pheomelanin, while highest and lowest values correspond to “bluish” or “greenish” individuals (closer to s and m vertices, respectively), i.e. with lower relative concentration of pheomelanin (Fig. 1). Because  $\theta$  is a polar variable and the Robinson projection is done onto the sphere, the values of  $-\pi$  and  $+\pi$  are virtually the same. Consequently in our analysis we employed the absolute value of  $\theta$ , because higher values of  $|\theta|$  represents darker, more eumelanic coloration and lower values of  $|\theta|$  represent less black feathers. Hence, higher values of  $r_A$  represent more color-saturated individuals. In most cases, both eu- and pheomelanin are present in the same MBC (Haase et al. 1992;

McGraw and Wakamatsu 2004; Ito et al. 2011), although dorsal feathers in the barn swallow predominantly contain eumelanin. McGraw et al. (2005) showed that melanin content was correlated to saturation in the barn swallow. Therefore, higher concentrations of eumelanin in the feather are reflected by higher saturation in black color. Variation in  $\Phi$  cannot be verbally described as it mainly represents the ultraviolet hue component, which cannot be perceived by the human eye.

### *Statistical procedures*

Repeatability of color variables was calculated following Lessells and Boag (1987) and Harper (1994). To test the condition-dependent hypothesis we examined all candidate models obtained from a generalized linear mixed model using scaled mass index as the dependent variable. We defined the global model including sex, age, the quadratic term of age (to account for a non-linear effect), tarsus length, tail length and the transformed color variables (i.e.,  $l\theta l$ ,  $\Phi$  and  $rA$ ) were included as input variables. We also included in the model all two-way interactions between sex and color variables and between age and color variables. We did not include the interaction between the quadratic term of age with the other input variables because it would result in over-parameterization. Year and individual identity were included as two cross-random effects. We also evaluated whether color variables predicted individual quality in a model where tail length was the response variable and age, the quadratic term of age, tarsus length, body condition, and color traits were included as fixed effects. We also included all two-way interactions between age and color traits in the global model. Year and individual identity were included as two cross-random effects. This model was only run on males, as tail length is a sexually selected trait in barn swallows (Møller 1994a).

To test our hypotheses of how MBC was correlated with survival rate we evaluated all possible candidate models obtained from a generalized linear mixed model. We defined a global model that included the colour variables, sex, age and the quadratic effect of age and body condition (i.e., scale mass index) and arrival date as input variables. We included in the

global model two-way interactions between age and color traits and sex and color traits to evaluate our competing hypotheses. We did not include the interaction between the quadratic term of age with the other input variables because it would result in over-parameterization. The response variable was the survival of individuals from one year to the next. Survival was coded as 1 if the individual survive to the next year or 0 if it did not survive. The global model was a linear mixed effect model in which we included these predictors as fixed effects and individual identity and year as random effects (intercepts).

Finally, we investigated if color variables predicted several components of fitness (i.e., arrival date, laying date and the number of fledglings produced in a given year). With this purpose, we developed several linear-mixed effects models. In the model in which arrival date was the response variable we included sex, age, the quadratic term of age, scaled mass index, tail length and color traits as fixed effects in the global model. We included all two-way interactions between age and color traits and sex and color. We did not include the interaction between the quadratic term of age with the other input variables because it would result in over-parameterization. Year and individual identity were included as random effects. We developed an additional models using laying date as response variable including only females because this trait is intimately related to female physiology. In this model we used arrival date, age, the quadratic term of age, scaled mass index, tail length and color traits as input variables. We also included all two-way interactions between age and color traits. We did not include the interaction between the quadratic term of age with the other input variables because it would result in over-parameterization. Year and individual identity were also included in the models as cross-random effects. Finally, we analyzed factors influencing the number of fledglings produced for known breeding pairs. In this analysis we included age, quadratic term of age, scaled mass index and color traits of both sexes as predictor variables. We also included all the two-way interactions between age of male and female and color traits for both males and females. We did not include the interaction between the quadratic term of age with the other input variables because it would result in over-parameterization. In this

analysis the identity of the nest of the breeding pair and year were included as two cross-random effects in the linear mixed effect model.

In all models, we first standardized input variables entering the global model by scaling them through centering by dividing their mean by two standard deviations, which allowed comparison on the same scale of coefficients of binary factors (e.g., sex) and covariates (Gelman 2008; Grueber et al. 2011). We started with a null model that included the intercept as a fixed effect. Model selection was carried out using the information theory approach (Burnham and Anderson 2002). Top models of all possible candidates were determined using Akaike's Information Criterion with a correction for small sample size (AICc). We calculated Akaike weight ( $w$ ) for each candidate model ( $i$ ) that can be interpreted as the probability that ( $i$ ) is the best model, given the data and set of candidate models. The final model was obtained by averaging the parameter estimates from top models at a cut-off criterion of  $AIC < 6.0$  (Richards 2008). We employed the library MuMIn (Bartón 2009) and the library lme4 (Bates and Maechler 2009) using R version 2.15.1 (R Core Team 2013). The lme4 library has a function that computes approximate confidence intervals by computing a likelihood profile and finding the appropriate cut-offs based on the likelihood ratio test. This estimates the confidence intervals of fixed-effect parameters based on the estimated local curvature of the likelihood surface. The confidence intervals have the same meaning as any other confidence interval if the procedure is repeated and more than 95% of the time the value of the parameter can be found within that interval. This model selection procedure was employed in all analyses.

## Results

We estimated repeatability of MBC by measuring the same feather three times, and we obtained a high repeatability for all three transformed color variables ( $\theta$ :  $F_{649,1300} = 6.72$ ,  $p < 0.001$ ;  $\Phi$ :  $F_{649,1300} = 16.11$ ,  $p < 0.001$ , and  $rA$ :  $F_{649,1300} = 9.24$ ,  $p < 0.001$ ) with values of  $R > 0.66$ . Thus we used the color variables averaged across the three measurements in the

present study. Color variables were poorly correlated among each other with the largest correlation coefficient only being 0.35 (Table 1).

#### *MBC and body condition*

We did not find a significant relationship between any component of MBC with the body condition measured with the scaled mass index while controlling for age and tail length of individuals. None of the confidence intervals of the estimates of the three color parameters or their interactions with sex excluded zero, indicating that color traits based on melanin did not predict body condition in barn swallows (Table 2). Only tail length predicted body condition as indicated by the exclusion of zero from the estimated confidence intervals for this trait (Table 2).

#### *MBC and male tail length*

Hue, expressed as the absolute value of theta ( $|\theta|$ ), explained a significant part of variation in male tail length while controlling for age and condition of males, which were two intrinsic traits that have previously been shown to affect male tail length in this species. This was because the confidence interval for these estimated parameters excluded zero (Table 3). Concretely, males with lower values of  $|\theta|$  (i.e., less black males) tend to have longer tail feathers than males with higher values of  $|\theta|$  (i.e., more black males) (Table 3).

#### *MBC and survival*

Hue, expressed as the absolute value of theta ( $|\theta|$ ) was correlated differently with survival in male and female barn swallows as shown by the significant interaction between sex and absolute theta ( $|\theta|$ ) on survival. The estimated confidence interval for this predictor did not include zero (Table 4). Specifically, females with a darker coloration of their back feathers (i.e., those that were more eumelanic) had a higher probability of survival from one year to the next than females with less black coloration of their dorsal feathers. In contrast, this relationship

was not found in males (Fig. 2).

### *MBC, migration and reproduction*

Color saturation measured in the back feathers predicted arrival date of male and female barn swallows, while controlling for other confounding variables such as age, tail length and body condition. Individuals with more saturated melanin color arrived earlier at the breeding grounds (Table 5, Fig. 3). Furthermore, color saturation predicted laying date in females, while controlling for other confounding variables such as age, body condition and arrival date (Table 6, Fig. 4). Confidence intervals for these two parameters excluded zero, which indicated that they were correlated with arrival and laying date, respectively. Age, sex and tail length were correlated with arrival date, which in turn was correlated with laying date (Tables 5 - 6).

In the analysis of annual total number of fledglings produced by the breeding pair, females with higher absolute hue ( $|\theta|$ ) (i.e., with blackish feathers) raised more fledglings than those with lower values of  $|\theta|$  (i.e., less black feathers) (Fig. 5). Confidence intervals for these color parameters excluded zero, which indicated that they were correlated with the number of fledglings produced by the breeding pair. Age of the male in the breeding pair also affected the number of fledglings produced by the breeding pair, as shown by the exclusion of zero from the estimates of confidence intervals (Table 7). This was not confounded by other variables that have previously been shown to correlate with the number of fledglings produced such as body condition or tail length.

### **Discussion**

We found that MBC did not reflected body condition when we investigated how each color component was related to the scale mass index of male and female barn swallow. Furthermore, we found that back feather coloration, expressed as the absolute value of theta ( $|\theta|$ ), was inversely related to tail length in males, with paler males tending to have longer tails, opposite to what we should expect if MBC reflected condition. Interestingly, we found that

MBC was related to several fitness components and that this relationship differed between sexes. Specifically, MBC measured on the back feathers was related to differently survival in males and females. In addition, females having a more saturated coloration of the back feathers arrived earlier at the breeding grounds and started laying eggs earlier during the reproductive season than females having a less saturated coloration of their back feathers. The component of coloration related to hue was also related to the number of fledglings produced a given breeding season by the breeding pair. Concretely, the value of  $l\theta l$  of the back feathers of females was positively related to the number of fledglings while controlling for age, body condition, tail length and color of their mates. However, we did not find that the value of  $l\theta l$  of the back feathers of males was related to the number of fledgling produced while controlling for age, body condition, tail length and colour of their mates.

MBC was related to several fitness traits in females but not in males. For instance, more eumelanic females survived better from one year to the next than less melanic ones, whereas that was not the case for males. Furthermore, females with more saturated coloration of their back feathers laid their clutches earlier than those with less saturated dorsal feathers. Moreover, females with blackish feathers produced more fledglings than females with less black feathers, while that was not the case for males. Thus females investing more in MBC experience fitness gains. Previous studies showed that animals with more eumelanic color are more successful in terms of several life-history traits. The melanogenesis biochemical pathway is very complex and many external enzymes and hormones are involved. Recently, pleiotropy has been suggested as a mechanism producing covariation between MBC and other phenotypic and behavioral traits (Roulin and Ducrest 2013; Roulin 2015). Ducrest et al. (2008) reviewed the mechanisms of the melanocortin systems and its role in the pleiotropic effects of melanogenesis on a wide range of phenotypical and behavioral traits in vertebrates. They showed the existence of links between melanogenesis and a number of fitness-related traits including sexual behavior and exocrine gland activity, aggressiveness, immune function, stress response and energy homeostasis, among others. These effects were mediated by

melanocortins, which are peptide hormones derived from prohormone proopiomelanocortin including hormones such as MSH or ACTH. These melanocortin peptides do not only bind to the MC1R that control melanogenesis, but they also bind in the other melanocortin receptors (MC2-5R) that are located in different parts of the body and regulate different behavioural and physiological functions, as the ones mentioned above. The authors provided evidence that darker wild vertebrates are more aggressive, sexually more active and more resistant to stress than lighter individuals. According to this evidence, some of these pleiotropic effects could explain our results. The advanced arrival and laying date observed in darker barn swallows could be due to enhanced body condition because of an improved stress response, better immune system, or higher regulatory control of energy caused by the interaction between the melanogenesis pathway and the former physiological functions.

However, we did not find these fitness benefits in reproductive traits in males. Males seems to trade investment in darker coloration against investment in tail length since we found a negative relationship between the length of the tail in males and the value of theta, with less eumelanic males tending to have longer tails. These extra costs (long tail and color investment) would affect male survival more strongly than female survival. These results are in line with a recent study with four different European populations of barn swallows showing that short-lived males have longer tails when young than long-lived males, while that was not the case for females. This finding suggested a trade-off between investment in production of long tails and survival, and it is consistent with tail length being a secondary sexual character that is costly to produce (Balbontín et al. 2011). The present study suggested a similar result with males but not females paying a cost from producing long tails and producing eumelanic back feathers at the same time. Similar sexually different signaling patterns in coloration have previously been reported in other species. For instance, more eumelanic (i.e., heavily spotted) female barn owls (*Tyto alba*) had higher probability of survival from one year to the next than less eumelanic (i.e., less spotted) females. However, spottiness in males seems to be slightly counter selected, i.e. selection strongly favors females with large spots and weakly favors

males with few spots (Roulin and Altwegg 2007; Roulin et al. 2010). Other studies of barn swallows that measured MBC in the ventral region of the body have showed that paler (i.e., with relative higher concentration of eumelanin) males survive better from one year to the next than reddish ones (i.e., with relative higher concentration of pheomelanin). Moreover, these authors also showed that there was no relationship between MBC and survival in females (Saino et al. 2013b). Therefore, the differences in the results of our and the latter study might be due to the different nature of coloration measured in each study. Saino et al. (2013b) measured MBC in the ventral region of the body, while we measured it in the dorsal region. Probably, covariation between coloration and fitness traits may largely depend on the body region where color is measured (Saino et al. 2013a). The amount of melanin present in the ventral region of the body is smaller than that of the dorsal region and this might partly explain the difference between these two studies. Furthermore, the ratio between the two forms of melanin (i.e., eu-/pheomelanin) is also very different between these two body regions, as eumelanin causes black coloration that is predominant in the dorsal region of the body, while pheomelanin leads to buffy brown colors, which are predominant in the ventral region of the body (McGraw 2008). This is very important, as differences in the pheo-eumelanin ratio are responsible for differences in the reflectance spectrum of the ornament, and they might incur different costs.

Males and females with more saturated coloration of their back feathers arrived earlier at their breeding areas than those with less saturated dorsal feathers. Early arrival may lead to higher reproductive success in multi-brooded species such as barn swallows (Møller 1994a, b; Kokko 1999). Although the relationship between fitness components and MBC has previously been established, as far as we know, this is the first study showing that MBC covaries with the date that individuals arrive to their breeding areas. Total melanin content could explain why both sexes arrive earlier to the breeding areas. It is well known that eumelanic feathers are more resistant to abrasion, parasites and breakage (Bonser 1995; Kose and Møller 1999). Thus the condition of the plumage could have consequences for components of fitness.

Feathers with less melanin more readily would become worn or break and this should affect flight ability (Barbosa et al. 2003) and hence arrival date and start of laying, as this trait is positively correlated with arrival date in barn swallows (Møller 1994a, b). These advances in breeding timing would also improve reproductive success (Møller 1994a, b; Kokko 1999). Future observational and experimental studies should investigate the relationship between ectoparasites and MBC of the feather to test if this type of coloration helped to maintain the feather in good condition and how this could explain the benefits of early arrival and other fitness traits.

According to our results, MBC was not condition-dependent. Inter-individual variation in coloration can be generated by both genetic factors and variation in body condition. MBC is strongly genetically regulated (Majerus 1998; Badyaev and Hill 2000). The genetic component of inter-individual variation in this kind of coloration is very high in birds with values of heritability above 0.53 (Roulin and Ducrest 2013). Therefore, it is not surprising we did not find a direct relationship between coloration and our index of body condition. In a recent paper, Roulin (2015) distinguish between condition-dependent and condition-related signalling to explain covariation between coloration and body condition. Condition-dependent signalling is used when condition causally affects the expression, maintenance or display of the ornament. Condition-related signalling applies when the degree of coloration correlates with aspects of body condition, i.e. the overall capacity to maintain the optimal functionality of body systems. Our data points to the latter option, as we did not find a direct, causal effect of body condition on MBC, while MBC covaried with survival, tail length and other condition-related traits.

To summarize, the intensity of black coloration of the dorsal feathers in barn swallows was related to survival and reproductive success in females, but not in males. Males seem to trade between investment in coloration and tail length. Darker individuals of both sexes arrived earlier to the breeding grounds than paler ones. Finally MBC was not directly condition-

dependent. These correlations among life-history parameters and among-individual differences in coloration have already been shown in a within-individual level by Dreiss and Roulin (2010). However, the melanogenesis biochemical pathway is very complex and many enzymes and hormones are involved (Ducrest et al. 2008). Future experimental studies are necessary to elucidate if MBC honestly signals individual fitness and survival, or if it is due to pleiotropic effects of the melanocortin system and/or genetic correlations among traits.

### **Acknowledgements**

This study was funded by grants from the Spanish Ministry of Economy and Competition (CGL2009-08976 and CGL2012-36665). Ignacio García Hermosell and Luz García-Longoria were supported by FPI grants from the Spanish Ministry of Science and the Ministry of Economy and Competition of Spain, respectively. Javier Balbontín benefited from a stay at the University of Extremadura with funds provided by a research project of the Spanish Ministry of Education and Science (CGL2012-36665).

### **References**

- Andersson M (1994) Sexual Selection. Princeton University Press, Princeton, New Jersey
- Antonov A, Stokke BG, Vikan JR, Fossøy F, Ranke PS, Røskaft E, Moksnes A, Møller AP, Shykoff JA (2010) Egg phenotype differentiation in sympatric cuckoo *Cuculus canorus* gentes. *J Evol Biol* 23:1170–82
- Badyaev AV, Hill GE (2000) Evolution of sexual dichromatism: contribution of carotenoid- versus melanin-based coloration. *Biol J Linn Soc* 69:153–172
- Balbontín J, de Lope F, Hermosell IG, Mousseau TA, Møller AP (2011) Determinants of age-dependent change in a secondary sexual character. *J Evol Biol* 24:440–448
- Balbontín J, Møller AP, Hermosell IG, Marzal A, Reviriego M, de Lope F (2009) Individual responses in spring arrival date to ecological conditions during winter and migration in a migratory bird. *J Anim Ecol* 78:981–989
- Barbosa A, Merino S, Cuervo JJ, de Lope F, Møller AP (2003) Feather damage of long

- tails in Barn Swallows *Hirundo rustica*. *Ardea* 91:85–90
- Bartón K (2009) MuMIn: multi-model inference. R package, version 0.12.2. <http://r-forge.r-project.org/projects/mumin/>
- Bates D, Maechler M (2009) lme4: Linear mixed-effects models using Eigen and Eigenfaces. R 431 package, version 0.999375-31
- Bokony V, Liker A (2005) Melanin-based black plumage coloration is related to reproductive investment in cardueline finches. *Condor* 107:775–787
- Bokony V, Liker A, Székely T, Kis J (2003) Melanin-based plumage coloration and flight displays in plovers and allies. *Proc R Soc B* 270:2491–2497
- Bonser RHC (1995) Melanin and the abrasion resistance of feathers. *Condor* 97:590–591
- Burham KP, Anderson DR (2002) Model selection and inference: A practical information-theoretic approach. Springer, New York
- Burt EHJ, Ichida JM (2004) Gloger's rule, feather-degrading bacteria, and color variation among song sparrows. *Condor* 106:681–686
- de Lope F (1983) La avifauna de las Vegas Bajas del Guadiana. *Doñana Acta Vertebrata* 10:91–121
- Dreiss AN, Roulin A (2010) Age-related change in melanin-based coloration of Barn owls (*Tyto alba*): Females that become more female-like and males that become more male-like perform better. *Biol J Linn Soc* 101:689–704
- Ducrest AL, Keller L, Roulin A (2008) Pleiotropy in the melanocortin system, coloration and behavioural syndromes. *Trends Ecol Evol* 23:502–10
- Endler JA, Westcott DA, Madden JR, Robson T (2005) Animal visual systems and the evolution of color patterns: sensory processing illuminates signal evolution. *Evolution* 59:1795–818
- Gelman A (2008) Scaling regression inputs by dividing by two standard deviations. *Stat Med* 27:2865–2873
- Goldsmith TH (1990) Optimization, constraint, and history in the evolution of eyes. *Q Rev*

Biol 65:281–322

Goldstein G, Flory KR, Browne BA, Majid S, Ichida JM, Burt EHJ (2004) Bacterial degradation of black and white feathers. *Auk* 121:656–659

Goodwin TW (1984) *The Biochemistry of Carotenoids. Vol 2. Animals.* Chapman and Hall, New York

Gordo O, Sanz JJ, Lobo JM (2007) Environmental and geographical constraints on common swift and barn swallow spring arrival patterns throughout the Iberian Peninsula. *J Biogeogr* 34:1065–1076

Griffith SC, Parker TH, Olson VA (2006) Melanin- versus carotenoid-based sexual signals: is the difference really so black and red? *Anim Behav.* 71:749–763

Grueber CE, Nakagawa S, Laws RJ, Jamieson IG (2011) Multimodel inference in ecology and evolution: Challenges and solutions. *J Evol Biol* 24:699–711

Haase E, Ito S, Sell A, Wakamatsu K (1992) Melanin Concentrations in Feathers from Wild and Domestic Pigeons. *J Hered* 83:64–67

Harper DGC (1994) Some comments on the repeatability of measurements. *Ringing Migr* 15:84–90

Hearing VJ (1993) Unraveling the melanocyte. *Am J Hum Genet* 52:1–7

Hill GE, Brawner WR (1998) Melanin-based plumage coloration in the house finch is unaffected by coccidial infection. *Proc R Soc B* 265:1105–1109

Ito S, Nakanishi Y, Valenzuela RK, Brilliant MH, Kolbe L, Wakamatsu K (2011) Usefulness of alkaline hydrogen peroxide oxidation to analyze eumelanin and pheomelanin in various tissue samples: application to chemical analysis of human hair melanins. *Pigment Cell Melanoma Res* 24:605–13

Ito S, Wakamatsu K (2006) Chemistry of Melanins. In: Nordlund JJ, Boissy VJ, Hearing VJ, et al (eds) *The Pigmentary System. Physiology and Pathophysiology*, 2nd edn. Blackwell Publishing, Oxford, UK, pp 282–310

Jacquin L, Lenouvel P, Haussy C, Ducatez S, Gasparini J (2011) Melanin-based

- coloration is related to parasite intensity and cellular immune response in an urban free living bird: the feral pigeon *Columba livia*. *J Avian Biol* 42:11–15
- Kingma SA, Szentirmai I, Székely T, Bokony V, Bleeker M, Liker A, Komdeur J (2008) Sexual selection and the function of a melanin-based plumage ornament in polygamous penduline tits *Remiz pendulinus*. *Behav Ecol Sociobiol* 62:1277–1288
- Kokko H (1999) Competition for early arrival in migratory birds. *J Anim Ecol* 68:940–950
- Kose M, Møller AP (1999) Sexual selection, feather breakage and parasites: the importance of white spots in the tail of the barn swallow (*Hirundo rustica*). *Behav Ecol Sociobiol* 45: 430–436
- Lessells CM, Boag PT (1987) Unrepeatable repeatabilities: A common mistake. *Auk* 104:116–121
- Majerus M (1998) *Melanism, Evolution in action*. Oxford University Press, Oxford
- Mateos-González F, Senar JC (2012) Melanin-based trait predicts individual exploratory behaviour in siskins, *Carduelis spinus*. *Anim Behav* 83:229–232
- McGraw KJ (2006) Mechanics of melanin -based coloration. In: Hill GE, McGraw KJ (eds) *Bird Coloration Vol I: Mechanisms and Measurements*. Harvard University Press, Cambridge, MA, pp 243–294
- McGraw KJ (2008) An update on the honesty of melanin-based color signals in birds. *Pigment Cell Melanoma Res* 21:133–138
- McGraw KJ, Hill GE (2000) Differential effects of endoparasitism on the expression of carotenoid- and melanin-based ornamental coloration. *Proc R Soc B* 267:1525–1531
- McGraw KJ, Safran RJ, Evans MR, Wakamatsu K (2004) European barn swallows use melanin pigments to color their feathers brown. *Behav Ecol* 15:889–891
- McGraw KJ, Safran, RJ, Wakamatsu K (2005) How feather colour reflects its melanin content. *Funct Ecol* 19:816–821
- McGraw KJ, Wakamatsu K (2004) Melanin basis of ornamental feather colors in male

Multi-focus approach to life histories in passerines

Zebra Finches. *Condor* 106:686–690

Møller AP (1992) Sexual Selection in the Monogamous barn Swallow (*Hirundo rustica*). 2. Mechanisms of Sexual Selection. *J Evol Biol* 5:603–624

Møller AP (1994a) Sexual selection and the Barn swallow. Oxford University Press, Oxford, UK

Møller AP (1994b) Phenotype-dependent arrival time and its consequences in a migratory bird. *Behav Ecol Sociobiol* 35:115–122

Møller AP, Biard C, Blount JD, Houston DC, Ninni P, Saino N, Surai PF (2000) Carotenoid-dependent signals: Indicators of foraging efficiency, immunocompetence or detoxification ability? *Poultry Avian Biol Rev* 11:137-159

Møller AP, de Lope F, Saino N (2005) Reproduction and migration in relation to senescence in the barn swallow *Hirundo rustica*: A study of avian “centenarians”. *Age* 27:307–318

Parker TH, Stansberry BM, Becker CD, Gipson PS (2003) Do melanin- or carotenoid-pigmented plumage ornaments signal condition and predict pairing success in the Kentucky warbler? *Condor* 105: 663-671

Peig J, Green AJ (2009) New perspectives for estimating body condition from mass/length data: the scaled mass index as an alternative method. *Oikos* 118:1883–1891

Peters A, Delhey K, Andersson S, van Noordwijk H, Förchler MI (2008) Condition-dependence of multiple carotenoid-based plumage traits: an experimental study. *Funct Ecol* 22:831–839

Peters A, Delhey K, Johnsen A, Kempenaers B (2007) The condition-dependent development of carotenoid-based and structural plumage in nestling blue tits: males and females differ. *Am Nat* 169:S122–S136

Prota G (1992) *Melanins and Melanogenesis*. Academic Press, New York

R Core Team (2013) *R: A Language and Environment for Statistical Computing*. R

Foundation for Statistical Computing, Vienna, Austria

Richards SA (2008) Dealing with overdispersed count data in applied ecology. *J Appl Ecol* 45:218–227

Roulin A (1999) Nonrandom pairing by male barn owls (*Tyto alba*) with respect to a female plumage trait. *Behav Ecol* 10:688–695

Roulin A (2015) Condition-dependence, pleiotropy and the handicap principle of sexual selection in melanin-based colouration. *Biol Rev*. doi: 10.1111/brv.12171

Roulin A, Altwegg R (2007) Breeding rate is associated with pheomelanism in male and with eumelanism in female barn owls. *Behav Ecol* 18:563–570

Roulin A, Altwegg R, Jensen H, Steinsland I, Schaub M (2010) Sex-dependent selection on an autosomal melanic female ornament promotes the evolution of sex ratio bias. *Ecol Lett* 13:616–626

Roulin A, Dijkstra C, Riols C, Ducrest AL (2001a) Female- and male-specific signals of quality in the barn owl. *J Evol Biol* 14:255–266

Roulin A, Ducrest AL (2013) Genetics of colouration in birds. *Sem Cell Dev Biol* 24:594–608

Roulin A, Jungi TW, Hedi P, Dijkstra, C (2000) Female barn owls (*Tyto alba*) advertise good genes. *Proc R Soc B* 267:937–941

Roulin A, Riols C, Dijkstra C, Ducrest AL (2001b) Female plumage spottiness signals parasite resistance in the barn owl (*Tyto alba*). *Behav Ecol* 12:103–110

Saino, N, Canova L, Costanzo A, Rubolini D, Roulin A, Møller, AP (2013a) Immune and stress responses covary with melanin-based coloration in the barn swallow. *Evol Biol* 40:521–531

Saino N, Romano M, Rubolini D, Ambrosini R, Caprioli M, Milzani A, Costanzo A, Colombo G, Canova L, Wakamatsu K (2013b) Viability is associated with melanin-based coloration in the barn swallow (*Hirundo rustica*). *PLoS ONE* 8:e60426

Saino N, Romano M, Rubolini D, Teplitsky C, Ambrosini R, Caprioli M, Canova L,

- Wakamatsu K (2013c) Sexual dimorphism in melanin pigmentation, feather coloration and its heritability in the barn swallow (*Hirundo rustica*). PLoS ONE 8:e58024
- Simon JD, Peles D, Wakamatsu K, Ito S (2009) Current challenges in understanding melanogenesis: bridging chemistry, biological control, morphology, and function. Pigment Cell Melanoma Res 22:563–79
- Stoddard MC, Prum RO (2008) Evolution of avian plumage color in a tetrahedral color space: a phylogenetic analysis of new world buntings. Am Nat 171:755–76
- Stoehr AM (2006) Costly melanin ornaments: The importance of taxon? Funct Ecol 20:276–281
- Tarof SA, Dunn PO, Whittingham LA (2005) Dual functions of a melanin-based ornament in the common yellowthroat. Proc R Soc B 272:1121–1127
- Thorpe RS (1975) Quantitative handling of characters useful in snake systematics with particular reference to intraspecific variation in the ringed snake *Natrix natrix* (L). Biol J Linn Soc 7:27-43
- Zink RM, Van Renssen JV, Jr (1986) Evolutionary processes and patterns of geographic variations in birds. In: Johnston RF (ed) Current Ornithology Vol 4. Plenum Press, New York, pp 1–69

TABLES

**Table 1.** Pearson correlations among transformed color variables for 565 barn swallows

	Phi ( $\Phi$ )	Achieved r (rA)
Absolute theta ( $ \theta $ )	0.24**	0.14**
Phi ( $\Phi$ )		0.35**

**Table 2.** Final model of the effects of sex, age, age squared, tail length and color traits on body condition of 585 barn swallows

Term	Estimate	Standard error	Confidence interval	Relative importance
(Intercept)	18.140	0.179	(17.79, 18.49)	
Tail length	0.414	0.163	(0.095, 0.734)	0.86
$\Phi$	0.282	0.152	(-0.017, 0.581)	0.47
$r_A$	-0.225	0.163	(-0.545, 0.095)	0.19
$ \theta $	0.139	0.145	(-0.146, 0.424)	0.10
Sex	-0.072	0.248	(-0.560, 0.415)	0.17
Age <sup>2</sup>	0.100	0.265	(-0.421, 0.621)	0.09
Age	0.078	0.256	(-0.425, 0.582)	0.11
Sex * $\Phi$	0.398	0.289	(-0.168, 0.965)	0.03
Age * $\Phi$	0.370	0.309	(-0.236, 0.976)	0.01

The global model was a linear mixed-effect model that included individual identity and year as cross-random effects. Significant factors in the models are highlighted in bold. Final model was obtained after averaging 26 top models from 213 candidate models

**Table 3.** Final model of the effects of age, age-squared, body condition and color traits on tail length in 297 male barn swallows

Term	Estimate	Standard error	Confidence interval	Relative importance
(Intercept)	98.097	0.529	(97.056, 99.137)	
Age	12.178	1.709	(8.814, 15.542)	1.00
Age <sup>2</sup>	-6.782	1.632	(-9.994, -3.569)	1.00
Condition	1.687	0.580	(0.546, 2.828)	1.00
lθl	-1.223	0.609	(-2.421, -0.025)	0.90
Age * lθl	-0.460	1.440	(-3.294, 2.374)	0.51
r <sub>A</sub>	0.402	0.605	(-0.788, 1.592)	0.56
Age * r <sub>A</sub>	-0.812	0.903	(-2.589, 0.964)	0.30
Φ	-0.190	0.607	(-1.384, 1.004)	0.52
Age * Φ	0.423	1.152	(-1.844, 2.690)	0.27

The global model was a linear mixed-effect model that included individual identity and year as two cross-random effects. Final model was obtained after averaging 27 top models from 29 candidate models. Significant factors in the models are highlighted in bold

**Table 4.** Final model of the effects of sex, age, body condition, arrival date and color traits on the survival of 463 adult Spanish barn swallows

Term	Estimate	Standard error	Confidence interval	Relative importance
(Intercept)	-0.629	0.458	(-1.530, 0.272)	
Sex	0.071	0.226	(-0.376, 0.524)	0.97
Age	0.081	0.848	(-1.667, 1.848)	0.90
Arrival	0.367	0.297	(-0.004, 0.964)	0.76
$r_A$	-0.386	0.274	(-0.930, 0.063)	0.89
$ \theta $	0.266	0.258	(-0.237, 0.776)	0.99
Sex * $ \theta $	1.357	0.557	(0.404, 2.399)	0.97
Age * $r_A$	0.629	0.662	(-0.017, 2.110)	0.60
Age <sup>2</sup>	-0.452	0.852	(-3.154, 0.868)	0.40
$\Phi$	0.267	0.279	(-0.141, 0.889)	0.71
Age * $\Phi$	0.407	0.602	(-0.170, 2.078)	0.43
Sex * $r_A$	0.073	0.263	(-0.608, 1.239)	0.23
Age * $ \theta $	0.110	0.351	(-0.722, 1.579)	0.26
Condition	-0.011	0.109	(-0.507, 0.402)	0.21
Sex * $\Phi$	0.001	0.184	(-0.943, 0.961)	0.14

The global model was a linear mixed-effect model that included individual identity and year as two cross-random effects. Final model was obtained after averaging 238 top models from 214 candidate models of the effects of color traits. Significant factors in the models are highlighted in bold

**Table 5.** Final model of the effects of sex, age, age squared, body condition, tail length, color traits and their interactions on arrival date in 585 barn swallows

Term	Estimate	Standard error	Confidence interval	Relative importance
(Intercept)	73.795	3.025	(67.854, 79.736)	
Sex	-5.610	2.579	(-10.675, -0.545)	1.00
Age	-45.978	6.313	(-58.377, -33.579)	1.00
Age <sup>2</sup>	36.264	6.144	(24.197, 48.331)	1.00
Condition	1.317	1.705	(-2.031, 4.667)	0.71
$\Phi$	3.121	1.941	(-0.692, 6.934)	1.00
$r_A$	-4.596	1.911	(-8.349, -0.842)	1.00
Tail length	-7.638	2.658	(-12.859, -2.416)	1.00
$ \theta $	0.219	1.778	(-3.275, 3.712)	1.00
Sex * $\Phi$	0.872	3.696	(-6.388, 8.132)	0.83
Sex * $r_A$	-1.538	3.664	(-8.735, 5.658)	0.84
Sex * $ \theta $	-0.795	3.525	(-7.717, 6.129)	0.81
Age * $\Phi$	-0.795	4.236	(-9.115, 7.525)	0.85
Age * $r_A$	4.190	3.650	(-2.979, 11.359)	0.93
Age * $ \theta $	4.333	3.509	(-2.560, 11.226)	0.95

The global model was a linear mixed-effect model that included individual identity and year as two cross-random effects. Final model was obtained after averaging 21 top models from 214 candidate models. Significant factors in the models are highlighted in bold

**Table 6.** Final model on laying date of arrival date, age, age squared, body condition, color variables, tarsus length and their interactions in 221 female barn swallows

Term	Estimate	Standard error	Confidence interval	Relative importance
(Intercept)	87.584	1.389	(84.846, 90.323)	
Age	-9.705	5.381	(-20.307, 0.898)	1.00
Age <sup>2</sup>	7.057	5.206	(-3.204, 17.318)	0.96
Arrival	14.303	1.565	(11.219, 17.388)	1.00
Condition	-2.476	1.450	(-5.334, 0.383)	0.89
$\Phi$	0.458	1.605	(-2.715, 3.611)	0.97
$r_A$	-3.417	1.580	(-6.532, -0.304)	1.00
$ \theta $	0.549	1.377	(-2.165, 3.262)	0.89
Age * $\Phi$	5.508	3.675	(-1.736, 12.751)	0.92
Age * $r_A$	-1.218	3.746	(-8.600, 6.164)	0.79
Age * $ \theta $	-2.929	3.361	(-9.555, 3.696)	0.76

The global model was a linear mixed-effect model that included individual identity and year as two cross-random effects. Final model was obtained after averaging 11 top models from 210 candidate models. Significant factors in the models are highlighted in bold

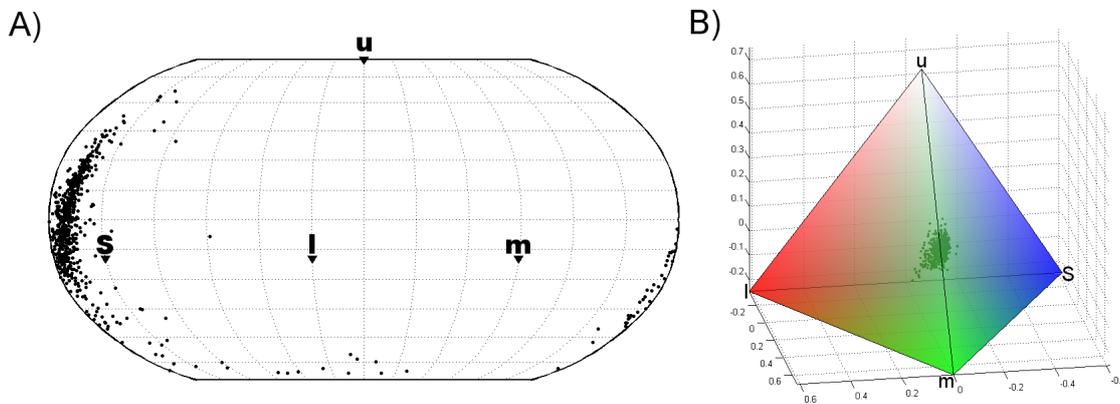
**Table 7.** Final model of the total number of fledglings produced by 151 pairs of barn swallows

Term	Estimate	Standard error	Confidence interval	Relative importance
(Intercept)	6.234	0.483	(5.289, 7.189)	
Age ♀	1.206	1.259	(-1.281, 3.694)	0.85
Age ♂	4.445	1.616	(1.251, 7.639)	1.00
Age <sup>2</sup> ♀	0.731	1.392	(-2.019, 3.480)	0.65
Age <sup>2</sup> ♂	-3.276	1.571	(-6.381, -0.172)	0.99
lθl ♀	0.809	0.409	(0.0002, 1.6186)	0.85
Age ♀ : lθl ♀	-0.273	0.969	(-2.189, 1.643)	0.29
Arrival ♀	-0.357	0.483	(-1.312, 0.597)	0.23
Φ ♂	-0.177	0.432	(-1.031, 0.678)	0.32
Age ♂ : Φ ♂	-1.069	0.975	(-2.995, 0.856)	0.20
lθl ♂	-0.055	0.581	(-1.202, 1.093)	0.29
Age ♂ : lθl ♂	0.595	1.485	(-2.340, 3.529)	0.17
Φ ♀	-0.048	0.452	(-0.941, 0.845)	0.19
Condition ♀	-0.116	0.411	(-0.928, 0.695)	0.12
Condition ♂	0.097	0.422	(-0.737, 0.931)	0.12
r <sub>A</sub> ♀	0.092	0.413	(-0.724, 0.907)	0.16
r <sub>A</sub> ♂	0.068	0.443	(-0.807, 0.943)	0.23
Age ♀ : Φ ♀	0.491	0.914	(-1.316, 2.297)	0.07
Age ♂ : r <sub>A</sub> ♂	0.753	0.835	(-0.896, 2.402)	0.11
Age ♀ : r <sub>A</sub> ♀	-0.036	0.913	(-1.851, 1.760)	0.05

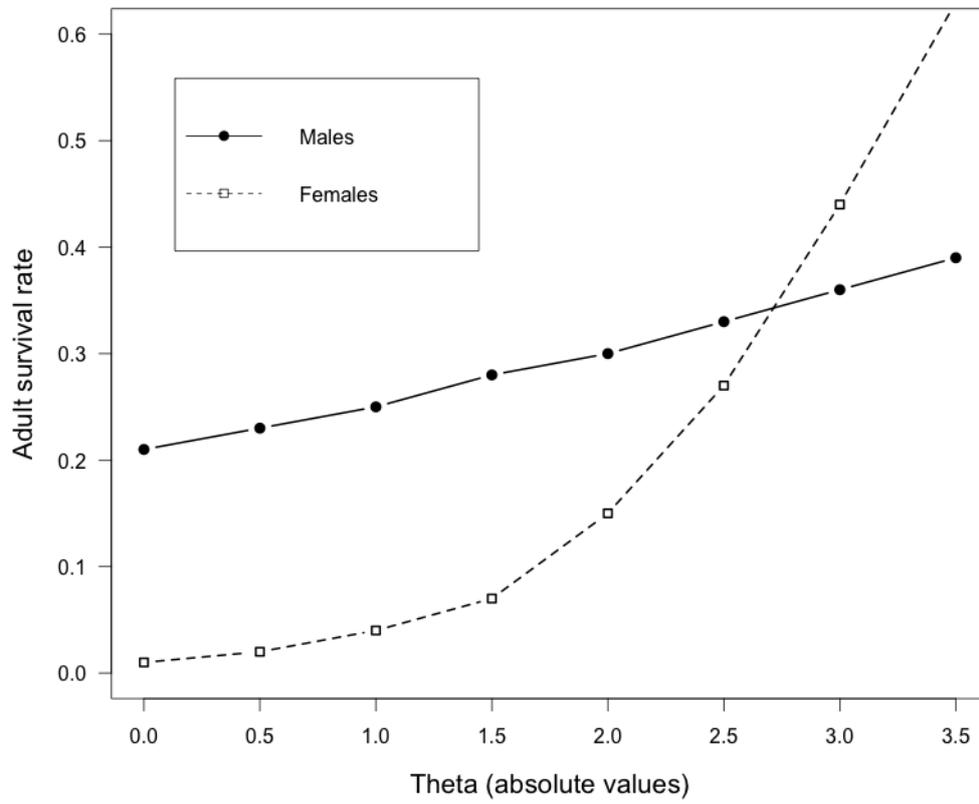
The global model was a linear mixed-effect model that included individual identity and year as two cross-random effects. Final model was obtained after averaging 660 top models from 220 candidate models of the effects of color traits. Significant factors in the models are highlighted in bold

## FIGURES AND LEGENDS

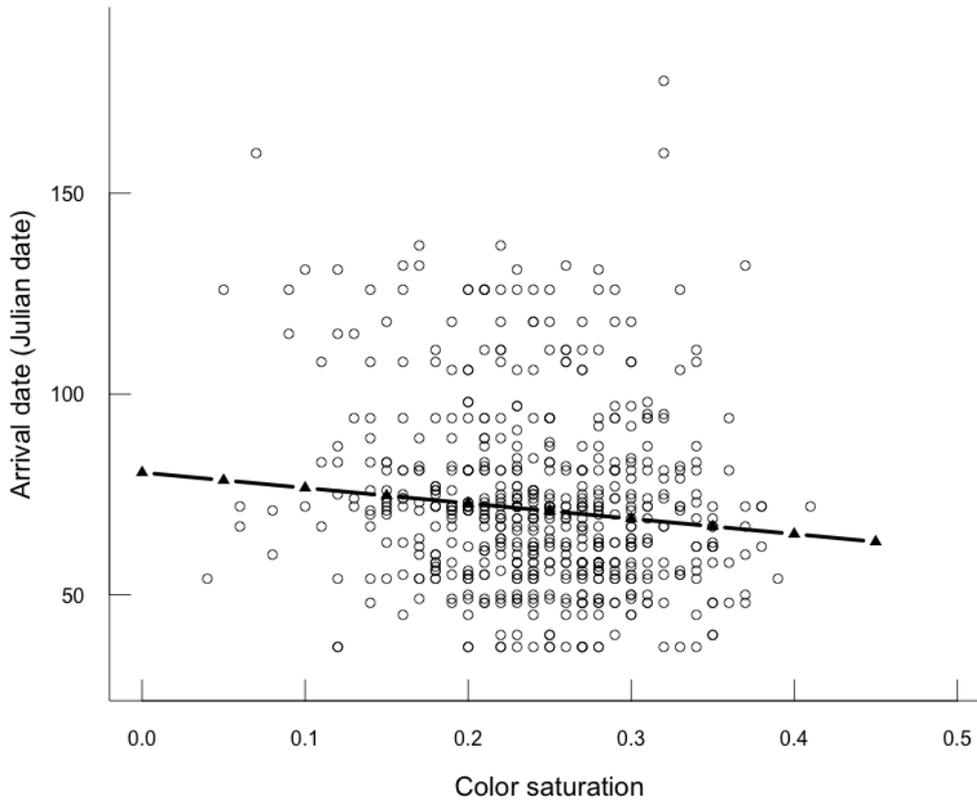
**Fig. 1.** (A) Robinson projection of color hue of dorsal feathers from 651 Spanish barn swallows. The distribution of dots indicates the variation in hue among the colors of the dorsal feathers of each individual, given by  $\theta$ , and  $\Phi$ , which are acting as coordinates of latitude and longitude, respectively (Endler et al. 2005; Stoddard and Prum 2008). Values of  $\theta$  range from  $-\pi$  to  $\pi$ , and values of  $\Phi$  from  $-\pi/2$  to  $\pi/2$ , from left to right and up to down in the graphics, respectively. The hue vectors are projected onto a sphere centered at the achromatic origin, and the sphere is depicted using the Robinson projection. Black triangles indicate projection of the vertices  $u$ ,  $s$ ,  $l$  and  $m$  from the tetrahedral color space (B)



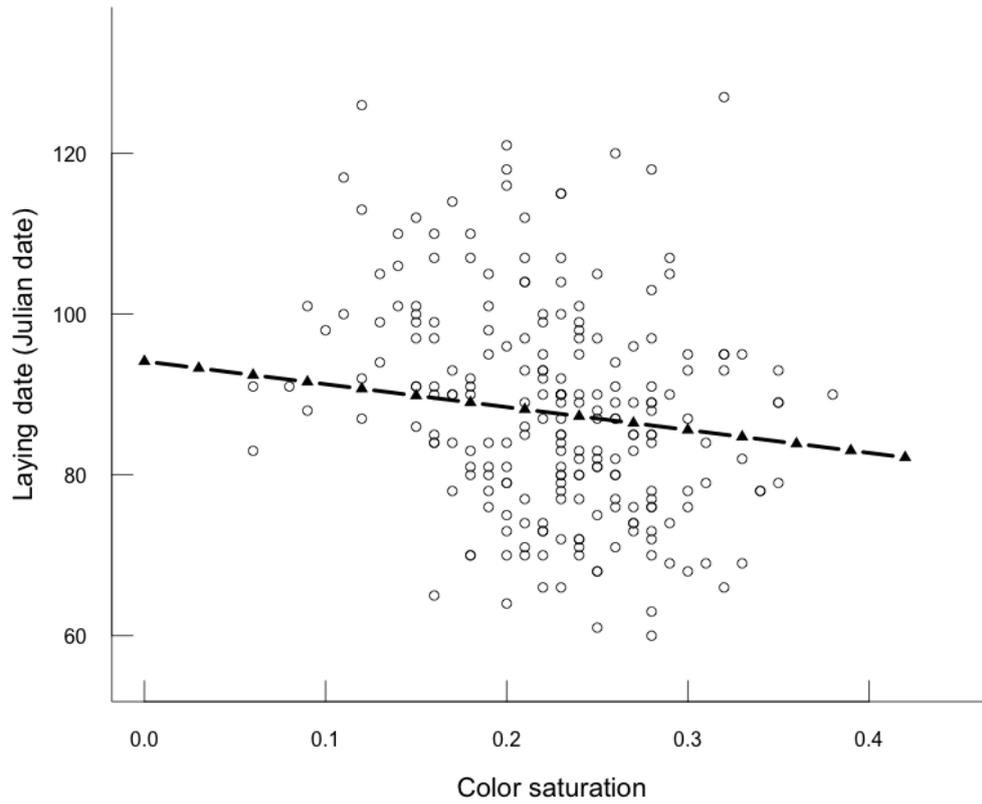
**Fig. 2.** The effect of coloration of dorsal feathers on survival in 486 barn swallows using the predicted values by our model



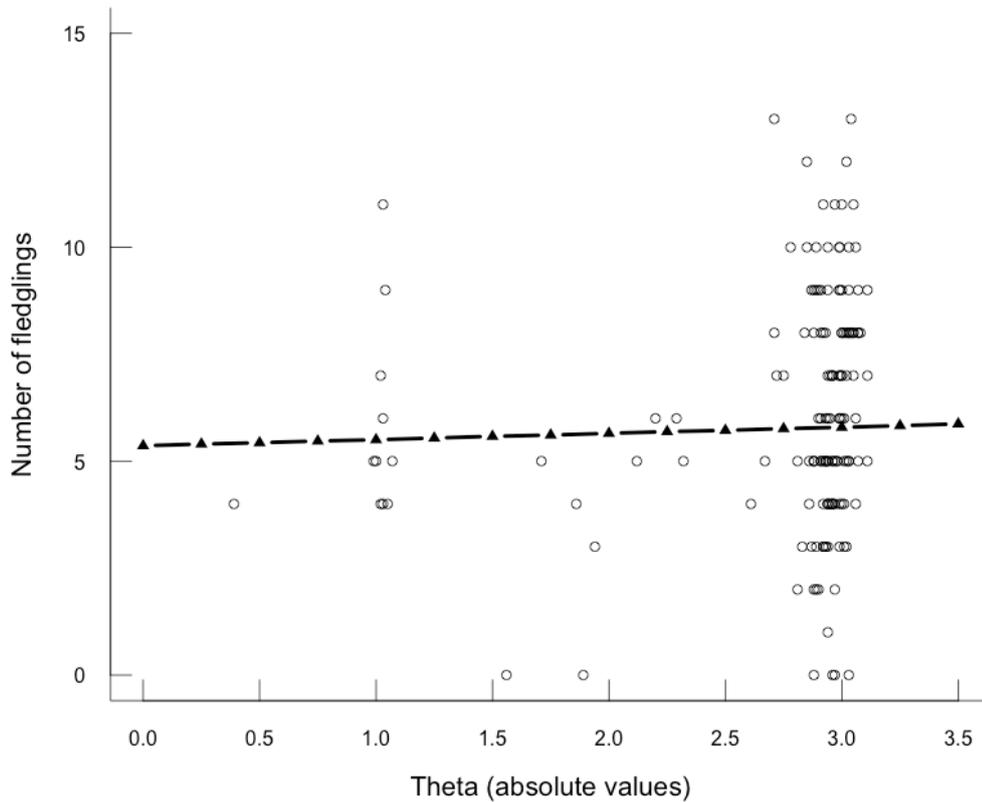
**Fig. 3.** Relationship between arrival date and degree of color saturation ( $rA$ ) in the dorsal feathers of 585 Spanish barn swallows (day 1 = 1st of January). Points correspond with the observed values and the line represents the values predicted by the model



**Fig. 4.** Relationship between date of first laying and degree of color saturation (rA) in dorsal feathers of 218 female Spanish barn swallows (day 1 = 1st of January). Points correspond with the observed values and the line represents the values predicted by the model



**Fig. 5.** Relationship between the number of fledglings raised by a pair of barn swallows during the breeding season and the absolute value of hue ( $|\theta|$ ) for the dorsal feathers of the female member of 152 pairs of Spanish barn swallows. Points correspond with the observed values and the line represents the values predicted by the model



### Capítulo 3

#### **La infección por malaria y la tasa de crecimiento de las plumas predicen el éxito reproductivo en aviones comunes.**

Effectos de arrastre ocurren cuando los eventos que ocurren en una estación influyen en el desempeño individual en la estación siguiente. Los parásitos sanguíneos (por ejemplo, *Plasmodium* y *Haemoproteus*) tienen un fuerte efecto negativo en la condición corporal de sus anfitriones y pueden ralentizar la tasas de crecimiento de las plumas en los cuarteles de invierno. Sucesivamente, estos costes en la muda invernal podrían reducir el éxito reproductivo en las siguientes épocas de cría. En aviones comunes *Delichon urbica* capturados y estudiados en un lugar de cría en Europa utilizamos la ptilocronología para medir la tasa de crecimiento de las rectrices mudadas en el área de invernada en África, y evaluamos el estado de la infección por parásitos sanguíneos transmitidos en los cuarteles de invierno. Encontramos una asociación negativa entre el estado de infección por hameosporídios parásitos y la tasa inferida de crecimiento de las rectrices. La baja tasa de crecimiento y la infección parasitaria sanguínea estuvieron relacionados con un retraso en la fecha de puesta en los cuarteles de cría europeos. Además, el tamaño de puesta y el número de volantones estuvieron negativamente relacionados con la fecha de puesta retrasada y con la infección parasitaria sanguínea. Estos resultados subrayan la importancia de los parásitos sanguíneos y la tasa de crecimiento de las plumas como posibles mecanismos que impulsan los efectos de arrastre para explicar las diferencias en la eficacia biológica en poblaciones salvajes de aves migratorias.

## **Malaria infection and feather growth rate predict reproductive success in house martins**

Alfonso Marzal, Maribel Reviriego, Ignacio G. Hermosell, Javier Balbontín, Staffan Bensch, Carmen Relinque, Laura Rodríguez, Luz Garcia-Longoria, Florentino de Lope

Carry-over effects take place when events occurring in one season influence individual performance in a subsequent season. Blood parasites (e.g. *Plasmodium* and *Haemoproteus*) have strong negative effects on the body condition of their hosts and could slow the rate of feather growth on the wintering grounds. In turn, these winter moult costs could reduce reproductive success in the following breeding season. In house martins *Delichon urbica* captured and studied at a breeding site in Europe, we used ptilochronology to measure growth rate of tail feathers moulted on the winter range in Africa, and assessed infection status of blood parasites transmitted on the wintering grounds. We found a negative association between haemosporidian parasite infection status and inferred growth rate of tail feathers. A low feather growth rate and blood parasite infections were related to a delay in laying date in their European breeding quarters. In addition, clutch size and the number of fledglings were negatively related to a delayed laying date and blood parasite infection. These results stress the importance of blood parasites and feather growth rate as potentially mechanisms driving carry-over effects to explain fitness differences in wild populations of migratory birds.

## Introduction

Annual periodicity provokes seasonal changes in environmental conditions of temperate areas to which animals must adapt in order to reproduce and survive. Migration, the regular seasonal journey undertaken by many different groups of animals, is a response to such environmental hazards in search of mild ecological conditions like warmer temperatures and more food availability (Lack 1968; Gauthreaux 1982). In birds, this usually involves the movement of billions of individuals flying vast distances between a breeding site and another non-breeding area, where they spend the rest of the year (Greenberg and Marra 2005; Newton 2008). In consequence, migratory individuals spend different periods of their annual cycle in widely separated and ecologically disparate locations. These periods are linked, so individuals can carry over effects from one season to the next (Webster et al. 2002; Harrison et al. 2011). In this line, Marra et al. (1998) showed in songbirds that winter habitat can influence the departure dates from winter quarters and subsequent dates of arrival and condition on the breeding grounds. Also, Norris et al. (2004) showed that the reproductive success of long-distance migratory American redstarts (*Setophaga ruticilla*) is influenced by the quality of the winter habitat, which is located thousands of kilometres from the temperate breeding grounds. Moreover, Saino et al. (2004) reported in barn swallows *Hirundo rustica* that higher winter food availability resulted in earlier arrival on the breeding grounds, increased frequency of second clutches and more fledged offspring per season.

In bird species, feathers are constantly exposed to degrading agents such as physical abrasion (Butler and Johnson 2004), bacterial activity (Burt and Ichida 1999) and ultraviolet light (Bergman 1982). Moulting, the regular replacement of feathers, has evolved as an important strategy in avian life-histories to repair this damage and to maintain plumage functions. The moulting process involves changes in nutritional and energetic demands of birds (Klaassen 1995; Murphy 1996), and important reallocation of resources among organs and functions (Murphy and Taruscio 1995; Murphy 1996; Nava et al. 2001). Accordingly, both the duration and timing of moulting is usually adjusted within the annual cycle of birds to minimise the overlap with other

energetically demanding activities such as breeding and migration (Jenni and Winkler 1994; Yuri and Rohwer 1997; de la Hera et al. 2009a). In this context, Rohwer et al. (2011) have recently shown a trade-off between moult and breeding in the black-footed albatross (*Phoebastria nigripes*), where adults with overly worn primary flight feathers suffered reduced fledging success in the current breeding season and were likely to skip the next breeding season. In addition, Nilsson and Svensson (1996) showed experimentally that a delay of breeding may compromise future reproduction and survival in blue tits (*Cyanistes caeruleus*), suggesting that these long-term effects may be the result of the late breeding following the clutch removal interfering with the moult that occurs after breeding. Also, feather quality might play a key role in moult strategies in birds. For instance, Dawson et al. (2000) demonstrated experimentally that newly grown primary feathers of male European starlings (*Sturnus vulgaris*) that re-grew quickly were of poorer quality. Similarly, Serra (2001) showed in grey plovers *Pluvialis squatarola* a higher quality of primaries that were re-grown at a slow speed.

Moult patterns can be affected by parasite infection. In this sense, Langston and Hillgarth (1995) showed that Laysan albatrosses *Phoebastria immutabilis* infected with oesophageal parasites replaced fewer primaries, grew fewer primaries simultaneously and began their moult later than uninfected individuals. Avian malaria and related haemosporidian parasites are known to provoke detrimental effects on their hosts by decreasing body condition (Valkiūnas 2005; Palinauskas et al. 2008). Moreover, some studies have shown blood parasite infections associated with moult status of their avian hosts. Morales et al. (2007) showed that the proportion of female pied flycatchers *Ficedula hypoleuca* infected with *Haemoproteus* at an initial breeding stage was higher among females with a moult–breeding overlap compared with females with no overlap. Also, Tarello (2007) reported a stop or delayed moult in falcons infected with *Haemoproteus*. In contrast, Allander and Sundberg (1997) did not find any relationship between moult score and *Haemoproteus* intensity in captive male yellowhammers *Emberiza citrinella*. However, whether avian blood parasites affect the growth rate of an individual feather still remains unknown.

Here, we propose an easy and quick way of using ptilochronology under field conditions to measure growth rate in outermost tail feathers based on daily feather growth shown by the number of growth bars in tail streamers. The house martin *Delichon urbica* is a small colonial hirundine which moults its wing and tail feathers in Africa (Cramp 1988; Turner and Rose 1989). This migratory species gets most of its blood parasite infections in Africa, but carry them back to Europe as chronic infections. This assumption is based on the absence of blood parasite infection in more than 400 fledgling and juvenile house martins analysed before their first migration (Marzal, unpublished data). Here, we analyse whether the presence of malaria infection during the breeding season is associated with the feather growth rate during moult the preceding winter in Africa. Also, we address the carry-over effects of these two events taking place at the African winter grounds (rectrix growth rate and haemosporidian infection) on the breeding success in Europe. If blood parasite infection provokes detrimental effects on daily elongation of rectrix of house martins, we would expect a lower rectrix growth rate in infected birds. In addition, since events on wintering grounds can affect breeding performance, we would also predict a positive effect of rectrix growth rate and a negative effect of haemosporidian infection on martins.

## **Materials and methods**

### *Study site and collecting samples*

The study was carried out in a colony of house martins in the surroundings of Badajoz (38°52'N, 7°05'W), southwest Spain, during an 11-year period (2000–2010).

Since breeding parameters differ from the first to the second clutches in house martins (Christe et al. 2001), as well as the effects of malaria parasites (Christe et al. 2002), only data from first clutches were used in this study. From February to July, we followed reproductive events and collected blood samples from breeding individuals. Nests were inspected every second day to determine the start of laying. When the clutch was finished, as shown by no more eggs being added to the clutch, we captured adult house martins at dawn in their nests

Multi-focus approach to life histories in passerines

and recorded body mass with a Pesola spring balance to the nearest 0.5 g. Feathers were inspected for the presence of chewing lice by holding the extended tail against the light and counting parasites (see Christe et al. 2002 for detailed information). We took a blood sample in a capillary tube for measurement of haematocrit. After centrifugation, blood cells were stored in 500 µL of 96 % ethanol at room temperature.

#### *Measurement of feather growth rate*

In the field, the length of the right outermost tail feather was measured to the nearest 1 mm with a ruler. In addition, the number of growth bars was estimated by holding the extended tail against the light and counting the number of dark bands. Bird feathers have a series of light and dark bands perpendicular to the feather rachis. Each light and dark band taken together (one growth bar) represents 24 h of growth (Riddle 1908; Michener and Michener 1938; Brodin 1993; Grubb 2006). Thus, the number of dark bands indicates the number of days employed in moulting these feathers.

In order to assess the accuracy of data obtained in the field, we plucked the right rectrix of 89 randomly chosen house martins after field measurements. We measure the number of growth bars and the length of right outermost rectrix feather in a gel documentation system in the laboratory following the instructions from Shawkey et al. (2003). To visualise growth bars, we placed the feather in the light cabinet. We positioned a ruler near to the feather for use as a scale marker. Growth bars were most clearly visible when one of the two cabinet lights was shaded using a piece of black cardboard. Once contrast and resolution were optimised, a digital image of the feather was obtained. We measured the number of growth bars and the length of rectrix minus the calamus using ImageJ software (Abramoff et al. 2004).

#### *Molecular detection of blood parasite infections*

Haemosporidian parasites (*Plasmodium spp.* and *Haemoproteus spp.*) were detected from

blood samples using molecular methods (Bensch et al. 2000; Waldenström et al. 2004). DNA from the avian blood samples were extracted in the laboratory using a standard chloroform/isoamylalcohol method (Sambrook et al. 2002). Diluted genomic DNA (25 ng/ll) was used as a template in a polymerase chain reaction (PCR) assay for detection of the parasites using nested-PCR protocols described by Waldenström et al. (2004). The amplification was evaluated by running 2.5 ll of the final PCR on a 2 % agarose gel. All PCR experiments contained one negative control for every eight samples. In the very few cases of negative controls showing signs of amplification (never more than faint bands in agarose gels), the whole PCR-batch was run again to make sure that all positives were true. Although we did not sequence positive samples, a previous study had revealed that *Haemoproteus* is the most prevalent blood parasite genus infecting house martins from this population (Marzal et al. 2008).

#### *Statistical procedures*

To assess the concordance in continuous data between paired measures of the same value, we calculated the concordance correlation coefficient,  $qc$ , between the number of growth bars and length of outermost rectrix feather from field data and laboratory data (Zar 1996; Lin 2000). The concordance correlation coefficient was obtained using the online statistical calculator tool provided by NZ Ministry of Health, the Foundation for Research, Science and Technology (FRST), and Environmental Diagnostics Ltd (<http://www.niwa.co.nz/our-services/online-services/statistical-calculators>).

We used one-way ANOVA to test for significant differences between uninfected and infected house martins for the length of rectrix feather, the number of growth bars, breeding success and three individual measures of body condition (body mass, haematocrit value and the abundance of chewing lice on the tail feather).

We used general linear models (GLM) to investigate the effect of sex, year (i.e.

Multi-focus approach to life histories in passerines

environmental variation), body mass (body condition), haematocrit, the abundance of chewing lice and infection status (uninfected or infected) on the rectrix growth rate of house martins. Sex, year and infection status were included as fixed factors in the model. There was missing information for some individuals, which resulted in slightly varying sample sizes in the different analyses. The total dataset included each individual only once to avoid pseudoreplication (Hurlbert 1984).

We also used GLM with laying date, clutch size and number of fledglings as dependent variables. In these models, infection status (uninfected, infected), sex, year, haematocrit, body mass, number of chewing lice and rectrix growth rate were included as independent variables in each case. Since, timing of breeding could affect breeding success in hirundines (Balbontín et al. 2007, 2009) laying date was also included as predictor, when clutch size and number of fledglings were the response variables. We used a backward stepwise procedure to eliminate all non-significant terms ( $P > 0.05$ ) from our starting maximal model. Only the final models were presented. All analyses were performed using PASW Statistics 18 statistical package for Windows.

## **Results**

### *Concordance between field and laboratory measurements*

We measured the length of rectrix and the number of growth bars in the same feather both in the field and in the laboratory in order to assess the agreement between measurements. We evaluated the accuracy in these measurements by using the concordance correlation coefficient. The results showed a substantial agreement between measurements both in length of rectrix [sample concordance correlation coefficient (qc) =0.956] and in the number of growth bars [sample concordance correlation coefficient (qc) =0.962]. In addition, we found a substantial agreement between measurements of rectrix growth rate in field and in the laboratory [sample concordance correlation coefficient (qc) =0.957].

*Blood parasites and body condition*

We analysed 444 blood samples from house martins in search for blood parasites. We found 155 (35 %) uninfected individuals and 289 (65 %) individuals infected with blood parasites. The length of rectrix feather was smaller in infected house martins than in uninfected individuals [mean length (SD): uninfected =60.90 (2.66); infected = 59.85 (2.71); ANOVA  $F_{1, 443} = 15.201$ ,  $P < 0.001$ ]. Infected house martins had more growth bars than uninfected birds [mean number of growth bars (SD): uninfected = 19.20 (3.32); infected =20.25 (2.53); ANOVA  $F_{1, 443} = 13.944$ ,  $P < 0.001$ ]. Infected house martins had lower body mass than uninfected individuals [mean body mass (SD): uninfected =16.73 (1.75); infected =16.23 (1.47); ANOVA  $F_{1, 443} = 9.95$ ,  $P = 0.002$ ]. In contrast, there were no differences between infected and uninfected house martins in the abundance of chewing lice [mean intensity of chewing lice (SD): uninfected =8.88 (10.11); infected =8.70 (9.92); ANOVA  $F_{1, 443} = 0.03$ ,  $P = 0.85$ ] or haematocrit levels [mean haematocrit (SD): uninfected =53.97 (3.42); infected =53.47 (3.71); ANOVA  $F_{1, 443} = 1.97$ ,  $P = 0.16$ ].

*Blood parasites and rectrix growth rate*

The GLM showed that infection status and year explained significant variation in rectrix growth rate, while controlling for other potentially influencing variables such as sex, abundance of chewing lice, haematocrit and body mass. Specifically, non-infected individuals had higher rectrix growth rate than infected individuals [mean feather growth rate (SD): uninfected =3.24 (0.57) mm/day; infected =2.99 (0.43) mm/day; estimate (SE) =0.042 (0.12)]. None of the interaction terms were statistically significant ( $P > 0.10$ ) in all models (Fig. 1; Table 1).

*Blood parasites, rectrix growth rate and reproduction investment*

Prevalence of blood parasites, year and rectrix growth rate explained significant variation in laying date. In contrast, neither body mass, haematocrit, abundance of chewing lice or sex explained a significant variation in laying date (Table 2). Uninfected birds initiated clutches

earlier than infected birds [mean laying date (SD) (Julian date): uninfected =104.59 (19.30); infected =114.47 (18.74); estimate (SE) =-5.087 (5.762)] (Fig. 2a). Moreover, individuals that had moulted their rectrix feathers faster also initiated their clutches earlier [GLM; estimate slope (SE) =-5.78 (2.19); Table 2; Fig. 3].

We found an effect of year, prevalence of blood parasites and laying date in clutch size (Table 3). Uninfected birds laid larger clutches compared with infected birds [mean clutch size (SD): uninfected =4.56 (0.71); infected =4.34 (0.72); estimate (SE) =0.132 (0.26)] (Fig. 2b). Those birds that initiated their reproduction earlier also laid larger clutches [estimate slope (SE) =0.007 (0.003)].

Finally, we found that prevalence of blood parasites, year and laying date influenced the number of fledglings, while controlling for other confounding variables such as body mass, sex, haematocrit and abundance of chewing lice (Table 4). However, rectrix growth rate did not affect the number of fledging produced [GLM; estimate slope (SE) =0.216 (0.17),  $F = 1.55$ ,  $P [0.05]$ . Uninfected birds also fledged more nestlings than infected house martins [mean number of nestlings (SD): uninfected =3.71 (1.06); infected =3.08 (1.30); estimate (SE) =0.532 (0.435)] (Fig. 2c). Those birds that initiated their reproduction earlier also bred more nestlings [estimate (SE) =-0.010 (0.004)]. Uninfected house martins also had higher breeding success (number of fledglings divided by clutch size) [mean breeding success (SD): uninfected =0.82 (0.20); infected =0.71 (0.28); ANOVA  $F_{1, 414} = 16.07$ ,  $P \setminus 0.001$ ].

## Discussion

Here, we show for the first time an association between malaria and related haemosporidian parasites infection and the growth rate of rectrix feather of birds. Applying a ptilochronology-modified technique, the main findings of this study were that: (1) rectrix growth rate in wintering house martins was associated with higher occurrence of blood parasite infection, (2) a low rectrix growth rate and blood parasite infection was associated with a delay in laying date in their European breeding quarters, (3) clutch size and the number of reared chicks were

negatively related to haemosporidian infection and a late laying date, and (4) rectrix growth rate and reproductive success varied significantly between the study years. Next, we will discuss these results in detail.

Previous studies have applied ptilochronology to estimate moult speed of both migratory and sedentary birds by measuring the width of growth bars (de la Hera et al. 2009b). Here, we used ptilichronology under field conditions to study the feather growth rate during the preceding winter to address its consequences for the reproductive performance. This non-invasive methodology requires only that individuals are captured once, and it is not limited to the moulting period. Given that a bird carries a regenerated feather until the next moult, the capture and study of moult can be studied whenever and wherever the bird is capture.

Moult is an unfavourable period for birds, since it reduces flight performance (Williams and Swaddle 2003), increases the exposure to predators (Lind 2001) and increases costs of thermoregulation (Ginn and Melville 1983). We found that rate of rectrix growth was negatively related to blood parasite infection, where infected individuals had a lower daily feather elongation. To the best of our knowledge, this is the first study showing an association between blood parasite infection and the rate of feather elongation. We propose two non-mutually exclusive hypotheses to explain this observation. First, the association between feather growth rate and blood parasites infection could be a consequence of the nutrients directly consumed by the parasites. In this sense, it is known that malaria parasites obtain essential amino acids from host plasma and digestion of host haemoglobin (Sherman 1979; Martin and Kirk 2007). The renewal of the feathers is one of the most remarkable metabolic feats that birds undergo during the course of their annual cycle, involving large amounts of amino acids, the main chemical compound of feathers (Murphy et al. 1990). In haemosporidian species, the synthesis of new malaria proteins during infection requires substantial amounts of leucine, valine and isoleucine (Sherman 1977), which are three of the four most abundant essential amino acids in the composition of bird feathers (Murphy et al.

1990). In consequence, the removal of essential amino acids from the avian host by blood parasites could provoke this decrease in feather growth rate. Alternatively, these differences in daily feather elongation could be caused by the need to mount an immune response against the blood parasites. Because both avian moult and immune response are nutrient-demanding processes (Murphy 1996; Martin et al. 2003; Klasing 2004), there may be a reallocation of resources and, consequently, trade-offs between physiological activities (Wikelski and Ricklefs 2001; Monaghan 2004), where the rate of feather growth of infected house martins is decreased, given that it is necessary to assign resources to mount an immune response. In this line, Amat et al. (2007) experimentally showed that regenerated feathers of immune-challenged greenfinches *Carduelis chloris* were more asymmetric in length than regenerated feathers from control birds. Moreover, Sanz et al. (2004) showed that immunised male pied flycatchers *Ficedula hypoleuca* delayed the onset of post-nuptial primary moult compared to control males, suggesting that the activation of the immune system decreased the resources necessary for initiating post-nuptial moult. Recently, Moreno-Rueda (2010) showed in male house sparrows *Passer domesticus* that the stimulation of the immune system with an antigen affected the moult by reducing its speed to half, showing a trade-off between moulting and the immune response. Further experimental investigations are needed to demonstrate conclusively the detrimental effect of haemosporidian infection on the feather growth rate of birds.

A 'seasonal interaction' can occur if events and conditions in one region or season affect populations and individuals in another (Webster and Marra 2005). The pattern of feather moult during winter may affect departure schedules from the wintering grounds, and thus migratory dates. Because most African moulting species (i.e. house martin) do not start the northward migration until the moult is complete (Cramp 1988; Møller et al. 2011; Turner and Rose 1989), a lower speed in feather growth could lead to a delay in timing of migration (Marra et al. 1998) and, consequently, a late arrival and start of reproduction in Europe (Saino et al. 2004). We found that the rate of feather growth and blood parasite infection in Africa was

associated with poorer breeding performance of house martins in their European breeding quarters. In agreement with our predictions, individuals moulting their outermost tail feathers more quickly advanced their laying date in their breeding areas. On the other hand, haemosporidian infected birds laid their clutches later in the breeding season. Given that, only uninfected house martins could moult their feather quickly and advance their arrival at their breeding grounds. Similar results were reported by Møller et al. (2004) in migratory barn swallows, where those infected with blood parasites delayed their arrival at Spanish breeding quarters.

These carry-over effects should lead to variations in reproductive success, because birds tend to show declining prospects of reproductive success as the breeding season advances (Pajuelo et al. 1992; Verhulst et al. 1995; Hansson et al. 2000; Arnold et al. 2004). For instance, Brown and Brown (1999) reported a decline in clutch size and annual reproductive success across the season in cliff swallows *Petrochelidon pyrrhonota*. In the same line, Sakraoui et al. (2005) showed a clear negative relation between clutch size and laying date in barn swallows. These observations are in agreement with our findings, since the delay in laying date was related to a decrease in clutch size and in the number of nestlings produced. We also found that blood parasite infection was associated with a decrease in clutch size and a reduced number of fledged chicks. Previous experimental studies on passerine birds reported similar detrimental effects of haematozoan parasites on breeding performance, negatively affecting brood size and breeding success (Merino et al. 2000; Marzal et al. 2005). In addition, the production of an immune response to fight against pathogens could be costly for house martins in terms of delay in egg laying and smaller brood sizes (Marzal et al. 2007).

Finally, we reported an inter-annual variation in rectrix growth rate, clutch size and the number of reared young. It is well established that variation in environmental factors can shape the evolution of life histories (Partridge and Harvey 1988). Previous studies have also

shown between year variation in estimated moult duration (Bensch and Grahn 1993; Smith and Sheeley 1993), while other studies have reported that moult speed did not vary across years (Borras et al. 2004). On the other hand, inter-annual variation in breeding success has also been reported in wild birds (Pajuelo et al. 1992; Cézilly et al. 1995; Forbes 2010). Such differences could be explained by annual variations in environmental factors such as climate or food availability that can affect moult phenology and/or key reproductive traits of birds (Bryant 1975; Murphy 1986; Smith and Sheeley 1993; Renfrew et al. 2011).

In conclusion, the results obtained in the present study reveal a negative association between haemosporidian parasite infection and the feather growth rate of house martins. Moreover, our findings also suggest carry-over effects between African winter quarters and European breeding sites, where timing of reproduction and reproductive success were negatively related to the rectrix growth rate and infection of blood parasites. It would be desirable that further studies would take into account blood parasite infection and feather growth rate as potentially mechanisms driving carry-over effects between winter quarters and breeding areas to explain fitness differences in wild populations of migratory birds.

### **Acknowledgments**

We thank to W. Douglas Robinson and two anonymous referees for their constructive comments on the manuscript. This study was funded by research projects of the Spanish Ministry of Education and Science (CGL2009-08976), Junta of Extremadura (PRI08A116) and University of Extremadura (A7-26). A.M., I.G.H. and L.G.L. were supported by grants from Spanish Ministry of Education and Science (JC2011-0405, BES-2007-15549 and BES-2010-030295, respectively). S.B. was supported by a grant from the Swedish Research Council (621-2007-5193). L.R. was supported by a collaboration grant of UEx. All the experiments comply with the current laws of Spain, where the experiments were performed.

## References

- Abramoff MD, Magelhaes PJ, Ram SJ (2004) Image processing with image. *J Biophotonics Int* 11:36–42
- Allander K, Sundberg J (1997) Temporal variation and reliability of blood parasite levels in captive yellow hammer males *Emberiza citrinella*. *J Avian Biol* 28:325–330
- Amat JA, Aguilera E, Visser GH (2007) Energetic and developmental costs of mounting an immune response in greenfinches (*Carduelis chloris*). *Ecol Res* 22:282–287
- Arnold JM, Hatch JJ, Nisbet ICT (2004) Seasonal declines in reproductive success of the common tern *Sterna hirundo*: timing or parental quality? *J Avian Biol* 35:33–45
- Balbontín J, Hermosell IG, Marzal A, Reviriego M, de Lope F, Møller AP (2007) Age-related change in breeding performance in early life is associated with an increase in competence in the migratory barn swallow (*Hirundo rustica*). *J Anim Ecol* 76:915–92
- Balbontín J, Møller AP, Hermosell IG, Marzal A, Reviriego M, de Lope F (2009) Divergent patterns of impact of environmental conditions on life history traits in two populations of a long-distance migratory bird. *Oecologia* 159:859–872
- Bensch S, Grahn M (1993) A new method for estimating individual speed of moult. *Condor* 95:305–315
- Bensch S, Stjernman M, Hasselquist D, Östman Ö, Hansson B, Westerdahl H, Pinheiro RT (2000) Host specificity in avian blood parasites: A study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. *Proc R Soc Lond B* 267:1583–1589
- Bergman G (1982) Why are the wings of *Larus f. fuscus* so dark? *Ornis Fenn* 59:77–83
- Borras A, Cabrera T, Cabrera J, Senar JC (2004) Inter-locality variation in speed of moult in the citril finch *Serinus citrinella*. *Ibis* 146:14–17
- Brodin A (1993) Radio-ptilochronology tracing radioactively labelled food in feathers. *Ornis Scand* 24:167–173
- Brown CR, Brown MB (1999) Fitness components associated with laying date in the cliff

Multi-focus approach to life histories in passerines

swallow. *Condor* 101:230–245

Bryant DM (1975) Breeding biology of the house martin *Delichon urbica* in relation to aerial insect abundance. *Ibis* 117:180–221.

Burt EH Jr, Ichida JM (1999) Occurrence of feather-degrading bacilli in the plumage of birds. *Auk* 116:364–372

Butler M, Johnson AS (2004) Are melanized feather barbs stronger? *J Exp Biol* 207:285–293

Cézilly F, Boy V, Green RE, Hirons GH, Johnson AR (1995) Interannual variation in greater flamingo breeding success in relation to water levels. *Ecology* 76:20–26

Christe P, de Lope F, González G, Saino N, Møller AP (2001) The influence of environmental conditions on immune responses, morphology and recapture probability of nestling house martins (*Delichon urbica*). *Oecologia* 126:333–338

Christe P, Møller AP, González G, de Lope F (2002) Intraseasonal variation in immune defence, body mass and hematocrit in adult house martin *Delichon urbica*. *J Avian Biol* 33:321–325

Cramp S (1988) The birds of the western palearctic vol 5. Oxford University Press, Oxford.

Dawson A, Hinsley SA, Ferns PN, Bonser RHC, Eccleston L (2000) Rate of moult affects feather quality: a mechanism linking current reproductive effort to future survival. *Proc R Soc Lond B* 267:2093–2098

De la Hera I, Díaz JA, Pérez-Tris J, Tellería JL (2009a) A comparative study of migratory behaviour and body mass as determinants of moult duration in passerines. *J Avian Biol* 40:461–465

De la Hera I, Pérez-Tris J, Tellería JL (2009b) Migratory behaviour affects the trade-off between feather growth rate and feather quality in a passerine bird. *Biol J Linn Soc* 97:98–105

Forbes S (2010) Family structure and variation in reproductive success in blackbirds. *Behav Ecol Sociobiol* 64:475–483

Gauthreaux JSA (1982) The ecology and evolution of avian migration systems. In: Farner DS,

- King JR, Parker KC (eds) Avian biology. Academic, New York, pp 93–163
- Ginn HB, Melville DS (1983) Molt in birds (BTO Guide 19). British Trust for Ornithology, Hertfordshire
- Greenberg R, Marra PP (2005) Birds of two worlds; the ecology and evolution of migration. Johns Hopkins University Press, Portland
- Grubbr TC Jr (2006) Ptilochronology: feather time and the biology of birds. Oxford University Press, New York
- Hansson B, Bensch S, Hasselquist D (2000) The quality and the timing hypotheses evaluated using data on great reed warblers. *Oikos* 90:575–581
- Harrison XA, Blount JD, Inger R, Norris DR, Bearhop S (2011) Carry-over effects as drivers of fitness differences in animals. *J Anim Ecol.* 80:4–18.
- Hurlbert SH (1984) Pseudo-replication and the design of ecological field experiments. *Ecol Monogr* 54:187–211
- Jenni L, Winkler R (1994) Molt and ageing of European passerines. Academic, London
- Klaassen M (1995) Molt and basal metabolic costs in males of two subspecies of stonechats: the European *Saxicola torquata rubicula* and the east African *S. t. axillaris*. *Oecologia* 104:424–432
- Klasing KC (2004) The cost of immunity. *Acta Zool Sin* 50:961–969
- Lack D (1968) Ecological adaptations for breeding in birds. Methuen, London.
- Langston NE, Hillgarth N (1995) The extent of primary molt varies with parasites in Laysan albatrosses: a possible role in life history tradeoffs between current and future reproduction. *Proc R Soc Lond B* 261: 239–243
- Lin LI-K (2000) A note on the concordance correlation coefficient. *Biometrics* 56:324–325
- Lind J. 2001. Escape flight in moulting tree sparrows (*Passer montanus*). *Funct Ecol.* 15:29–35.
- Marra PP, Hobson KA, Holmes RT (1998) Linking winter and summer events in a migratory bird by using stable-carbon isotopes. *Science* 282:1884–1886

- Martin RE, Kirk K (2007) Transport of the essential nutrient isoleucine in human erythrocytes infected with the malaria parasite *Plasmodium falciparum*. *Blood* 109:2217–2224
- Martin LB II, Scheuerlein A, Wikelski M (2003) Immune activity elevates energy expenditure of house sparrows: a link between direct and indirect costs? *Proc R Soc Lond B* 270:153–158
- Marzal A, de Lope F, Navarro C, Møller AP (2005) Malarial parasites decrease reproductive success: an experimental study in a passerine bird. *Oecologia* 142:541–545
- Marzal A, Reviriego M, de Lope F, Møller AP (2007) Fitness costs of an immune response in the house martin (*Delichon urbica*). *Behav Ecol Sociobiol* 61:1573–1580
- Marzal A, Bensch S, Reviriego M, Balbontin J, de Lope F (2008) Effects of malaria double infection in birds: one plus one is not two. *J Evol Biol* 21:979–987
- Merino S, Moreno J, Sanz JJ, Arriero E (2000) Are avian blood parasites pathogenic in the wild? A medication experiment in blue tits (*Parus caeruleus*). *Proc R Soc Lond B* 267:2507–2510
- Michener H, Michener JR (1938) Bars in flight feathers. *Condor* 40:149–160
- Møller AP, de Lope F, Saino N (2004) Parasitism, immunity, and arrival date in a migratory bird, the barn swallow. *Ecology* 85:206–219
- Møller AP, Nuttall R, Piper SE, Szép T, Vickers EJ (2011) Migration, moult and climate change in barn swallows *Hirundo rustica* in South Africa. *Clim Res* 47:201–205
- Monaghan P (2004) Resource allocation and life history strategies in birds. *Acta Zool Sin* 50:942–947
- Morales J, Moreno J, Merino S, Sanz JJ, Tomás G, Lobato E, Martínez-de la Puente J. (2007) Early moult improves local survival and reduces reproductive output in female pied flycatcher. *Ecoscience* 14:31–39
- Moreno-Rueda G (2010) Experimental test of a trade-off between moult and immune response in house sparrows *Passer domesticus*. *J Evol Biol* 23:2229–2237
- Murphy MT (1986) Temporal components of reproductive variability in eastern kingbirds

- (*Tyrannus tyrannus*). *Ecology* 67:1483–1492
- Murphy ME (1996) Energetics and nutrition of molt. In: Carey C (ed) *Avian energetics and nutritional ecology*. Plenum, New York, pp 158–198
- Murphy ME, Taruscio TG (1995) Sparrows increase their rates of tissue and whole-body protein synthesis during the annual molt. *Comp Biochem Physiol A Mol Integr Physiol* 111:385–396
- Murphy ME, King JR, Taruscio TG, Geupel GR (1990) Amino acid composition of feather barbs and rachises in three species of pygoscelid penguins: nutritional implication. *Condor* 92:913–921
- Nava MP, Veiga JP, Puerta M (2001) White blood cell counts in house sparrows (*Passer domesticus*) before and after moult and after testosterone treatment. *Can J Zool* 79:145–148
- Newton I (2008) *The migration ecology of birds*. Academic, London.
- Nilsson JÅ, Svensson E (1996) The cost of reproduction: a new link between current reproductive effort and future reproductive success. *Proc R Soc Lond B* 263:711–714
- Norris DR, Marra PP, Kyser TK, Sherry TW, Ratcliffe LM (2004) Tropical winter habitat limits reproductive success on the temperate breeding grounds in a migratory bird. *Proc R Soc Lond B* 271:59–64
- Pajuelo L, de Lope F, da Silva E (1992) Biología de la reproducción del avión común (*Delichon urbica*) en Badajoz, España. *Ardeola* 39:15–23
- Palinauskas V, Valkiunas G, Bensch S, Bolshakov VC (2008) Effects of *Plasmodium relictum* (lineage P-SGS1) on experimentally infected passerine birds. *Exp Parasitol* 120:372–380
- Partridge L, Harvey PH (1988) The ecological context of life history evolution. *Science* 241:1449–1455
- Renfrew RB, Frey SJK, Klavins J (2011) Phenology and sequence of the complete prealternate molt of bobolinks in South America. *J Field Ornithol* 82:101–113

- Riddle O (1908) The genesis of fault-bars in feathers and the cause of alternation of light and dark fundamental bars. *Biol Bull* 14:328–370
- Rohwer S, Viggiano A, Marzluff JM (2011) Reciprocal tradeoffs between molt and breeding in Albatrosses. *Condor* 113:61–71
- Saino N, Szép T, Romano M, Rubolini D, Spina F, Møller AP (2004) Ecological conditions during winter predict arrival date at the breeding quarters in a trans-saharan migratory bird. *Ecol Lett* 7:21–25
- Sakraoui R, Dadci W, Bańbura J, Chabi Y (2005) Breeding biology of barn swallows *Hirundo rustica* in Algeria, North Africa. *Ornis Fenn* 82:33–43
- Sambrook J, Fritsch FJ, Maniatis T (2002) *Molecular cloning, a laboratory manual*. Cold Spring Harbor Laboratory Press, New York
- Sanz JJ, Moreno J, Merino S, Tomás G (2004) A trade-off between two resource-demanding functions: post-nuptial moult and immunity during reproduction in male pied flycatcher. *J Anim Ecol* 73:441–447
- Serra L (2001) Duration of primary moult affects primary quality in grey plovers *Pluvialis squatarola*. *J Avian Biol* 32:377–380
- Shawkey MD, Beck ML, Hill GE (2003) Use of a gel documentation system to measure feather growth bars. *J Field Ornithol* 74:125–128
- Sherman IW (1977) Amino acid metabolism and protein synthesis in malarial parasites. *Bull World Health Org* 55:265–276
- Sherman IW (1979) Biochemistry of *Plasmodium* (malarial parasites). *Microbiol Rev* 43:453–495
- Smith L, Sheeley D (1993) Molt patterns of wintering northern pintails in the southern high plains. *J Wildl Manag* 57:229–238
- Tarello W (2007) Clinical signs and response to primaquine in falcons with *Haemoproteus tinnunculi* infection. *Vet Rec* 161:204–205
- Turner AK, Rose C (1989) *A handbook to the swallows and martins of the world*. Christopher

Helm, London

Valkiūnas G (2005) Avian malaria parasites and other haemosporidia. CRC, Boca Raton

Verhulst S, Balen JHV, Tinbergen JM (1995) Seasonal decline in reproductive success of the great tit: variation in time or quality? *Ecology* 76:2392–2403

Waldenström J, Hasselquist D, Östman Ö, Bensch S (2004) A new nested PCR method very efficient in detecting *Plasmodium* and *Haemoproteus* infections from avian blood. *J Parasitol* 90:191–194

Webster MS, Marra PP (2005) Importance of understanding migratory connectivity In: Greenberg Russell S. *Birds of two worlds: ecology and evolution of migration*. Johns Hopkins University Press, Baltimore, pp 199–209

Webster MS, Marra PP, Haig SM, Bensch S, Holmes RT (2002) Links between worlds: unraveling migratory connectivity. *Trends Ecol Evol* 17:76–83

Wikelski M, Ricklefs R (2001) The physiology of life-histories. *Trends Ecol Evol* 16:479–481

Williams EV, Swaddle JP (2003) Molt, flight performance, and wingbeat frequency during take-off in european starlings. *J Avian Biol* 34:371–378

Yuri T, Rohwer S (1997) Molt and migration in the northern roughwinged swallow. *Auk* 114:249–262

Zar JH (1996) *Biostatistical analysis*, 3rd edn. Prentice Hall, Upper Saddle River

TABLES

**Table 1.** Results from the GLM explaining variation in the feather growth rate for individual house martins *Delichon urbica*.

Independent variable	Square-sum III	df	F	P
Prevalence	0.463	1	3.945	0.048
Year	58.778	10	50.065	<0.001

Prevalence of blood parasites infection, year, sex, body mass, haematocrit and chewing lice were the predictor variables. Sample size was 444 individuals. Only statistical significant terms ( $P < 0.05$ ) are shown.

**Table 2.** Factors explaining variation in laying date of house martins.

Independent variable	Square-sum III	df	F	P
Prevalence	3,422.827	1	14.841	<0.001
Year	29,391.602	10	12.744	<0.001
Feather growth rate	1,621.924	1	7.033	0.008

A backward stepwise procedure was used in a GLM analysis with prevalence of blood parasite, sex, year, body mass, haematocrit, number of chewing lice and feather growth rate as predictor variables. Only independent variables selected by the backward stepwise procedure are listed. Sample size was 439 individuals.

**Table 3.** Factors explaining variation in clutch size of house martins

Independent variable	Square-sum III	df	F	P
Prevalence	2.818	1	5.742	0.017
Year	19.718	10	4.018	<0.001
Laying date	7.988	1	16.278	<0.001

A backward stepwise procedure was used in a GLM analysis with prevalence of blood parasite, sex, year, body mass, haematocrit, number of chewing lice, laying date and feather growth rate as predictor variables. Only independent variables selected by the backward stepwise procedure are listed. Sample size was 439 individuals.

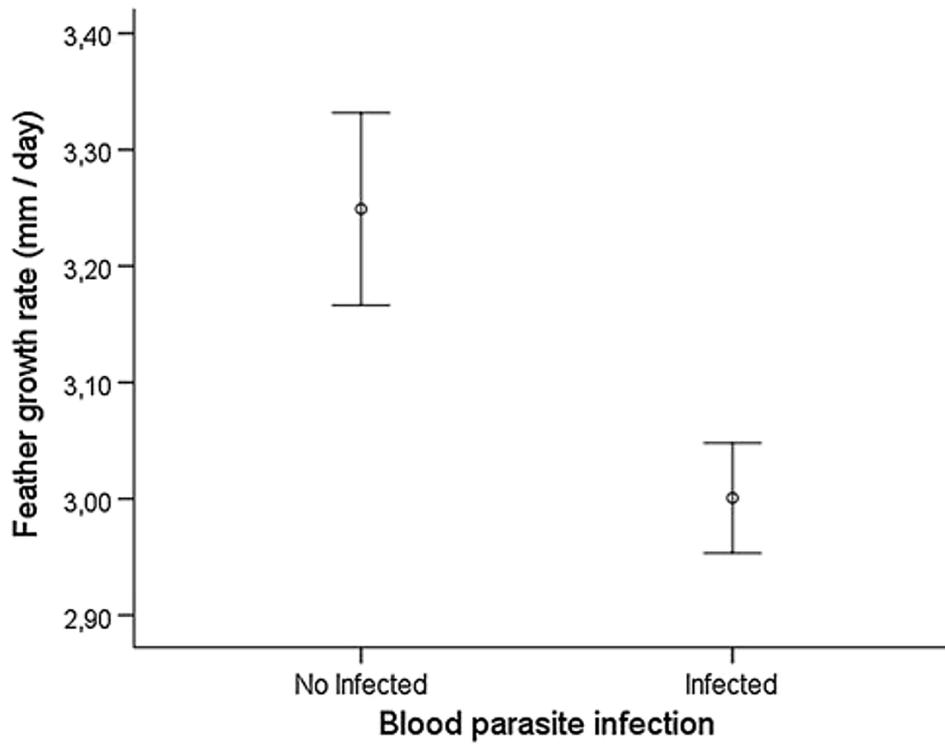
**Table 4.** Factors explaining variation in the number of fledglings produced by house martins

Independent variable	Square-sum III	df	F	P
Prevalence	34.277	1	26.370	<0.001
Year	55.239	10	4.250	<0.001
Laying date	15.221	1	11.710	0.001

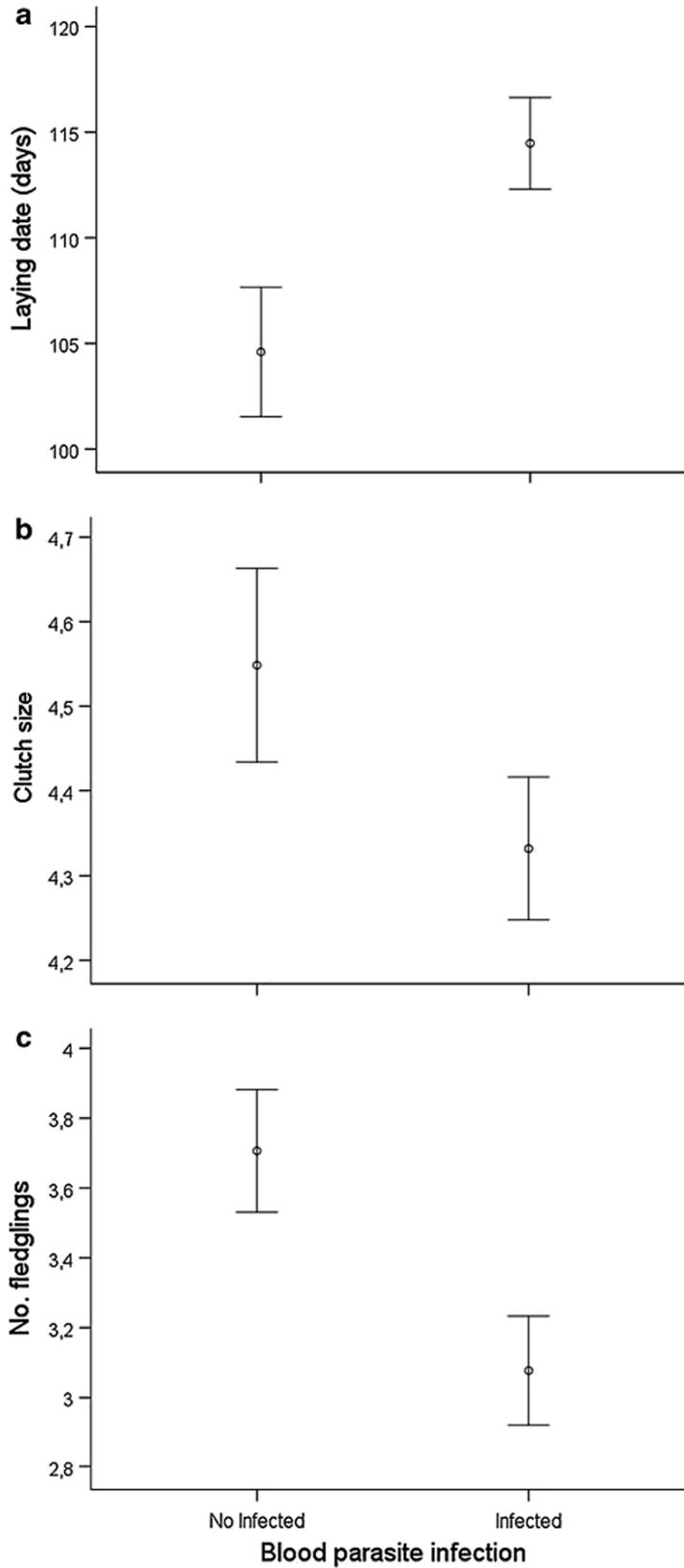
A backward stepwise procedure was used in a GLM analysis with prevalence of blood parasite, sex, year, body mass, haematocrit, number of chewing lice, laying date and feather growth rate as predictor variables. Only independent variables selected by the backward stepwise procedure are listed. Sample size was 410 individuals.

FIGURES AND LEGENDS

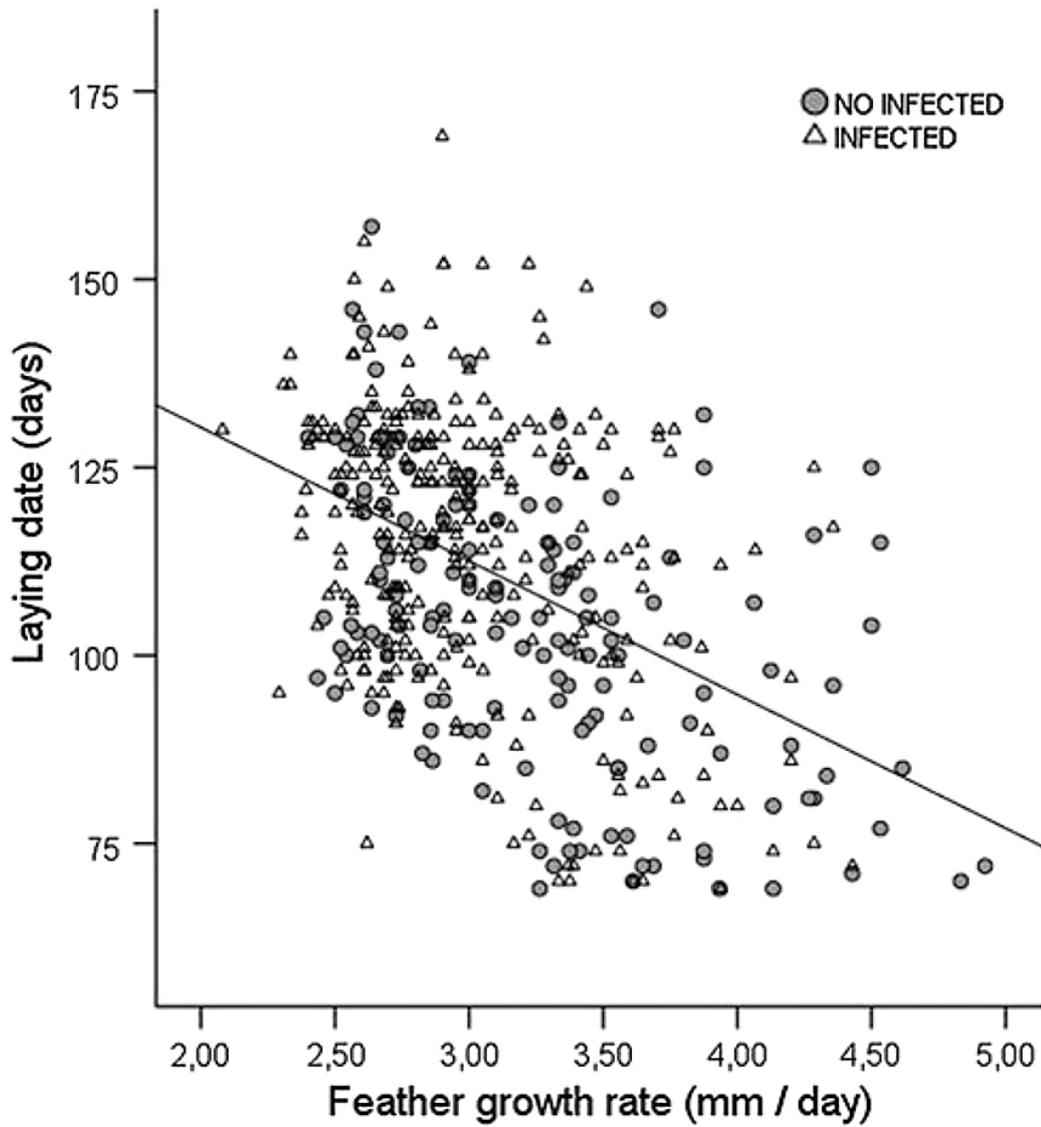
**Figure 1.** Feather growth rate for uninfected (n = 155) and infected house martins *Delichon urbica* (n = 289). Error bars show means  $\pm$  95 % of confidence interval



**Figure 2.** Reproductive parameters for uninfected (n = 155) and infected house martins (n = 289) of a laying date, b clutch size and c number of fledglings. Error bars show means  $\pm$  95 % of confidence interval



**Figure 3.** Scatter plot showing the relationship between the feather growth rate and laying date for uninfected (open symbol, n = 155) and infected house martins (closed symbol, n = 289)



## Capítulo 4

### **Variación en la morfometría de los espermatozoides y en la competencia espermática entre poblaciones de Golondrina común (*Hirundo rustica*).**

Los espermatozoides varían enormemente en tamaño y forma entre distintas especies del reino animal. Se cree que la selección sexual postcopulatoria es la principal fuerza evolutiva que impulsa esta diversidad. En contraste, se sabe poco acerca de cómo el tamaño del espermatozoide varía entre poblaciones de una misma especie. Aquí investigamos la variación geográfica en el tamaño del esperma en la Golondrina común *Hirundo rustica*, un paseriforme socialmente monógamo con una amplia distribución de cría Holártica. Incluimos muestras de siete poblaciones y tres subespecies: cinco poblaciones de la ssp. *rustica* en Europa (República Checa, Italia, Noruega, España y Ucrania), una población de la ssp. *transitiva* de Israel, y una población de la ssp. *erythrogaster* de Canadá. Todos los rasgos de los espermatozoides (longitud de la cabeza, longitud de la parte central, longitud de la cola y longitud total) variaron significativamente entre poblaciones. La variación entre las poblaciones europeas de *rustica* fue mucho más baja que las diferencias entre subespecies, indicando que los rasgos del esperma reflejan distancia filogenética. También evaluamos la relación entre el coeficiente de variación entre machos de la longitud total del esperma y los niveles de paternidad fuera de la pareja entre distintas poblaciones de una misma especie. Estudios recientes han encontrado una fuerte relación negativa entre variación del tamaño de los espermatozoides y paternidad fuera de la pareja entre especies. Aquí mostramos una relación negativa similar entre seis poblaciones de golondrinas, lo que sugiere que la varianza en la longitud del espermatozoide en los machos en una población está modelada por la fuerza de la selección sexual postcopulatoria.

**Variation in sperm morphometry and sperm competition among barn swallow (*Hirundo rustica*) populations**

Terje Laskemoen, Tomas Albrecht, Andrea Bonisoli-Alquati, Jaroslav Cepak, Florentino de Lope, Ignacio G. Hermosell, Lars Erik Johannessen, Oddmund Kleven, Alfonso Marzal, Timothy A. Mousseau, Anders P. Møller, Raleigh J. Robertson & Geir Rudolfsen, Nicola Saino, Yoni Vortman, Jan T. Lifjeld

Spermatozoa vary greatly in size and shape among species across the animal kingdom. Postcopulatory sexual selection is thought to be the major evolutionary force driving this diversity. In contrast, less is known about how sperm size varies among populations of the same species. Here, we investigate geographic variation in sperm size in barn swallows *Hirundo rustica*, a socially monogamous passerine with a wide Holarctic breeding distribution. We included samples from seven populations and three subspecies: five populations of ssp. *rustica* in Europe (Czech, Italy, Norway, Spain, and Ukraine), one population of ssp. *transitiva* in Israel, and one population of ssp. *erythrogaster* in Canada. All sperm traits (head length, midpiece length, tail length, and total length) varied significantly among populations. The variation among the European *rustica* populations was much lower than the differences among subspecies, indicating that sperm traits reflect phylogenetic distance. We also performed a test of the relationship between the coefficient of between-male variation in total sperm length and extrapair paternity levels across different populations within a species. Recent studies have found a strong negative relationship between sperm size variation and extrapair paternity among species. Here, we show a similar negative relationship among six barn swallow populations, which suggests that the variance in male sperm length in a population is shaped by the strength of stabilizing postcopulatory sexual selection.

## Introduction

Spermatozoa show enormous variation in size and shape across animal taxa (e.g., Cohen 1977). The evolutionary forces shaping this variation have received increasing attention but are not fully understood (Birkhead et al. 2009). Polyandry, i.e., females mating with more than one male, is common across the animal kingdom, and this constitutes a potentially powerful source of postcopulatory sexual selection on sperm traits, either through sperm competition (Parker 1970) or cryptic female choice (Eberhard 1996).

It is well documented that spermatozoa vary considerably in size both within (e.g., Ward 1998; Morrow and Gage 2001b; Laskemoen et al. 2007) and among species (e.g., Gage 1998; Calhim et al. 2007; Kleven et al. 2008; Lüpold et al. 2009). Although geographical variation in sperm size within species has been studied in a range of species, the patterns are complex and not clear. For example, in guppies (*Poecilia reticulata*), Elgee et al. (2010) found that males from populations with high risk of predation had faster swimming sperm and sperm with longer midpieces than males from populations with lower risk of predation. However, total sperm length did not differ between guppy populations (Elgee et al. 2010). Studies of several drosophilid flies have found significant variation in sperm size among populations (Snook 2001; Pitnick et al. 2003; Joly et al. 2004). Yellow dung flies (*Scatophaga stercoraria*) have been shown to have longer sperm at high temperature when raised experimentally in laboratory conditions (Blanckenhorn and Hellriegel 2002). However, another study of the same species did not find differences in sperm length in three natural populations (Hosken et al. 2003). A comparative study of sperm morphology of rhacophorid frogs included samples of the species (*Chirixalus eiffingeri*) from both Japan and Taiwan and documented significant differences in sperm size between these two populations (Kuramoto 1996). A study of quacking frogs (*Crinia georgiana*) documented significant variation in sperm size and relative number of sperm among four populations (Hettyey and Roberts 2006). The sea urchin (*Strongylocentrotus droebachiensis*) was found to have high diversity in sperm traits, except total sperm length, among three different populations (Manier and Palumbi 2008). Minoretti

and Baur (2006) documented significant differences in sperm size among four populations of the land snail (*Arianta arbustorum*). In birds, two recent studies have investigated geographic variation in sperm size, both documenting significant differences among populations (Lüpold et al. 2011; Schmoll and Kleven 2011). In red-winged blackbirds (*Agelaius phoeniceus*), sperm morphology varied significantly among the 17 study sites in continental United States (Lüpold et al. 2011). Lüpold et al. (2011) also found a gradual increase in sperm size from southwest to northeast of the breeding range, and further, a negative relationship between sperm length and body size. Schmoll and Kleven (2011) investigated variation in sperm size within and between two populations of coal tits (*Parus ater*) and documented significant variation in total sperm length and sperm head length between the two populations. These two species are characterized by little or moderate gene flow, which could help explain geographical differences in sperm traits with high heritability. A call is thus made for investigation of variation in sperm size among populations in species with a higher degree of gene flow among populations.

Recently, comparative studies of passerine birds have shown that the intraspecific variation in total sperm length and other sperm components are negatively associated with the risk of sperm competition (Calhim et al. 2007; Immler et al. 2008; Kleven et al. 2008; Lifjeld et al. 2010). This can be interpreted as evidence of stronger stabilizing selection on males producing an optimal sperm type when the risk of sperm competition increases. However, this relationship has not been investigated among populations of the same species. It is well known that levels of extrapair paternity vary among populations (Petrie and Kempenaers 1998), and in this study, we ask if this variation amongst populations in sperm competition could also be reflected in between-male variation in sperm size.

The barn swallow (*Hirundo rustica*) is a socially monogamous passerine with a broad Holarctic breeding distribution (Møller 1994b; Turner 2006). The barn swallow has been thoroughly studied over the last decades and is considered a model species for studies on

sexual selection, mate choice, and sperm competition (e.g., Møller 1988; Møller 1994a; Møller 1994b; Saino et al. 1997; Safran et al. 2005; Kleven et al. 2006; Lifjeld et al. 2011). Currently, six different subspecies of barn swallows are recognized (Turner 2006; Dor et al. 2010). However, some of these subspecies are geographically isolated, preventing gene flow (Turner 2006). A recent phylogeny of barn swallows and other *Hirundo* species found that the European *H. rustica rustica* and East-Mediterranean barn swallows *H. rustica transitiva* cluster together, as do the Asian and American barn swallows (Dor et al. 2010). Dor et al. (2010) also reported pairwise molecular distances within and between these clades, showing low distance within the European-Mediterranean clade and substantially greater distance between the Asian-American and the European-Mediterranean clades. Furthermore, there is evidence from barcoding of the mitochondrial cytochrome c oxidase I (CO1) gene showing that the North American barn swallow *H. rustica erythrogaster* differs almost 2 % in genetic distance from the European barn swallow (Johnsen et al. 2010).

Here, we investigate geographic variation in sperm morphometry in barn swallows. We examined sperm morphometry in seven different barn swallow populations representing three subspecies: five populations of European (Czech, Italy, Norway, Spain and Ukraine), one population of East-Mediterranean (Israel), and one North American population (Canada). Based on the prior knowledge of genetic variation (Dor et al. 2010; Johnsen et al. 2010), we predicted that European and Mediterranean barn swallows would exhibit more similar sperm morphometry when compared to North American barn swallows. We also tested the hypothesis that sperm size variation is negatively related to the risk of sperm competition across populations.

## **Material and methods**

### *Field procedures*

We captured and sampled male barn swallows from seven different populations; Czech Republic (2009), Italy (2011), Norway (2008 and 2009), Spain (2010 and 2011), Ukraine

(2010), Israel (2010 and 2011), and Canada (2006). See supplementary Table S1 for detailed sampling location information. Notably, European barn swallows migrate through the breeding areas of East-Mediterranean barn swallows in Israel, which could potentially cause sampling errors in this area. However, all birds sampled in Israel were earlier banded and belonged to a monitored population of East-Mediterranean barn swallows. To prevent pseudo-replication of already sampled birds, all unbanded birds were banded with unique aluminium bands. We obtained ejaculate samples either by gently massaging the males' cloacal protuberance following a modified method from Wolfson (1952) or from fecal samples following Immler and Birkhead (2005). The ejaculate or fecal sample was immediately fixed in a 5 % formalin solution and stored until slide preparation. After sampling, all birds were released in the immediate vicinity of their respective colonies.

### *Sperm morphometry*

For each sperm sample, a small aliquot of approximately 15  $\mu$ l was applied on a microscope slide, allowed to air-dry, and subsequently gently rinsed with distilled water and airdried again. We measured the head, midpiece, and tail ( $\pm 0.1 \mu$ m) of ten intact spermatozoa per male. Measuring ten sperms per male has been shown to give representative estimations of an individual's mean sperm length (Laskemoen et al. 2007). Total length is the sum of all three separate sperm traits. For one individual, we measured the same ten sperms twice to establish the repeatability of our measurements, following Lessells and Boag (1987). The measurements were highly repeatable (head:  $F_{9,10}=6.83$ ,  $P=0.003$ ,  $r=0.74$ ; midpiece:  $F_{9,10}=114.0$ ,  $P<0.001$ ,  $r=0.98$ ; tail:  $F_{9,10}=105.8$ ,  $P<0.001$ ,  $r=0.98$ ). For the samples of North American barn swallows, we used a Zeiss AxioCam HRc camera mounted on a Zeiss AxioPlan 2 light microscope to obtain digital images of spermatozoa at a magnification of 200x. Further, the morphometric measurements were conducted using the software Zeiss AxioVision 4.1 (© Carl Zeiss Vision GmbH, Germany). For all other samples, we used a Leica DFC420 camera mounted on a Leica DM6000 B digital light microscope to obtain digital

images at magnifications of 200x. The morphometric measurements were conducted using Leica Application Suite (version 2.6.0 R1). The use of two different microscope setups could potentially cause artificial differences. In order to establish whether the different microscope systems gave different results, we measured ten randomly chosen individuals of the North American barn swallows on the Leica microscope system as well. No significant differences were observed (average head length, 14.3 vs. 14.3  $\mu\text{m}$ ; average midpiece length, 59.8 vs. 59.9  $\mu\text{m}$ ; average tail length, 14.4 vs. 14.2  $\mu\text{m}$ , Zeiss vs. Leica systems, respectively; paired t tests: all  $t < 0.57$ , all  $P > 0.58$ ). To avoid observer effects, one person (TL) conducted all morphometric measurements. In an earlier study of sperm characteristics of Ukrainian barn swallows, Møller et al. (2008) found differences in both sperm size and proportion of abnormal sperm between birds sampled near the Chernobyl area and those sampled at control areas southwest of Chernobyl. Therefore, we tested for differences in sperm traits between the four Ukrainian localities in the present dataset. We found no significant differences in any of the measured sperm traits (ANOVA, all  $F_{3,21} < 1.46$ , all  $P > 0.25$ ). Hence, we pooled all individuals from Ukraine as one population. Notably, the Ukrainian samples included in the present study are not the same as in Møller et al. (2008).

#### Extrapair paternity data

We extracted data on extrapair paternity from five populations from the literature (Canada, Italy, Israel, Spain, and Ukraine). In addition, we obtained unpublished data on extrapair paternity from the Czech population (T. Albrecht, J. Kreisinger, and R. Michalkova unpublished data). Notably, all extrapair paternity data originate from the same populations from which we obtained sperm samples. A summary of paternity data and sample sizes are presented in Table 1.

#### Statistical analyses

We included all sperm measurements, i.e., ten sperms per male, and used General Linear

Mixed Models (GLMM) with population as fixed factor and individual as random factor in our tests for differences in sperm traits among populations. For the traits that showed significant variation among populations, we estimated marginal means and conducted all possible pairwise comparisons, Bonferroni-adjusted for multiple comparisons. We calculated the coefficient of variation in sperm length between males ( $CV_{bm}$ ) for all populations using the formula:  $CV_{bm} = SD/mean \times 100$ . Furthermore, as the coefficient of variation for small sample sizes tends to be underestimated, we applied the formula recommended by Sokal and Rohlf (1995):  $CV_{adj} = CV \times (1 + (1/4n))$ . Hence, all  $CV_{bm}$  values reported here are adjusted for sample size. Statistical tests were conducted using Statistica v7.1 (StatSoft Inc), SPSSv19.0.0 (SPSS Inc.), and figures were drawn using Origin v7.0300 (OriginLab Corporation).

## Results

### *Sperm morphometry*

Sperm head length, midpiece length, and total length differed significantly among populations (GLMMs: headlength:  $F_{6,183} = 17.4$ ,  $P < 0.001$ ; midpiece length:  $F_{6,183} = 15.9$ ,  $P < 0.001$ ; total length:  $F_{6,183} = 10.3$ ,  $P < 0.001$ ). Thus, for these traits, we estimated marginal means and pairwise comparisons to reveal which populations differed (Table S2–S4). Descriptive statistics of sperm morphometry from all seven populations are presented in Table 2. Sperm tail length did not differ significantly among populations ( $F_{6,183} = 2.0$ ,  $P = 0.07$ ). Generally, the North American barn swallows differed most from the other populations in sperm morphometry, with longer sperm heads than individuals from all the other populations and shorter midpieces than all but the Italian and East-Mediterranean populations (Fig. 1a,b; Table S2, S3). Considering total sperm length, North American barn swallows generally had shorter sperm than the European populations but did not differ from the population of East-Mediterranean barn swallows (Fig. 1c; Table S4).

Two of our populations (Norway and Spain) included samples from two different seasons, and thus we tested for potential year-effects on sperm size. However, we found no

Multi-focus approach to life histories in passerines

significant effect of sample year on any of the sperm traits measured (Norway: all  $F_{1,24} < 0.69$ ,  $P > 0.42$ ; Spain: all  $F_{1,11} < 2.48$ , all  $P > 0.14$ ).

#### Among-male variation in sperm size and extrapair paternity levels

The populations showed somewhat different values of among male variation in sperm size ( $CV_{bm}$ ) (Table 2). When examining the relationship between  $CV_{bm}$  and percentage broods containing one or more extrapair young (EPB), we found a significant negative relationship (Pearson's:  $r = -0.96$ ,  $N = 6$ ,  $P = 0.003$ ) (Fig. 2a). The relationship between  $CV_{bm}$  and percentage extrapair young (EPY) also went in the predicted negative direction, although this was not statistically significant (Pearson's:  $r = -0.62$ ,  $N = 6$ ,  $P = 0.19$ ) (Fig. 2b). Hence, both tests were in the predicted direction with decreasing  $CV_{bm}$  being associated with increasing level of extrapair paternity (Lifjeld et al. 2010).

#### Discussion

Our analysis revealed significant differences in sperm morphometry among barn swallow populations and subspecies. North American barn swallows from Canada had significantly shorter sperm than European barn swallows from Czech, Norway, Spain, and Ukraine but did not differ significantly from European barn swallows from Italy and East-Mediterranean barn swallows from Israel. When examining the three different sperm traits included in our analyses (head length, midpiece length, and tail length), North American barn swallows were characterized by longer sperm heads than all other populations and shorter midpieces than all other populations except the East-Mediterranean one. Further, we found that between male variation in sperm length ( $CV_{bm}$ ) was negatively related to levels of extrapair paternity, both expressed as percentage broods containing extrapair young (EPB) and percentage extrapair young (EPY).

Based on the phylogenetic relationships between the three subspecies of barn swallows included in our analyses (Dor et al. 2010), we predicted that European and East-

Mediterranean barn swallows should be more similar to each other in sperm morphometry compared to North American barn swallows. Although our findings were not clear, some of the sperm traits coincided with this prediction, e.g., spermatozoa of North American barn swallows had longer heads and shorter midpieces than all other populations. Total sperm length showed a different pattern, with European barn swallows having significantly longer sperm than both East-Mediterranean and North American barn swallows, whereas the two latter did not differ significantly for this trait. Interestingly, sperm head size seems to be a trait that often differs among populations. It was the most variable sperm trait in the coal tit study (Schmoll and Kleven 2011). Also, in a recent comparison of wild and domesticated zebra finches (*Taeniopygia guttata*), sperm head length showed the greatest difference between the two groups (Immler et al. 2012). We do not know if genetic drift or selection is causing the differentiation in sperm morphometry among populations. Both factors can however be important for differentiation in sperm morphometry and are not mutually exclusive. Whereas the studies of coal tits and red-winged blackbirds documented significant differentiation in sperm size between relatively close populations (Lüpold et al. 2011; Schmoll and Kleven 2011), this study documented little differentiation in sperm size across the five European barn swallow populations. Another study on pied flycatchers (*Ficedula hypoleuca*) found a similar pattern as the present study, with no difference in sperm size between one German and two Norwegian populations (Lifjeld et al. 2012). The discrepancy between the coal tit and red-winged blackbird on one hand and the pied flycatcher and barn swallow studies on the other, might be explained by differences in gene flow among populations. Both coal tits and red-winged blackbirds are resident or short distance migratory birds, whereas pied flycatchers and barn swallows are long distance migrants with clear differences in dispersal distance (Paradis et al. 1999). Notably, the East-Mediterranean barn swallow is sedentary but still is subject to high levels of gene flow supposedly from migrating European barn swallows (Dor et al. 2012). Thus, gene flow could be more prominent in the two latter species.

Notably, it has been shown in Gouldian finches (*Erythrura gouldiae*) that males can

exhibit plasticity in sperm morphometry within the same breeding season when the social environment is altered (Immler et al. 2010). Males were found to increase the relative size of the sperm midpiece when placed in intermediate to high competitive environments and increase the size of the sperm tail when facing low to intermediate competitive environments (Immler et al. 2010). This could imply that the relative size of the sperm midpiece and tail could change across the breeding season in barn swallows as well. Similarly, a recent study of sperm size variation in house wrens (*Troglodytes aedon*) found that total sperm length was highly consistent across the breeding season, whereas the flagellum:head length ratio increased during the season, indicating phenotypic plasticity or adjustment in the relative size of sperm components (Cramer et al. 2012). However, repeated samples across the breeding season of North American and Czech barn swallows have shown very high repeatability of all sperm traits (own unpublished data).

Sperm head, midpiece, and flagellum length have been shown to be heritable in the zebra finch (Birkhead et al. 2005) and in several other taxa (e.g. Ward 2000; Morrow and Gage 2001a; Baer et al. 2006). Thus, plasticity in sperm morphometry within the same breeding season might be rather marginal and not biasing the population differences reported here.

To the best of our knowledge, this is the first study examining the relationship between sperm size variation and extrapair paternity levels across populations within the same species. A few studies have previously demonstrated that this relationship is significantly negative in passerine birds (Calhim et al. 2007; Kleven et al. 2008; Lifjeld et al. 2010). Recently, a similar negative relationship between sperm size variation and polyandry has been demonstrated in social insects (Fitzpatrick and Baer 2011). As extrapair paternity levels are known to vary among populations of the same species (e.g., Griffith et al. 2002), with barn swallows being one of the species showing considerable variation (Saino et al. 1999; Møller et al. 2003; Kleven et al. 2005; Vortman et al. 2011), we tested associations between  $CV_{bm}$  and

levels of extrapair paternity data across populations of this species. Both EPB and EPY were negatively related to  $CV_{bm}$ , as predicted from the aforementioned interspecific studies. The relationship between  $CV_{bm}$  and EPY was, however, not statistically significant. Although our sample size in the present dataset is small, we argue that this indicates that  $CV_{bm}$  and risk of sperm competition are negatively related also within species, further supporting the notion that risk of sperm competition induces stabilizing selection on sperm size.

The present study shows little variation in sperm size/ morphometry among European and East-Mediterranean barn swallows, and somewhat larger variation between European-East-Mediterranean and North American barn swallows. These results make sense in the light of the genetic difference and the large geographic distance between populations. The subspecies *rustica* and *transitiva* are genetically closely related (Dor et al. 2010), and they do not differ much in sperm morphometry. The subspecies *erythrogaster* is genetically quite distant from both *rustica* and *transitiva* (Dor et al. 2010; Johnsen et al. 2010), and this is also reflected in the observed sperm morphometry. In conclusion, sperm morphometry might give an indication of genetic distance, not only between species, but also between phylogenetic lines within species. Further, we found negative relationships between  $CV_{bm}$  and two measures of extrapair paternity, EPB and EPY, indicating that the same pattern as have been found among species (Calhim et al. 2007; Kleven et al. 2008; Lifjeld et al. 2010; Fitzpatrick and Baer 2011), also is present intraspecifically, and which strengthens the explanation that sperm competition exerts a stabilizing selection pressure on sperm size variation.

#### Acknowledgments

We are grateful to all people that assisted with field work, especially Frode Fossøy in Canada, Bjørn Aksel Bjerke in Norway, and Luz Garcia-Longoria in Spain. A special thanks to Gustav Thorsø Mohr for allowing us to trap inside the barn at Thorsø Herregård. We thank two anonymous reviewers for helpful comments on an earlier draft of the manuscript. This study was supported by funding from the Czech Science Foundation (to TA, project no.

P506/12/2472), a Fondazione Cariplo grant (to NS, grant no. 2009–3496), the Ministry of Culture of the Czech Republic (to JC, grant no. DKRVO 00023272), the Natural Sciences and Engineering Research Council of Canada (to RJR), the Norwegian Research Council (to JTL, OK, LEJ and TL), the Samuel Freeman Charitable Trust (to TAM), and the Spanish Ministry of Economy and Competitiveness (to IGH, FdL and AM, grant no. CGL 2012–36665).

### **Ethical standards**

All authors declare that the present study complies with the current laws and ethical standards of animal research in Canada, Czech Republic, Israel, Italy, Norway, Spain, and Ukraine.

### **References**

- Baer B, de Jong G, Schmid-Hempel R, Schmid-Hempel P, Høeg JT, Boomsma JJ (2006) Heritability of sperm length in the bumblebee *Bombus terrestris*. *Genetica* 127:11–23
- Birkhead TR, Pellatt EJ, Brekke P, Yeates R, Castillo-Juarez H (2005) Genetic effects on sperm design in the zebra finch. *Nature* 434:383–387
- Birkhead TR, Hosken DJ, Pitnick S (2009) *Sperm biology: an evolutionary perspective*. Academic Press, Oxford.
- Blanckenhorn WU, Hellriegel B (2002) Against Bergmann's rule: fly sperm size increases with temperature. *Ecol Lett* 5:7–10
- Calhim S, Immler S, Birkhead TR (2007) Postcopulatory sexual selection is associated with reduced variation in sperm morphology. *PLoS One* 2:e413
- Cohen J (1977) *Reproduction*. Butterworths, London
- Cramer ERA, Laskemoen T, Kleven O, Lifjeld JT (2012) Sperm length variation in House Wrens *Troglodytes aedon*. *J Ornithol*. DOI: 10.1007/s10336–012-0878-3
- Dor R, Safran RJ, Sheldon FH, Winkler DW, Lovette IJ (2010) Phylogeny of the genus *Hirundo* and the barn swallow subspecies complex. *Mol Phyl Evol* 56:409–418
- Dor R, Safran RJ, Vortman Y, Lotem A, McGowan A, Evans MR, Lovette IJ (2012) Population genetics and morphological comparisons of migratory European (*Hirundo rustica*

Multi-focus approach to life histories in passerines

*rustica*) and sedentary East-Mediterranean (*Hirundo rustica transitiva*) barn swallows.

J Hered 103:55–63

Eberhard WG (1996) Female control: sexual selection by cryptic female choice. Princeton University Press, Princeton, NJ

Elgee KE, Evans JP, Ramnarine IW, Rush SA, Pitcher TE (2010) Geographic variation in sperm traits reflects predation risk and natural rates of multiple paternity in the guppy. J Evol Biol 23:1331–1338

Ellegren H, Lindgren G, Primmer CR, Møller AP (1997) Fitness loss and germline mutations in barn swallows breeding in Chernobyl. Nature 389:593–596

Fitzpatrick JL, Baer B (2011) Polyandry reduces sperm length variation in social insects. Evolution 65:3006–3012

Gage MJG (1998) Mammalian sperm morphometry. Proc R Soc Lond B 265:97–103

Griffith SC, Owens IPF, Thuman KA (2002) Extra pair paternity in birds: a review of interspecific variation and adaptive function. Mol Ecol 11:2195–2212

Hettyey A, Roberts JD (2006) Sperm traits of the quacking frog, *Crinia georgiana*: intra- and interpopulation variation in a species with a high risk of sperm competition. Behav Ecol Sociobiol 59:389–396

Hosken DJ, Garner TWJ, Blanckenhorn WU (2003) Asymmetry, testis and sperm size in yellow dung flies. Funct Ecol 17:231–236

Immler S, Birkhead TR (2005) A non-invasive method for obtaining spermatozoa from birds. Ibis 147:827–830

Immler S, Calhim S, Birkhead TR (2008) Increased postcopulatory sexual selection reduces the intramale variation in sperm design. Evolution 62:1538–1543

Immler S, Pryke SR, Birkhead TR, Griffith SC (2010) Pronounced within-individual plasticity in sperm morphometry across social environments. Evolution 64:1634–1643

Immler S, Griffith SC, Zann R, Birkhead TR (2012) Intra-specific variance in sperm morphometry: a comparison between wild and domesticated Zebra Finches

*Taeniopygia guttata*. Ibis 154:480–487

Johnsen A, Rindal E, Ericson PGP, Zuccon D, Kerr KCR, Stoeckle MY, Lifjeld JT (2010) DNA barcoding of Scandinavian birds reveals divergent lineages in trans-Atlantic species. J Ornithol 151:565–578

Joly D, Korol A, Nevo E (2004) Sperm size evolution in *Drosophila*: inter- and intraspecific analysis. Genetica 120:233–244

Kleven O, Jacobsen F, Robertson RJ, Lifjeld JT (2005) Extrapair mating between relatives in the barn swallow: a role for kin selection? Biol Lett 1:389–392

Kleven O, Jacobsen F, Izadnegahdar R, Robertson RJ, Lifjeld JT (2006) Male tail streamer length predicts fertilization success in the North American barn swallow (*Hirundo rustica erythrogaster*). Behav Ecol Sociobiol 59:412–418

Kleven O, Laskemoen T, Fossøy F, Robertson RJ, Lifjeld JT (2008) Intraspecific variation in sperm length is negatively related to sperm competition in passerine birds. Evolution 62:494–499

Kuramoto M (1996) Generic differentiation of sperm morphology in treefrogs from Japan and Taiwan. J Herpetol 30:437–443

Laskemoen T, Kleven O, Fossøy F, Lifjeld JT (2007) Intraspecific variation in sperm length in two passerine species, the bluethroat *Luscinia svecica* and the willow warbler *Phylloscopus trochilus*. Ornis Fenn 84:131–139

Lessells CM, Boag PT (1987) Unrepeatable repeatabilities: a common mistake. Auk 104:116–121

Lifjeld JT, Laskemoen T, Kleven O, Albrecht T, Robertson RJ (2010) Sperm length variation as a predictor of extrapair paternity in passerine birds. PLoS One 5:e13456

Lifjeld JT, Kleven O, Jacobsen F, McGraw KJ, Safran RJ, Robertson RJ (2011) Age before beauty? Relationships between fertilization success and age-dependent ornaments in barn swallows. Behav Ecol Sociobiol 65:1687–1697

Lifjeld JT, Laskemoen T, Kleven O, Pedersen ATM, Lampe HM, Rudolfsen G, Schmoll T,

- Slagsvold T (2012) No evidence for precopulatory sexual selection in a passerine bird. PLoS One 7:e32611
- Lüpold S, Linz GM, Birkhead TR (2009) Sperm design and variation in the New World blackbirds (Icteridae). Behav Ecol Sociobiol 63:899–909
- Lüpold S, Westneat DF, Birkhead TR (2011) Geographical variation in sperm morphology in the red-winged blackbird (*Agelaius phoeniceus*). Evol Ecol 25:373–390
- Manier MK, Palumbi SR (2008) Intraspecific divergence in sperm morphology of the green sea urchin, *Strongylocentrotus droebachiensis*: implications for selection in broadcast spawners. BMC Evol Biol 8:283
- Minoretti N, Baur B (2006) Among- and within-population variation in sperm quality in the simultaneously hermaphroditic land snail *Arianta arbustorum*. Behav Ecol Sociobiol 60:270–280
- Møller AP (1988) Female choice selects for male sexual tail ornaments in the monogamous swallow. Nature 332:640–642
- Møller AP (1994a) Repeatability of female choice in a monogamous swallow. Anim Behav 47:643–648
- Møller AP (1994b) Sexual selection and the barn swallow. Oxford University Press, Oxford
- Møller AP, Brohede J, Cuervo JJ, de Lope F, Primmer C (2003) Extrapair paternity in relation to sexual ornamentation, arrival date, and condition in a migratory bird. Behav Ecol 14:707–712
- Møller AP, Mousseau TA, Lynn C, Ostermiller S, Rudolfson G (2008) Impaired swimming behaviour and morphology of sperm from barn swallows *Hirundo rustica* in Chernobyl. Mutat Res Genet Tox Environ Mutagen 650:210–216
- Morrow EH, Gage MJG (2001a) Artificial selection and heritability of sperm length in *Gryllus bimaculatus*. Heredity 87:356–362
- Morrow EH, Gage MJG (2001b) Consistent significant variation between individual males in spermatozoal morphometry. J Zool Lond 254:147–153

- Paradis E, Baillie SR, Sutherland WJ, Gregory RD (1999) Dispersal and spatial scale affect synchrony in spatial population dynamics. *Ecol Lett* 2:114–120
- Parker GA (1970) Sperm competition and its evolutionary consequences in the insects. *Biol Rev* 45:525–567
- Petrie M, Kempenaers B (1998) Extra-pair paternity in birds: explaining variation between species and populations. *Trends Ecol Evol* 13:52–58
- Pitnick S, Miller GT, Schneider B, Markow TA (2003) Ejaculate-female coevolution in *Drosophila mojavensis*. *Proc R Soc Lond B* 270:1507–1512
- Safran RJ, Neumann CR, McGraw KJ, Lovette IJ (2005) Dynamic paternity allocation as a function of male plumage color in barn swallows. *Science* 309:2210–2212
- Saino N, Primmer CR, Ellegren H, Møller AP (1997) An experimental study of paternity and tail ornamentation in the barn swallow (*Hirundo rustica*). *Evolution* 51:562–570
- Saino N, Primmer CR, Ellegren H, Møller AP (1999) Breeding synchrony and paternity in the barn swallow (*Hirundo rustica*). *Behav Ecol Sociobiol* 45:211–218
- Schmoll T, Kleven O (2011) Sperm dimensions differ between two coal tit *Parus ater* populations. *J Ornithol* 152:515–520
- Snook RR (2001) Absence of latitudinal clines in sperm characters in North American populations of *Drosophila subobscura* (Diptera: Drosophilidae). *Pan-Pac Entomol* 77:261–271
- Sokal RR, Rohlf FJ (1995) *Biometry: the principles and practice of statistics in biological research*. Freeman, New York
- Turner AK (2006) *The barn swallow*. T. & A. D, Poyser, London
- Vortman Y, Lotem A, Dor R, Lovette IJ, Safran RJ (2011) The sexual signals of the East-Mediterranean barn swallow: a different swallow tale. *Behav Ecol* 22:1344–1352
- Ward PI (1998) Intraspecific variation in sperm size characters. *Heredity* 80:655–659
- Ward PI (2000) Sperm length is heritable and sex-linked in the yellow dung fly (*Scathophaga stercoraria*). *J Zool Lond* 251:349–353

Multi-focus approach to life histories in passerines

Wolfson A (1952) The cloacal protuberance: a means for determining breeding condition in live male passerines. *Bird Band* 23:159–165

TABLES

**Table 1.** Summary of extrapair paternity data from six populations of barn swallows *Hirundo rustica* used in the present study

Country	EPY (95 % CI) <sup>a</sup>	EPB <sup>b</sup>	N young/N broods	Source
Czech	23.1 (15.2–31.1)	60.0	108/25	(T. Albrecht, J. Kreisinger)
Italy	29.0 (22.9–35.1)	52.0	214/52	(Saino et al. 1999)
Spain	17.8 (14.9–20.7)	32.4	674/170	(Møller et al. 2003)
Ukraine	30.0 (20.0–40.0)	50.0	80/18	(Ellegren et al. 1997)
Israel	15.3 (9.7–20.9)	43.9	161/41	(Vortman et al. 2011)
Canada	28.8 (25.9–31.7)	48.1	917/210	(Kleven et al. 2005)

a Percentage extrapair young with the 95 % lower and upper confidence limits of the estimate indicated

b Percentage broods containing one or more extrapair young

**Table 2.** Descriptive statistics of sperm morphometry in seven populations of barn swallows *Hirundo rustica* (see supplementary Table S2–S4 for pairwise comparisons between all populations)

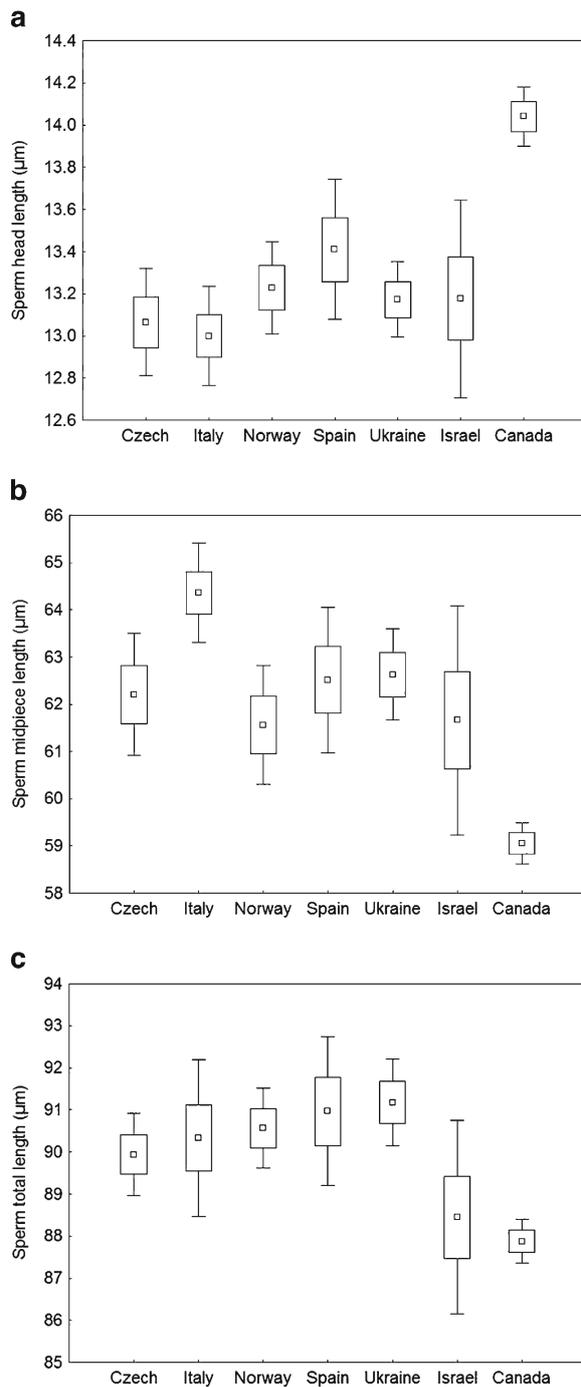
	Head length (µm)	Midpiece length (µm)	Tail length (µm)	Total length (µm)	CV <sub>dm</sub> <sup>a</sup>	CV <sub>wm</sub> <sup>b</sup>				
	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range				
Czech (n=20)	13.1±0.5	11.7–13.9	62.2±2.8	57.3–66.9	14.7±2.2	9.7–18.7	89.9±2.1	86.4–94.2	2.34	1.52
Italy (n=8)	13.0±0.3	12.6–13.4	64.4±1.3	62.9–66.4	13.0±2.6	10.2–18.7	90.3±2.2	88.0–94.8	2.54	1.65
Norway (n=26)	13.2±0.5	12.1–14.2	61.6±3.1	54.2–65.9	15.8±3.4	11.6–25.6	90.6±2.4	86.4–94.6	2.63	1.67
Spain (n=13)	13.4±0.5	12.8–14.6	62.5±2.5	58.3–66.0	15.0±2.2	11.5–20.3	91.0±2.9	85.3–96.9	3.29	1.66
Ukraine (n=25)	13.2±0.4	12.2–14.2	62.6±2.3	57.7–66.6	15.4±2.9	11.0–24.5	91.2±2.5	86.2–96.8	2.76	1.92
Israel (n=8)	13.2±0.6	12.4–14.0	61.7±2.9	57.5–66.0	13.6±1.2	11.5–15.5	88.5±2.8	84.3–92.2	3.13	2.24
Canada (n=90)	14.0±0.7	12.5–15.5	59.0±2.1	52.3–63.3	14.8±2.1	10.2–20.1	87.9±2.5	81.4–93.0	2.82	1.74

a Coefficient of variation of total sperm length calculated as SD/mean\*100 and adjusted for sample size following the formula (CV\*(1+(1/4n))) (Sokal and Rohlf 1995)

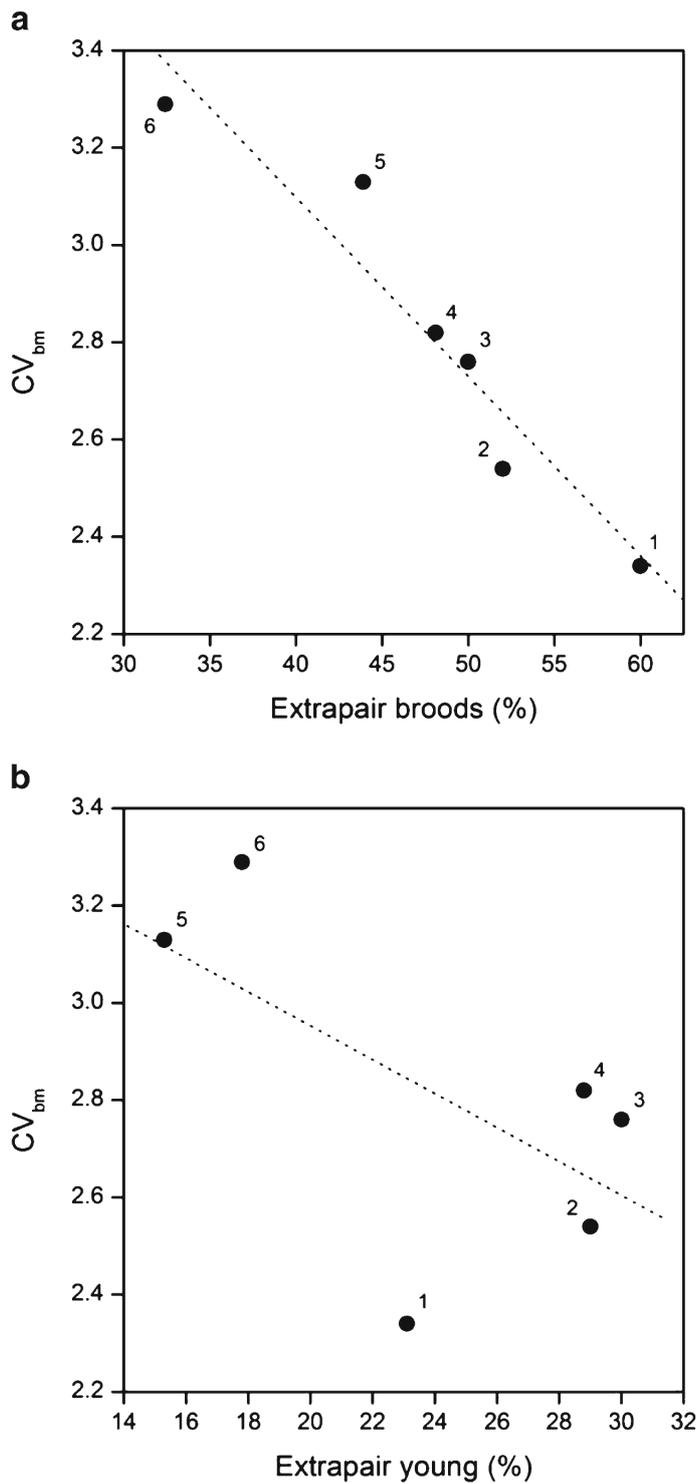
b Average coefficient of variation of total sperm length within males, based on ten sperms per male

FIGURES AND LEGENDS

**Figure 1.** Sperm a head, b midpiece, and c total length, in seven populations of barn swallows *Hirundo rustica*. Average values are indicated as small squares, boxes indicate  $\pm$ SE, and whiskers indicate  $\pm$ 95% confidence intervals. Sample sizes are; Czech n=20 males, Italy n=8 males, Norway n=26 males, Spain n=13 males, Ukraine n=25 males, Israel n=8 males, and Canada n=90 males. See “Results” section for test statistics



**Figure 2.** Relationship between a percentage broods containing extrapair young and coefficient of variation (adjusted for sample size) ( $CV_{bm}$ ) in sperm length between males and b percentage extrapair young and  $CV_{bm}$ , in six populations of barn swallows. Numbers represent the following populations; (1) Czech, (2) Italy, (3) Ukraine, (4) Canada, (5) Israel, and (6) Spain. See “Results” section for test statistics



## Capítulo 5

### **Los patrones de daño espermático en paseriformes de Chernobyl sugieren un trade-off entre la longitud del esperma y su integridad.**

La variación interespecífica en el tamaño del esperma es enigmática, pero generalmente se asume que refleja compromisos propios de cada especie con presiones selectivas. Entre las aves paseriformes, la longitud del esperma varía hasta tamaños 7 veces superiores, y el riesgo de competencia espermática parece impulsar la evolución de espermatozoides más largos. Sin embargo, se sabe poco acerca de los factores que favorecen los espermatozoides cortos o constriñen la evolución de esperma más largo. Aquí presentamos un análisis comparativo de las anomalías presentes en las cabezas de espermatozoides entre 11 especies de paseriformes de Chernobyl, que presumiblemente son el resultado de la irradiación crónica que siguió al accidente de 1986. La frecuencia de anomalías en el esperma varió entre el 15,7 y 77,3% entre especies, y fue más de cuatro veces más alta que en áreas no contaminadas. No obstante, las especies de zonas no contaminadas mostraron rangos de anomalías similares a los de Chernobyl, lo que apunta a algún factor intrínseco subyacente a la variación en el daño espermático entre especies. El análisis por microscopía electrónica de espermatozoides anormales reveló patrones de daño acrosómico consistentes con una reacción acrosómica prematura. La longitud del esperma, pero no el riesgo de competencia espermática explicó la variación en el daño espermático entre especies. Esto sugiere que los espermatozoides más largos son más susceptibles a una reacción prematura del acrosoma. Por tanto, hipotetizamos la existencia de un trade-off entre la longitud del esperma y la integridad del mismo que afecta a la evolución del esperma en paseriformes.

**Patterns of sperm damage in Chernobyl passerine birds suggest a trade-off between sperm length and integrity**

Ignacio G. Hermosell, Terje Laskemoen, Melissah Rowe, Anders P. Møller, Timothy A. Mousseau, Tomáš Albrecht, Jan T. Lifjeld

Interspecific variation in sperm size is enigmatic, but generally assumed to reflect species-specific trade-offs in selection pressures. Among passerine birds, sperm length varies seven-fold, and sperm competition risk seems to drive the evolution of longer sperm. However, little is known about factors favouring short sperm or constraining the evolution of longer sperm. Here we report a comparative analysis of sperm head abnormalities among 11 species of passerine bird in Chernobyl, presumably resulting from chronic irradiation following the 1986 accident. Frequencies of sperm abnormalities varied between 15.7 and 77.3% among species, and more than four-fold higher than in uncontaminated areas. Nonetheless, species ranked similarly in sperm abnormalities in unpolluted areas as in Chernobyl, pointing to some intrinsic factor underlying variation in sperm damage among species. Scanning electron microscopy of abnormal spermatozoa revealed patterns of acrosome damage consistent with premature acrosome reaction. Sperm length, but not sperm competition risk explained variation in sperm damage among species. This suggests that longer spermatozoa are more susceptible to premature acrosome reaction. Therefore, we hypothesize a trade-off between sperm length and sperm integrity affecting sperm evolution in passerine birds.

## Introduction

Spermatozoa display tremendous diversity in size and shape across the animal kingdom (Pitnick et al. 2009). Fertilization mode and post-copulatory sexual selection are two main factors assumed to shape this diversity among taxa (Snook 2005, Pitnick et al. 2009, Immler et al. 2011). Passerine birds have spermatozoa adapted to internal fertilization and sperm storage, with a seven-fold variation in length, i.e.  $\sim 40 - 290 \mu\text{m}$  (Jamieson 2006, Lifjeld et al. 2010, Immler et al. 2011). Post-copulatory sexual selection seems to promote the evolution of longer sperm (Kleven et al. 2009, Lifjeld et al. 2010, Immler et al. 2011) although the adaptive function of longer sperm in sperm competition is not well understood. Longer sperm have longer midpieces, which contain the fused mitochondrion and hence more energy resources (Rowe et al. 2013). However, energy does not seem to translate directly into higher swimming speeds (Kleven et al. 2009, Rowe et al. 2013), so other energy-dependent traits like longevity, could be important. Post-copulatory sexual selection also increases total investment in sperm production (Pitcher et al. 2005, Rowe and Pruett-Jones 2011), which is manifested in a disproportionate increase in sperm number compared to the increase in sperm size across species (Immler et al. 2011). This suggests that there is no clear trade-off between sperm size and number in passerine birds, due to some significant cost or constraint to the evolution of longer sperm.

Here we report a comparative study of sperm abnormalities in passerine birds providing evidence for reduced functional performance of longer sperm. Radioactive contamination following the Chernobyl accident in 1986 had a series of negative environmental effects, including sperm damage in mammals (Pomerantseva et al. 1997) and birds (Møller et al. 2005). We examined the frequency of abnormal sperm among 11 passerine species sampled around Chernobyl in 2010 and 2011. We also scored sperm abnormalities in samples from 10 of these species from uncontaminated areas elsewhere in Europe. We also tested whether the patterns of sperm damage co-varied with species-specific sperm lengths and sperm competition risk, as sperm competition enhances both sperm

quantity and quality among passerine birds (Rowe and Pruett-Jones 2011). Thus, we predicted that species with a high risk of sperm competition should have more robust sperm, in this case lower frequencies of radiation-induced damage. As sperm length is positively correlated with sperm competition risk (Kleven et al. 2009, Lifjeld et al. 2010, Immler et al. 2011), we would also expect species with longer sperm to have less damaged sperm. Finally, we used scanning electron microscopy (SEM) to study the nature of sperm abnormalities.

## **Material and methods**

We obtained sperm samples from 102 individuals belonging to 11 species of passerine birds in Chernobyl, Ukraine, during May-June 2010-2011, and 84 individuals from 10 of the same species in Norway and the Czech Republic (uncontaminated areas) during 2007-2013. A detailed sample list is given in Table S1. Ejaculates were obtained by cloacal massage, diluted in saline, and immediately fixed in 5% formaldehyde (Kleven et al. 2009). From each sample, a ~3 $\mu$ l aliquot was spread on a microscope slide and air-dried, then gently rinsed with distilled water and air-dried again. Digital images of the spermatozoa were captured (160x magnification) using a Leica DFC420 digital camera attached to a LEICA DM6000 B microscope, and analyzed with Leica Application Software. We typically examined 100 spermatozoa per sample, unless fewer sperm available, and scored them as “normal” or “abnormal” (i.e. different head shape or reduced head length). All images were scored by one person (IGH) to reduce observer error. Additional scoring of images from 10 samples (one sample randomly drawn from each species in uncontaminated areas), done blindly with respect to the first scorings, gave a high repeatability of the scored frequencies of abnormal sperm ( $R = 0.98$ ,  $F_{9,10} = 101.6$ ,  $p < 0.001$ ).

For SEM we selected samples from three species with relatively high frequencies of abnormal sperm. Formalin-fixed spermatozoa were prepared and examined as in Lifjeld et al. (2013). Mean sperm lengths for each species were obtained from the avian sperm collection database at the Natural History Museum, Oslo, only using measurements of intact sperm from

the control populations (10 species) or Chernobyl (*Luscinia luscinia*). The coefficient of variation in mean sperm length (CV<sub>bm</sub>) among males from the same populations was used as an index of sperm competition risk (Lifjeld et al. 2010). Species-specific values for sperm length and sperm competition risk are listed in Table S2.

Comparative analyses of the proportion of abnormal sperm were conducted using a generalized least-squares approach controlling for phylogenetic relatedness among species (i.e. PGLS; Supplementary Methods). For all parametric tests, proportions were arcsine square-root transformed, and sperm lengths were log<sub>10</sub>-transformed to approach normality.

## Results

Frequencies of abnormal sperm varied from 15.7 to 73.3% among Chernobyl species (N = 11), with an overall mean frequency of 39.6%. Among uncontaminated populations of the same species (N = 10), frequencies varied from 0.1 to 21.1%, with an overall mean frequency of 8.6%. The frequency of abnormal sperm was always higher in Chernobyl than in uncontaminated populations (paired  $t_9 = 7.68$ ,  $p < 0.001$ ; figure 1a), although for one outlier species, *Coccothraustes coccothraustes*, frequencies were quite similar. Sperm abnormalities tended to be positively correlated between contaminated and uncontaminated areas (Pearson  $r = 0.629$ ,  $p = 0.051$ ,  $n = 10$ ; figure 1a, with the outlier species excluded:  $r = 0.928$ ,  $p < 0.001$ ,  $n = 9$ ), which implies an intrinsic, species-specific factor underlying the consistent variation in sperm abnormalities between environments.

Variation in sperm abnormalities among Chernobyl populations was significantly associated with sperm length, i.e. species with longer sperm had more abnormal sperm (table 1, figure 1b). In contrast, there was no significant association with sperm competition risk (table 1). Among uncontaminated populations, neither sperm length nor sperm competition risk explained significant variation in sperm abnormalities (table 1), but sperm length had a significant effect when the outlier was excluded (PGLS,  $t = 2.77$ ,  $n = 9$ ,  $p = 0.028$ ).

SEM images of spermatozoa (figure 2) revealed that abnormalities were restricted to the acrosome, i.e. the anterior part of the head. The types of damage varied between a bent acrosomal tip (figure 2b), a reduced acrosome and acrosomal microtubule helix (figure 2g), an “emptied” acrosome with remnants of the plasma membrane (figure 2f), and an entirely missing acrosome (figure 2d). These changes reflect the various stages of the acrosome reaction, which normally takes place when the sperm penetrates the inner perivitelline layer of the ovum (Stepinska and Bakst 2006). Hence, we interpret the sperm head abnormalities as evidence of premature acrosome reaction.

## **Discussion**

Our study documented three novel patterns of sperm damage. First, all studied passerine birds in the Chernobyl area have elevated frequencies of sperm abnormality, even 25 years after the radioactive fallout. Background radiation in this environment has had significant negative impact on many birds and other taxa (Møller and Mousseau 2007, 2009). Studies of local barn swallow *Hirundo rustica* populations have revealed a series of physiological and morphological defects (Møller et al. 2007, Bonisoli-Alquati et al. 2010), including increased sperm abnormalities and reduced sperm swimming speed (Møller et al. 2005, 2008). Our results indicate that most passerine birds in the area are significantly affected by radiation-induced sperm damage.

Second, our SEM analyses identified premature acrosome reaction as the likely mechanism of the observed sperm damage. Correct timing of the acrosome reaction is crucial for fertilizing success of an ejaculate, and spermatozoa without an intact acrosome are unable to penetrate the inner perivitelline layer of the ovum (Stepinska and Bakst 2006). Our sperm samples were fresh ejaculates, so the conditions causing the defect must be related to the physiology of the male. Several factors are known to induce premature acrosome reaction in birds and mammals, including mutations (Brown et al. 1989), extracellular calcium and lead (Lemoine et al. 2008, Oliveira et al. 2009), and oxidative stress (Bansal and Bilaspuri 2011).

Interestingly, a link between oxidative stress and poor sperm performance has been indicated in barn swallows from Chernobyl (Møller et al. 2005, Bonisoli-Alquati et al. 2011), so we speculate that premature acrosome reaction is caused by oxidative stress during spermatogenesis.

Finally, species with longer sperm had more damaged sperm. This implies that longer sperm, under otherwise similar extracellular conditions, have a lower structural integrity of the acrosome, and may be more susceptible to lipid peroxidation of the plasma membranes (Bansal and Bilaspuri 2011). Oscine passerine birds are characterized by the acrosome being longer than the nucleus (Jamieson 2006), and longer acrosomes could be structurally less stable. However, it is not yet known whether species with longer sperm also have longer acrosomes. Regardless of the actual mechanism underlying the inferred premature acrosome reaction of longer sperm, the pattern reflects a possible trade-off between sperm length and sperm integrity, and a possible explanation for why there is no run-away selection for longer sperm in passerine birds as compared to other taxa (Immler et al. 2011).

### **Acknowledgments**

We thank various field assistants for help with sperm sampling and Antje Hoenen for help with SEM analyses. The work was supported by grants from the Research Council of Norway, the Czech Science Foundation, the Samuel Freeman Charitable Trust, the University of South Carolina, the Fulbright Program, and NATO.

### **References**

- Bansal AK, Bilaspuri GS (2011) Impacts of oxidative stress and antioxidants on semen functions. *Vet Med Int.* doi: 10.4061/2011/686137
- Bonisoli-Alquati A, Mousseau TA, Møller AP, Caprioli M, Saino N (2010) Increased oxidative stress in barn swallows from the Chernobyl region. *Comp Biochem Physiol A* 155:205-210

- Bonisoli-Alquati A, Møller AP, Rudolfsen G, Saino N, Caprioli M, Ostermiller S, Mousseau TA (2011) The effects of radiation on sperm swimming behavior depend on plasma oxidative status in the barn swallow (*Hirundo rustica*). *Comp Biochem Physiol A* 159:105-112
- Brown J, Cebra-Thomas JA, Bleil JD, Wassarman PM, Silver LM (1989) A premature acrosome reaction is programmed by mouse t haplotypes during sperm differentiation and could play a role in transmission ratio distortion. *Development* 106:769-773
- Immler S, Pitnick S, Parker GA, Durrant KL, Lüpold S, Calhim S, Birkhead TR (2011) Resolving variation in the reproductive tradeoff between sperm size and number. *Proc Natl Acad Sci USA* 108:5325-5330
- Jamieson BGM (2006) Avian spermatozoa: structure and phylogeny. In: Jamieson BGM (ed) *Reproductive Biology and Phylogeny of Birds. Part A*. Science Publishers Inc, Enfield, New Hampshire, USA; pp 249-511
- Kleven O, Fossøy F, Laskemoen T, Robertson RJ, Rudolfsen G, Lifjeld JT (2009) Comparative evidence for the evolution of sperm swimming speed by sperm competition and female sperm storage duration in passerine birds. *Evolution* 63:2466-2473
- Lemoine M, Grasseau I, Brillard JP, Blesbois E (2008) A reappraisal of the factors involved in in vitro initiation of the acrosome reaction in chicken spermatozoa. *Reproduction* 136:391-399
- Lifjeld JT, Laskemoen T, Kleven O, Albrecht T, Robertson RJ (2010) Sperm length variation as a predictor of extrapair paternity in passerine birds. *PLoS ONE* 5:e13456
- Lifjeld JT, Hoenen A, Johannessen LE, Laskemoen T, Lopes RJ, Rodrigues P, Rowe M (2013) The Azores bullfinch (*Pyrrhula murina*) has the same unusual and size-variable sperm morphology as the Eurasian bullfinch (*Pyrrhula pyrrhula*). *Biol J Linn Soc* 108:677-687
- Møller AP, Mousseau TA (2007) Species richness and abundance of forest birds in relation to

- radiation at Chernobyl. *Biol Lett* 3:483-486
- Møller AP, Mousseau TA (2009) Reduced abundance of insects and spiders linked to radiation at Chernobyl 20 years after the accident. *Biol Lett* 5:356-359
- Møller AP, Mousseau TA, de Lope F, Saino N (2007) Elevated frequency of abnormalities in barn swallows from Chernobyl. *Biol Lett* 3:414-417
- Møller AP, Mousseau TA, Lynn C, Ostermiller S, Rudolfson G (2008) Impaired swimming behaviour and morphology of sperm from barn swallows *Hirundo rustica* in Chernobyl. *Mutation Res* 650:210-216
- Møller AP, Surai P, Mousseau TA (2005) Antioxidants, radiation and mutation as revealed by sperm abnormality in barn swallows from Chernobyl. *Proc R Soc B* 272:247-252
- Oliveira H, Spanò M, Santos C, Pereira M (2009) Lead chloride affects sperm motility and acrosome reaction in mice. *Cell Biol Toxicol* 25:341-353
- Pitcher TE, Dunn PO, Whittingham LA (2005) Sperm competition and the evolution of testes size in birds. *J Evol Biol* 18:557-567
- Pitnick S, Hosken DJ, Birkhead TR (2009) Sperm morphological diversity. In Birkhead TR, Hosken DJ, Pitnick S (eds) *Sperm Biology: An Evolutionary Perspective*. Elsevier, Oxford, pp. 69-149
- Pomerantseva MD, Ramaiya LK, Chekhovich AV (1997) Genetic disorders in house mouse germ cells after the Chernobyl catastrophe. *Mutation Res* 381:97-103
- Rowe M, Laskemoen T, Johnsen A, Lifjeld JT (2013) Evolution of sperm structure and energetics in passerine birds. *Proc R Soc B* 280:2012.2616
- Rowe M, Pruett-Jones S (2011) Sperm competition selects for sperm quantity and quality in the Australian Maluridae. *PLoS ONE* 6:e15720
- Snook RR (2005) Sperm in competition: not playing by the numbers. *Trends Ecol Evol* 20:46-53
- Stepinska U, Bakst MR (2006) Fertilization. In Jamieson BGM (ed) *Reproductive Biology and Phylogeny of Birds. Part A*. Science Publishers Inc, Enfield, New Hampshire, USA.

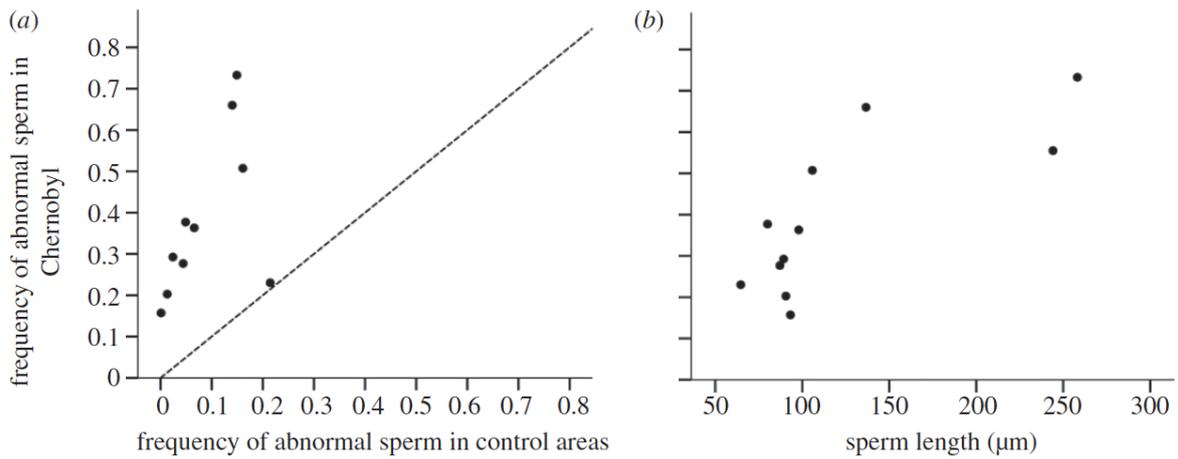
## TABLES

**Table 1.** PGLS analyses of the proportion of abnormal sperm in relation to total sperm length and sperm competition risk (CVbm of sperm length) among passerine species in Chernobyl, Ukraine, and uncontaminated areas (Norway and Czech Republic). In all tests, the phylogenetic parameter ( $\lambda$ ) was  $\sim 0$  and significantly different from 1, suggesting there was no strong phylogenetic signal in the relationships.

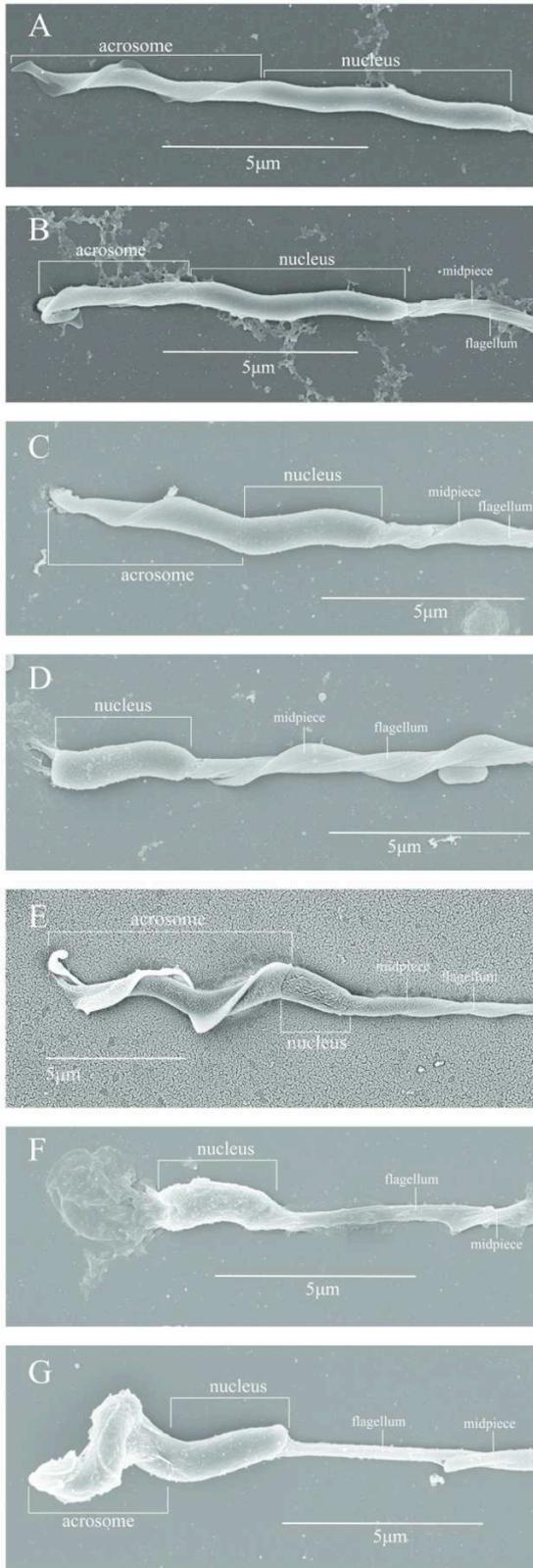
Area	predictor	slope	s.e.	t	p
Chernobyl ( $n = 11$ species)	Total sperm length	1.02	0.24	4.32	0.002
	Sperm competition risk	0.08	0.09	0.89	0.40
Uncontaminated ( $n = 10$ species)	Total sperm length	0.16	0.29	0.55	0.60
	Sperm competition risk	0.04	0.07	0.63	0.54

FIGURES AND LEGENDS

**Figure 1.** Frequency of sperm abnormality in Chernobyl passerine species plotted against (a) the frequency of sperm abnormality in uncontaminated areas, and (b) mean sperm length for the species. The line in (a) is  $y = x$ .



**Figure 2.** Scanning electron micrographs of normal and abnormal spermatozoa of three passerine species: barn swallow *Hirundo rustica* (a,b), common blackbird *Turdus merula* (c,d) and common chaffinch *Fringilla coelebs* (e-g).



## General results and discussion

In this study we have made an approach to different life history traits in passerine birds. Life histories are one of the main subjects of study in current Ecology research. The field is as wide as interesting, so we have just focused in some of the most active areas, like coloration, sperm competition and moult and migration. I have also tried to improve methodology in sex discrimination in a very well-known and widely used model species for Ecology studies, the European barn swallow.

In the first chapter we obtained two discriminant functions to sex adult barn swallows, one for a Spanish population and the other one for a Danish population. We employed just three of the most common morphological traits used in any study with barn swallows: the length of the inner tail feathers, the length of the rectrix feathers, and the length of the keel. The discriminant functions for the Spanish (1) and the Danish (2) population are, respectively:

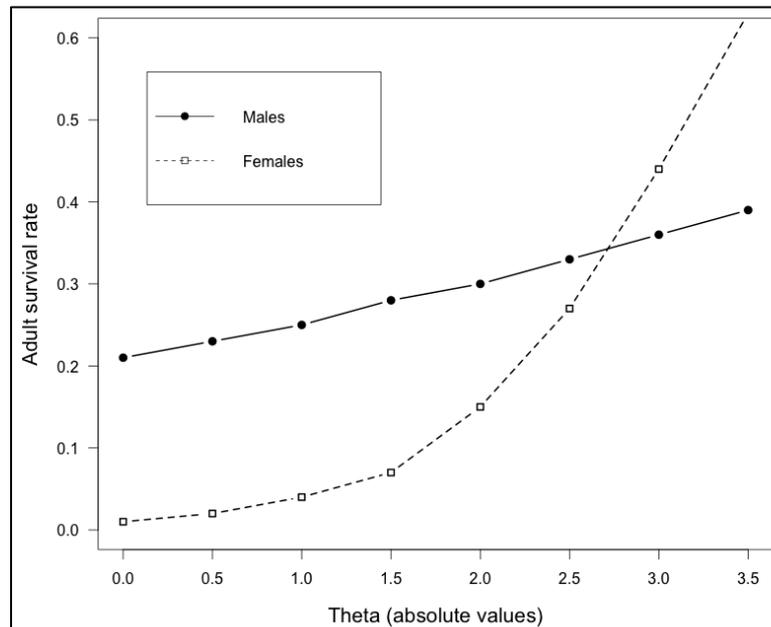
$$(1) D = 0.256 * KEEL + 0.158 * TAILOUT - 0.154 * TAILIN - 13.139$$

$$(2) D = 0.406 * KEEL + 0.125 * TAILOUT - 0.192 * TAILIN - 12.581$$

Any observer can measure these traits easily and with accuracy because they show low variability when measured by different persons (Møller et al 2006). Our methodology, with very little modifications, can be used in very distant geographical populations thus improving current discriminant methods for this species (Calleja et al. 2006).

In the following chapter, we explored the relationship between melanin based coloration from dorsal black feathers and fitness in the European barn swallow. The intensity of black coloration of the dorsal feathers in barn swallows was related to several fitness traits in females, but not in males.

Darker females survived better from one year to the next than less melanic ones, whereas that was not the case for males. Males seemed to trade between investment in coloration and tail length as color saturation was inversely related to tail length in males. These extra costs (long



tail and color investment) would affect male survival more strongly than female survival. This finding is consistent with tail length being a secondary sexual character that is costly to produce (Møller 1992; 1994a, b; Balbontín et al. 2011).

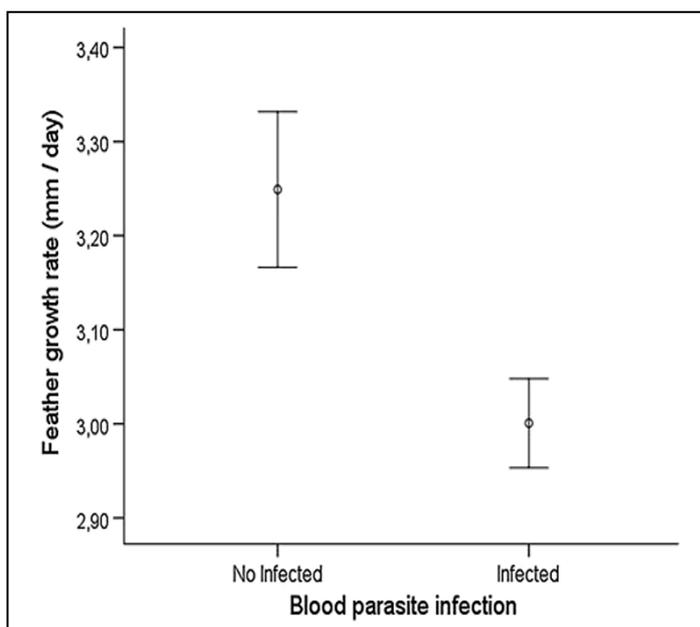
Furthermore, darker females started laying eggs earlier in the season than paler ones, and the number of fledgling was related to female coloration but not to male color. Ducrest et al. (2008) reviewed the mechanisms of the melanocortin systems and its role in the pleiotropic effects of melanogenesis on a wide range of phenotypical and behavioral traits in vertebrates, suggesting that darker vertebrates are more aggressive, sexually more active and more resistant to stress than lighter individuals. Recently, pleiotropy has been suggested as a mechanism producing covariation between MBC and other phenotypic and behavioral traits (Roulin and Ducrest 2013, Roulin 2015). These effects were mediated by melanocortins, which are peptide hormones derived from prohormone proopiomelanocortin. These melanocortin peptides do not only bind to the MC1R that control melanogenesis, but they also bind in the other melanocortin receptors (MC2-5R) that are located in different parts of the body and regulate different behavioural and physiological functions. Therefore, some of these pleiotropic effects could be the explanation to our results.

Darker individuals in both sexes arrived earlier to the breeding grounds than paler

ones. The total amount of melanin could be explaining these outcomes. Eumelaninic feathers are more resistant to abrasion, parasites and breakage (Bonser 1995; Kose and Møller 1999). Thus feathers with less melanin more readily would become worn or break and this should affect flight ability (Barbosa et al. 2003) and hence arrival date and start of laying, as this trait is positively correlated with arrival date in barn swallows (Møller 1994a, b). These advances in breeding timing would also improve reproductive success (Møller, 1994a, b; Kokko, 1999;).

Finally, this kind of coloration was not condition-dependent. In a recent paper, Roulin (2015) distinguish between condition-dependent and condition-related signalling. Condition-dependent signalling is used when body condition causally affects the ornament in any way. Condition-related signalling applies when the variation of coloration correlates with other aspects of body condition, i.e. the overall capacity to maintain the optimal functionality of body systems. Our data points to the latter option, as we did not find a causal effect of body condition on MBC, but MBC covaried with survival, tail length and other condition-related traits.

In the third chapter we studied the effect of malaria infection on feather growth rate and the possible carry-over effects in individual performance in the next breeding season.



We showed for the first time a negative association between malaria and related haemosporidian parasites infection and the growth rate of rectrix feather of birds, with infected individuals having a lower daily feather elongation. We propose two non-mutually exclusive hypotheses to explain this observation. First, this

association could be reflecting a direct consume of nutrients by parasites. In this sense, malaria parasites obtain essential amino acids from host plasma and digestion of host haemoglobin (Sherman 1979; Martin and Kirk 2007). As the renewal of the feathers is one of the most remarkable metabolic feats during annual cycle (Murphy et al. 1990) the removal of essential nutrients from the avian host by blood parasites could provoke this decrease in feather growth rate. Second, these differences in feather growth rate could be a consequence of the need to mount an immune response against the blood parasites. Because both avian moult and immune response are nutrient-demanding processes (Murphy 1996; Martin et al. 2003; Klasing 2004) there may be a reallocation of resource, incurring in trade-offs between both physiological activities (Wikelski and Ricklefs 2001; Monaghan 2004), with a subsequent increase in the immune response in detrimental of feather growth.

There was also a carry-over effect of malarial infection status on next breeding season performance: house martin with higher outermost tail feather growth rate in Africa advanced their laying date in their breeding areas in Europe. Also, haemosporidian infected birds laid their clutches later in the breeding season. Blood parasite infections decrease body condition of birds (Valkiūnas 2005; Palinauskas et al. 2008), and may slow down their feather replacement. Hence, only healthy, uninfected house martins are able to moult fast and arrive early to the breeding quarters. The delay on arrival rate was associated to a decrease in clutch size and number of nestling produced. These two traits were also negatively associated to blood parasite infection. These outcomes are in accordance to previous studies showing a decline in clutch size and annual reproductive success along the season (Brown and Brown 1999) and with late laying date (Sakraoui et al. 2005), and also with studies showing detrimental effects of haematozoan parasites on reproductive performance (Merino et al. 2000; Marzal et al. 2005).

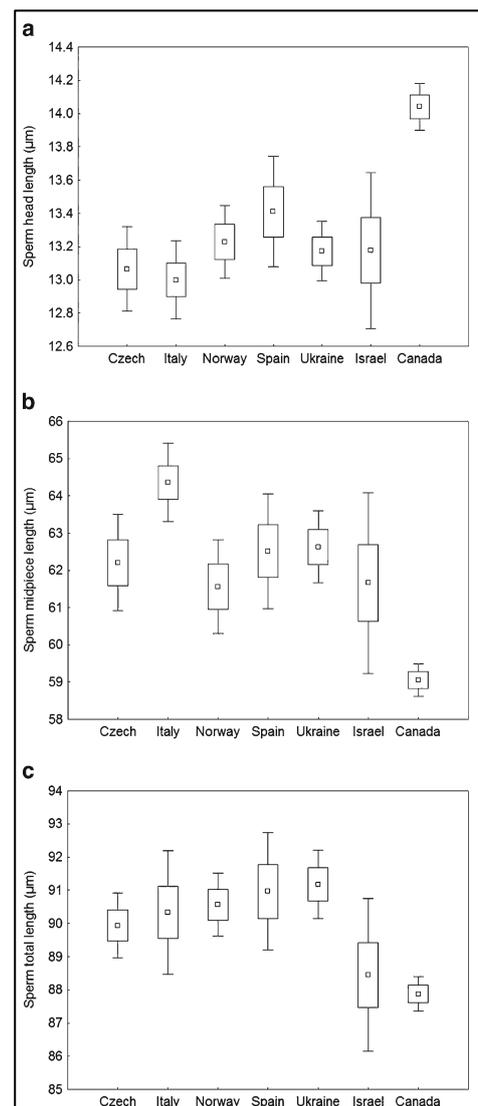
Finally, we reported an inter-annual variation in rectrix growth rate, clutch size and the number of reared young. Variation in environmental factors can shape the evolution of life

histories (Partridge and Harvey 1988). Therefore, factors such as climate or food availability that can affect moult phenology and/or key reproductive traits of birds could explain these results (Bryant 1975; Murphy 1986; Smith and Sheeley 1993; Renfrew et al. 2011).

In the forth chapter, our results revealed significant differences in sperm morphometry among barn swallow populations and subspecies. Based on the phylogenetic relationships (Dor et al. 2010), we predicted that European and East-Mediterranean barn swallows should be more similar to each other in sperm morphometry compared to North American population.

Spermatozoons from North American barn swallows had longer heads and shorter midpieces than those from all other populations. However, European barn swallows had significantly longer sperm than both East-Mediterranean and North American barn swallows. The two latter did not differ significantly for this trait.

Our results might be explained by differences in gene flow among populations. Barn swallows are long distance migrants with clear differences in dispersal distance (Paradis et al. 1999). Although the East-Mediterranean barn swallow population is sedentary, it still is subject to high levels of gene flow supposedly from migrating European barn swallows (Dor et al. 2012).

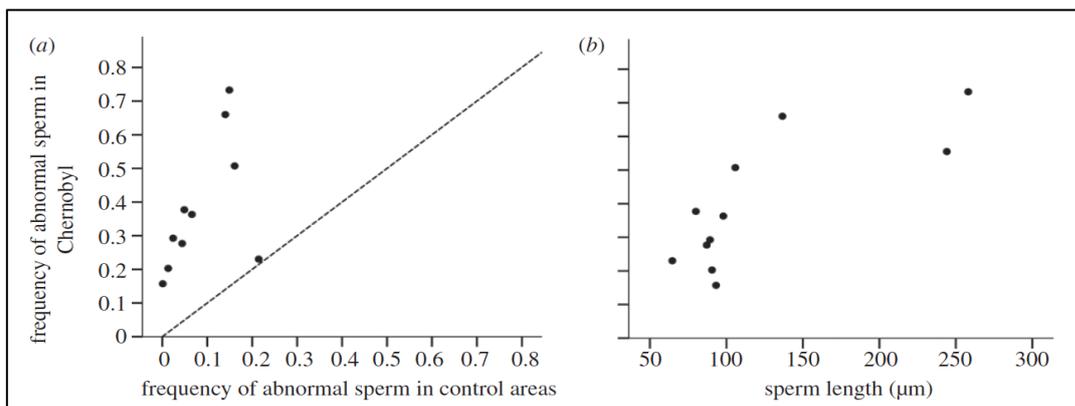


We also tested the relationship between CVbm and levels of extrapaternity data across populations. Both EPB and EPY were negatively correlated, although the relationship with EPB was not statistically significant. Although our sample size in the present dataset is small,

these results are consistent with previous studies that support the notions that risk of sperm competition induces stabilizing selection on sperm size (Calhim et al. 2007; Kleven et al. 2008; Lifjeld et al. 2010; Fitzpatrick and Baer 2011).

To the best of our knowledge, this is the first study examining the relationship between sperm size variation and extrapair paternity levels across populations within the same species. In the light of our results, we can conclude that sperm morphometry might provide an indication of genetic distance between phylogenetic lines within species. Our outcomes also strengthen the explanation that sperm competition exerts a stabilizing selection pressure on sperm size variation.

Finally, in the last chapter, we documented three novel patterns of sperm damage. First, all eleven species in the Chernobyl area showed high frequencies of sperm abnormalities 25 years after the accident. Our results suggest that most of passerine species in the area are negatively affected by radioactivity, as other studies have showed (Møller and Mousseau 2007, 2009; Møller et al. 2005, 2007, 2008; Bonisoli-Alquati et al. 2010).



Secondly, we identified premature acrosome reactions as the most likely mechanism provoking these abnormalities by using SEM analysis. We speculate that this outcome could be due to oxidative stress during spermatogenesis, as there is a link between oxidative stress and poor sperm performance in barn swallows (Møller et al. 2005, Bonisoli-Alquati et al. 2011).

And thirdly, we showed that species with longer sperm have more damaged sperm. This would imply that longer sperm have lower structural integrity of the acrosome and it is more susceptible to lipid peroxidation of the plasma membranes (Bansal and Bilaspuri 2011). This pattern reflects a possible trade-off between sperm length and sperm integrity, what might explain why there is no run-away selection for longer sperm in passerine birds compared to other species (Immler et al. 2011).

## References

- Balbontín J, de Lope F, Hermosell IG, Mousseau TA, Møller AP (2011) Determinants of age-dependent change in a secondary sexual character. *J Evol Biol* 24:440–448
- Bansal AK, Bilaspuri GS (2011) Impacts of oxidative stress and antioxidants on semen functions. *Vet Med Int*. doi: 10.4061/2011/686137
- Barbosa A, Merino S, Cuervo JJ, de Lope F, Møller AP (2003) Feather damage of long tails in Barn Swallows *Hirundo rustica*. *Ardea* 91:85–90
- Bonisoli-Alquati A, Mousseau TA, Møller AP, Caprioli M, Saino N (2010) Increased oxidative stress in barn swallows from the Chernobyl region. *Comp Biochem Physiol A* 155:205-210
- Bonisoli-Alquati A, Møller AP, Rudolfsen G, Saino N, Caprioli M, Ostermiller S, Mousseau TA (2011) The effects of radiation on sperm swimming behavior depend on plasma oxidative status in the barn swallow (*Hirundo rustica*). *Comp Biochem Physiol A* 159:105-112
- Bonser RHC (1995) Melanin and the Abrasion Resistance of Feathers. *Condor* 97:590–591
- Brown CR, Brown MB (1999) Fitness components associated with laying date in the cliff swallow. *Condor* 101:230–245
- Bryant DM (1975) Breeding biology of the house martin, *Delichon urbica* in relation to aerial insect abundance. *Ibis* 117:180–221
- Calhim S, Immler S, Birkhead TR (2007) Postcopulatory sexual selection is associated with reduced variation in sperm morphology. *PLoS One* 2:e413

- Calleja JA, Pone C, Díaz A, Leal A, Cruz A (2006) Biometría de la Golondrina común (*Hirundo rustica*) en la colonia reproductora de la Universidad Autónoma de Madrid. *Revista de Anillamiento* 16-17:59-62
- Dor R, Safran RJ, Sheldon FH, Winkler DW, Lovette IJ (2010) Phylogeny of the genus *Hirundo* and the barn swallow subspecies complex. *Mol Phyl Evol* 56:409–418
- Dor R, Safran RJ, Vortman Y, Lotem A, McGowan A, Evans MR, Lovette IJ (2012) Population genetics and morphological comparisons of migratory European (*Hirundo rustica rustica*) and sedentary East-Mediterranean (*Hirundo rustica transitiva*) barn swallows. *J Hered* 103:55–63
- Ducrest AL, Keller L, Roulin A (2008) Pleiotropy in the melanocortin system, coloration and behavioural syndromes. *Trends Ecol Evol* 23:502–10
- Fitzpatrick JL, Baer B (2011) Polyandry reduces sperm length variation in social insects. *Evolution* 65:3006–3012
- Immler S, Pitnick S, Parker GA, Durrant KL, Lüpold S, Calhim S, Birkhead TR (2011) Resolving variation in the reproductive tradeoff between sperm size and number. *Proc Natl Acad Sci USA* 108:5325-5330
- Klasing KC (2004) The cost of immunity. *Acta Zool Sin* 50:961–969
- Kleven O, Laskemoen T, Fossøy F, Robertson RJ, Lifjeld JT (2008) Intraspecific variation in sperm length is negatively related to sperm competition in passerine birds. *Evolution* 62:494–499
- Kokko H (1999) Competition for early arrival in migratory birds. *J Anim Ecol* 68:940–950
- Kose M, Møller AP (1999) Sexual selection, feather breakage and parasites: the importance of white spots in the tail of the barn swallow (*Hirundo rustica*). *Behav Ecol Sociobiol* 45:430–436
- Lifjeld JT, Laskemoen T, Kleven O, Albrecht T, Robertson RJ (2010) Sperm length variation as a predictor of extrapair paternity in passerine birds. *PLoS One* 5:e13456
- Martin RE, Kirk K (2007) Transport of the essential nutrient isoleucine in human erythrocytes

- infected with the malaria parasite *Plasmodium falciparum*. *Blood* 109:2217–2224
- Martin LB II, Scheuerlein A, Wikelski M (2003) Immune activity elevates energy expenditure of house sparrows: a link between direct and indirect costs? *Proc R Soc Lond B* 270:153–158
- Marzal A, de Lope F, Navarro C, Møller AP (2005) Malarial parasites decrease reproductive success: an experimental study in a passerine bird. *Oecologia* 142:541–545
- Merino S, Moreno J, Sanz JJ, Arriero E (2000) Are avian blood parasites pathogenic in the wild? A medication experiment in blue tits (*Parus caeruleus*). *Proc R Soc Lond B* 267:2507–2510
- Monaghan P (2004) Resource allocation and life history strategies in birds. *Acta Zool Sin* 50:942–947
- Murphy MT (1986) Temporal components of reproductive variability in eastern kingbirds (*Tyrannus tyrannus*). *Ecology* 67:1483–1492
- Murphy ME (1996) Energetics and nutrition of molt. In: Carey C (ed) *Avian energetics and nutritional ecology*. Plenum, New York, pp 158–198
- Murphy ME, King JR, Taruscio TG, Geupel GR (1990) Amino acid composition of feather barbs and rachises in three species of pygoscelid penguins: nutritional implication. *Condor* 92:913–921
- Møller AP (1992) Sexual Selection in the Monogamous barn Swallow (*Hirundo rustica*). 2. Mechanisms of Sexual Selection. *J Evol Biol* 5:603–624
- Møller AP (1994a) *Sexual selection and the Barn swallow*. Oxford University Press, Oxford, UK
- Møller AP (1994b) Phenotype-dependent arrival time and its consequences in a migratory bird. *Behav Ecol Sociobiol* 35:115–122
- Møller AP, Chabi Y, Cuervo JJ, de Lope F, Kilpimaa J, Kose M, Matyjasiak P, Pap PL, Saino N, Sakraoui R, Schifferli L, Hirschheydt J (2006) An analysis of continentwide patterns of sexual selection in a Passerine bird. *Evolution* 60:856–868

- Møller AP, Mousseau TA (2007) Species richness and abundance of forest birds in relation to radiation at Chernobyl. *Biol Lett* 3:483-486
- Møller AP, Mousseau TA (2009) Reduced abundance of insects and spiders linked to radiation at Chernobyl 20 years after the accident. *Biol Lett* 5:356-359
- Møller AP, Mousseau TA, de Lope F, Saino N (2007) Elevated frequency of abnormalities in barn swallows from Chernobyl. *Biol Lett* 3:414-417
- Møller AP, Mousseau TA, Lynn C, Ostermiller S, Rudolfson G (2008) Impaired swimming behaviour and morphology of sperm from barn swallows *Hirundo rustica* in Chernobyl. *Mutation Res* 650:210-216
- Møller AP, Surai P, Mousseau TA (2005) Antioxidants, radiation and mutation as revealed by sperm abnormality in barn swallows from Chernobyl. *Proc R Soc B* 272:247-252
- Palinauskas V, Valkiunas G, Bensch S, Bolshakov VC (2008) Effects of *Plasmodium relictum* (lineage P-SGS1) on experimentally infected passerine birds. *Exp Parasitol* 120:372–380
- Paradis E, Baillie SR, Sutherland WJ, Gregory RD (1999) Dispersal and spatial scale affect synchrony in spatial population dynamics. *Ecol Lett* 2:114–120
- Partridge L, Harvey PH (1988) The ecological context of life history evolution. *Science* 241:1449–1455
- Renfrew RB, Frey SJK, Klavins J (2011) Phenology and sequence of the complete prealternate molt of bobolinks in South America. *J Field Ornithol* 82:101–113
- Roulin A (2015) Condition-dependence, pleiotropy and the handicap principle of sexual selection in melanin-based colouration. *Biol Rev*. doi: 10.1111/brv.12171
- Roulin A, Ducrest AL (2013) Genetics of colouration in birds. *Semin Cell Dev Biol* 24:594–608
- Sakraoui R, Dadci W, Bañbura J, Chabi Y (2005) Breeding biology of barn swallows *Hirundo rustica* in Algeria, North Africa. *Ornis Fenn* 82:33–43
- Sherman IW (1979) Biochemistry of *Plasmodium* (malarial parasites). *Microbiol Rev* 43:453–495

Multi-focus approach to life histories in passerines

Smith L, Sheeley D (1993) Molt patterns of wintering northern pintails in the southern high plains. *J Wildl Manag* 57:229–238

Valkiūnas G (2005) Avian malaria parasites and other haemosporidia. CRC, Boca Raton

Wikelski M, Ricklefs R (2001) The physiology of life-histories. *Trends Ecol Evol* 16:479–481

## Conclusions

- ❖ There are major morphological differences between male and female barn swallows and these differences can be assessed by mean of Discriminant Analysis Functions, even for distant populations.
- ❖ The intensity of black coloration of the dorsal feathers in barn swallows was related to survival and reproductive success in females, but not in males.
- ❖ Darker female barn swallows survive better to the next year, begin laying eggs earlier and raise more fledglings than paler females.
- ❖ Darker individuals of both sexes in barn swallows arrive earlier to their breeding grounds than paler males.
- ❖ House martins with high infections by blood parasites show lower rectrix growth rate, arrive later to the breeding quarters, have smaller clutch size and rear less chicks than non-infected ones.
- ❖ Variation in sperm size and morphometry among European and East-Mediterranean populations is small, meanwhile this variation is somewhat larger between those populations and the North-America population.
- ❖ The inter-male variation in sperm morphometry is related to extra-paternity levels among the populations of barn swallows, same as across species.
- ❖ All studied species of passerines in Chernobyl show higher frequencies of sperm abnormalities than in control populations and they are like due to premature acrosome reaction.
- ❖ Species with longer sperm have lower structural integrity of the acrosome and present more damaged sperm.

## Papers

List of papers included in this doctoral dissertation:

Hermosell IG, Balbontín J, Marzal A, Reviriego M, de Lope F. 2007. Sex determination in barn swallows *Hirundo rustica* by means of discriminant analysis in two European populations. *Ardeola* 54: 93-100. Impact Factor: 0.593

Laskemoen T, Albretch T, Hermosell IG, Johannessen LE, Kleven O, Møller AP, Marzal A, Robertson RJ, Rudolfsen G, Saino N, Vortman Y, Liefjeld JT. 2013. Variation in sperm morphometry and sperm competition among barn swallow (*Hirundo rustica*) populations. *Behav Ecol Sociobiol* 67: 301-309. Impact Factor: 3.179

Marzal A, Reviriego M, Hermosell IG, Balbontín J, Bensch S, Relinque C, Rodríguez L, García-Longoria L, de Lope F. 2012. Malaria infection and feather growth rate predict reproductive success in House martins. *Oecología* 171:853-861. Impact Factor: 3.412

Hermosell IG, Laskemoen T, Rowe M, Møller AP, Mousseau TA, Albrecht T and Liefjeld JT. 2013. Patterns of sperm damage in Chernobyl passerine birds suggest a trade-off between sperm length and integrity. *Biol Lett* 9:20130530. Impact Factor: 3.348

Hermosell IG, Balbontín J, de Lope F, Møller AP, Reviriego M, García-Longoria L, Relinque C, Marzal A. 2014. Melanin-based colorations as a reliable indicator of reproductive success in barn swallows. *Behav Ecol Sociobiol* (under review)

The papers above will not be part of any other doctoral dissertation.

## SEX DETERMINATION IN BARN SWALLOWS *HIRUNDO RUSTICA* BY MEANS OF DISCRIMINANT ANALYSIS IN TWO EUROPEAN POPULATIONS

Ignacio G. HERMOSELL\*<sup>1</sup>, Javier BALBONTÍN\*, Alfonso MARZAL\*\*\*,  
Maribel REVIRIEGO\* and Florentino DE LOPE\*

**SUMMARY.**—*Sex determination in barn swallows *Hirundo rustica* by means of discriminant analysis in two European populations.*

**Aims:** Differences in morphology in adult barn swallows were investigated using bone and feather measurements taken from birds from a northern (Danish) and a southern (Spanish) European population. Discriminant Function Analysis was used to help in discriminating the sex of individuals of both populations.

**Location:** Badajoz (Spain) and Kraghede (Denmark).

**Methods:** Two discriminant functions were obtained using biometric data from adult individuals, one for each European population. These functions were validated using external data from a sample of an Italian and Ukrainian barn swallow populations.

**Results:** Three morphometric variables: the length of the outermost tail feathers, the length of the inner tail feathers and the length of the keel were retained in the discriminant function in the two populations studied. Overall, these functions allowed us to determine the sex of adult birds with 90.1 % and 91.9 % accuracy for the Spanish and the Danish populations, respectively. External validation using a sample from the Italian population to validate Spanish discriminant function and from the Ukrainian population to validate the Danish discriminant function showed that 91 % of Italian and 86 % of Ukrainian swallows were correctly classified.

**Conclusions:** The two discriminant functions obtained would be highly valuable in future work carried out on this species because it would allow researchers to determine the sex of individuals shortly after arrival to the breeding areas without having to wait for the beginning of courtship or incubation for reliable sexing.

*Key words:* Barn swallow, biometry, discriminant, sexing.

**RESUMEN.**—*Determinación del sexo en la Golondrina Común *Hirundo rustica* por medio de análisis discriminante en dos poblaciones europeas.*

**Objetivos:** Se investigaron diferencias en morfología de ejemplares adultos de golondrina común usando medidas esqueléticas y de plumas tomadas de una población del norte (danesa) y otra del sur (española) de Europa. Se usó el Análisis de Función Discriminante para diferenciar el sexo de los individuos de ambas poblaciones.

**Localidad:** Badajoz (España) y Kraghede (Dinamarca).

**Métodos:** Se obtuvieron dos funciones discriminantes usando los datos biométricos de los individuos, una para cada población. Estas funciones fueron validadas usando datos externos procedentes de una muestra de una población italiana y otra ucraniana.

\* Departamento de Biología Animal, Universidad de Extremadura, Badajoz, E-06071 Spain.

\*\* Department of Animal Ecology, Ecology Building, Lund University, S-223 62 Lund Sweden.

<sup>1</sup> Corresponding author: nachogh@unex.es

**Resultados:** Tres variables morfométricas: longitud de la rectriz externa, longitud de la rectriz interna y longitud de la quilla, fueron retenidas en las funciones discriminantes de las dos poblaciones estudiadas. Dichas funciones nos permitieron determinar el sexo de aves adultas con un 90,1 % y un 91,9 % de exactitud para las poblaciones española y danesa, respectivamente. Una validación externa usando una muestra de la población italiana para validar la función discriminante española y de la ucraniana para validar la función discriminante danesa mostró que el 91 % de las italianas y el 86 % de las ucranianas fueron correctamente clasificadas.

**Conclusiones:** Las dos funciones discriminantes obtenidas serían muy valiosas en futuros trabajos llevados a cabo con esta especie, dado que permitiría a los investigadores determinar el sexo de los individuos apenas llegasen a las áreas de cría sin tener que esperar al cortejo o la incubación para un sexado fiable.

*Palabras clave:* biometría, discriminante, Golondrina común, sexado.

## INTRODUCTION

Observation of breeding behaviour in the field was formerly used by researchers as a method to identify the sex of non-dimorphic birds (Samuel, 1971; Tella and Torre, 1993; Delestrade, 2001). Later, with development of computer software, multivariate statistical methods were employed to sex size dimorphic species. For instance, morphological data have been used to build discriminant functions to sex several bird species (Bortolotti, 1984a, 1984b; Garcelon *et al.*, 1985; Edwards and Kochert, 1987; Ferrer and Delecourt, 1992; Bosch, 1996; Balbontín *et al.*, 2001; Bertellotti *et al.*, 2002; Grecian *et al.*, 2003; Devlin *et al.*, 2004; Sarasola and Negro, 2004; Figuerola *et al.*, 2006). Recently, advances in genetics have allowed sexing of individuals using molecular methods (Ellegren, 1996; Griffiths *et al.*, 1998). However, these techniques are expensive, require resources unavailable for many researchers and they cannot be used rapidly in the field.

The barn swallow *Hirundo rustica* has been used as a model species in many avian studies that require knowledge of the sex of individuals with accuracy (de Lope and Møller, 1993; Møller 1993, 1994a; Cuervo *et al.*, 1996a, 1996b; Kose *et al.*, 1999; Møller, 2003, 2004; Garamszegi *et al.*, 2005; Cuervo and Møller, 2006; Balbontín *et al.*, 2007). In many of these investigations individuals have been sexed after observation of presence (females) or absence (males) of a brood patch (Saino and Møller,

1995; Møller, 2002). However, many experimental studies require knowing the sex of individuals at first capture after arrival at the breeding grounds (de Lope and Møller, 1993; Cuervo *et al.*, 1996b, 2003).

Despite the difference in tail length between males and females, both sexes overlap in a narrow range that might bias sex assignment by pure observation, especially in southern populations, due to the clinal variation in sexual size dimorphism in length of the outermost tail feathers (de Lope, 1985; Møller, 1995). The use of secondary features is also used to assign sex in this species: females usually have lighter red-brown throat patch (Ninni and Møller, unpublished data), whiter belly feathers, smaller white patches in the tail feathers (Kose and Møller, 1999) and tips of tail feathers much wider than males (Møller *et al.*, 1995). But only very experienced observers can sex individuals accurately using these traits. So it would be of special interest for researchers using this small passerine bird as a model species to have a reliable field method to discriminate sexes with precision.

## MATERIAL AND METHODS

### *Study area*

Our research group has been studying populations of barn swallows in several farms from 1976 to 2006 on the outskirts of Badajoz, south-

west Spain (39° N, 7° W). Danish barn swallows data were collected at Kraghede (57° 12' N, 10° 00' E), Denmark, during 1984 - 2006. The areas were mainly agricultural with scattered groups of trees in Spain (de Lope, 1983) and plantations, ponds and hedgerows in Denmark (Møller, 1994b). Barn swallows breed in rooms in farm houses, getting permanent access through open doors and windows.

### *Field procedures*

Just after arrival of birds and before the breeding season we captured adults at dawn by using mist nets placed across windows and doors of the breeding rooms. Each bird was identified with a numbered metal ring and a combination of coloured PVC rings, so we could recognize each adult visually. All birds were also provided with an individual combination of colour markings on their belly feathers using stamp ink. Individuals were sexed from the presence (females) or absence (males) of a brood patch and from observation of breeding behaviour during the courtship and incubation period.

We took several morphological measurements for each individual: length of bill's exposed culmen (CULMEN), tarsus (TAR), keel (KEEL), wing (WING), wingspan (WSPAN), length of the outermost tail feathers (TAILOUT) and length of the inner tail feathers (TAILIN). BILL, TAR and KEEL were measured with a digital caliper to the nearest 0.01 mm; WING, WSPAN, TAILOUT and TAILIN were measured with a graduated ruler to the nearest mm. All birds were measured by the same person every year (FdeL in Spain and APM in Denmark).

We also tracked all reproductive events and identified visually each individual in the pairs using binoculars from inside a hide, to avoid disturbance during reproduction. Once females start laying, we did daily observation sessions inside a hide, observing each occupied

nest, with the aim of identifying individuals based on colour rings and ink marks. We were able to identify each individual in the pair and their nest for the majority of the occupied nest sites.

Only those individuals with all their morphological data recorded and whose sex was well known by researchers were used in the analysis. Final sample was 806 individuals (391 males and 415 females) in the Spanish population from one of our study farms and 2115 individuals (1030 males and 1085 females) in the Danish one.

### *Statistical analysis*

Multivariate analysis of variance (MANOVA) was used to evaluate differences in morphological traits between sexes and locations. Discriminant Function Analysis (DFA) was used to obtain a mathematical model for sexing birds based on morphometric data. We used a leaving-one-out resampling method (jackknife) to test the performance of the discriminant functions.

We also validated the two discriminant functions using external data taken from other Spanish and Danish barn swallows colonies. Specifically, for the Spanish swallows we used the sample from one of our study colonies "Potosi" (280 males and 301 females,  $n = 581$ ) to obtain the Spanish discriminant function validating it using a sample of individuals captured in other colonies ( $n = 223$ ). For the Danish population, we used 918 males and 973 females ( $n = 1891$ ) leaving a separated sample of the same colony to evaluate the Danish discriminant function ( $n = 223$ ).

Additionally, we also validated the discriminant functions of both populations from a sample of individuals taken from a different geographical area with the aim of knowing if researchers that use this species as a model on their studies could use reliably these discriminant functions to sex their birds. Con-

TABLE 1

Difference in the size of barn swallows between location and gender using Multivariate Analysis of Variance (MANOVA). Shown statistic is the Hotelling's trace (error df = 2911).

[*Diferencias en tamaño en golondrinas entre localidades y sexos, usando Análisis Multivariante de la Varianza (MANOVA). El estadístico mostrado es la traza de Hotelling (error gl = 2911).*]

	Value	F	Hypthesis df	P
Location	0.687	285.898	7	< 0.001
Sex	1.242	516.618	7	< 0.001
Location * sex	0.021	8.671	7	< 0.001

cretely, we validated the discriminant function obtained from the Spanish population with a sample of an Italian population ( $n = 100$ ) and the discriminant function obtained from the Danish population with a sample of a Ukrainian population ( $n = 100$ ).

Statistical analysis was performed using SPSS 13.01 for Windows software (Lachenbruch and Goldstein, 1979; Huberty and Lowman, 1997).

## RESULTS

Multivariate analysis of variance (MANOVA) showed that there is difference in size between the Spanish and Danish barn swallows. This analysis showed there are differences in size between males and females and between populations, as it is stated by the significant interaction between location and sex (Table 1).

The most dimorphic traits in both populations were the length of the keel, the length of the inner tail and the length of the outermost tail feather. All morphometric data from both populations are summarized in Table 2.

The discriminant function for adult Spanish barn swallow was:

$$D = 0.256 * KEEL + 0.158 * TAILOUT - 0.154 * TAILIN - 13.139$$

Values of  $D > 0$  correspond to males and values  $D < 0$  to females. This function classified correctly 90.2 % of individuals using a jackknife validation procedure. Individuals with discriminant scores between -0.450 and 0.450 should remain unsexed (17 % of total sample), if the total error committed is wanted to be reduced at 5 % level.

The discriminant function for adult Danish barn swallows was:

$$D = 0.406 * KEEL + 0.125 * TAILOUT - 0.192 * TAILIN - 12.581$$

It classified correctly 91.6 % of individuals using a jackknife validation procedure. The percentage of reliability was 91.9 % when other data from the same population were used. Individuals with scores between -0.300 and 0.300 should remain unsexed (10 % of total sample), if the total error committed is wanted to be reduced at 5 % level.

Reliability of the two discriminant functions was similar when we used a sample of individuals taken either from our own study areas (but not used to obtain the discriminant functions) or from populations located in a different geographical area. The Spanish function classified correctly 91.0 % and 90.1 % of Italian and Spanish individuals and the Danish function classified correctly 86.0 % and 91.9 % of Ukrainian and Danish individuals (Table 3).

TABLE 2

Morphometric measurements for males and females from both populations (mean  $\pm$  SD, sample in brackets).  
 [Medidas morfométricas para machos y hembras de ambas poblaciones (media  $\pm$  DT, muestra entre paréntesis).]

	SPAIN		DENMARK	
	Males (280)	Females (301)	Males (918)	Females (973)
CULMEN	7.50 $\pm$ 0.53	7.47 $\pm$ 0.54	7.79 $\pm$ 0.49	7.88 $\pm$ 0.52
KEEL	21.17 $\pm$ 0.90	20.50 $\pm$ 0.92	21.46 $\pm$ 0.74	20.74 $\pm$ 0.76
TAR	11.06 $\pm$ 0.48	11.15 $\pm$ 0.49	11.09 $\pm$ 0.56	11.08 $\pm$ 0.58
WING	121.23 $\pm$ 2.96	119.88 $\pm$ 3.08	126.43 $\pm$ 2.90	124.39 $\pm$ 2.82
TAIL_OUT	99.51 $\pm$ 7.33	85.23 $\pm$ 5.02	109.62 $\pm$ 8.92	91.37 $\pm$ 6.05
TAIL_IN	43.23 $\pm$ 2.14	43.57 $\pm$ 1.64	43.97 $\pm$ 1.64	44.51 $\pm$ 1.69
WSPAN	320.53 $\pm$ 9.35	317.21 $\pm$ 9.38	332.35 $\pm$ 11.64	327.91 $\pm$ 6.29

TABLE 3

Validation of the two discriminant functions (Spanish and Danish) using external data from the same study area at the Spanish and Danish populations and from two different geographical area on an Italian and Ukrainian barn swallows populations.

[Validación de las dos funciones discriminantes (española y danesa) usando datos externos del mismo área de estudio de las poblaciones española y danesa, y de dos áreas geográficas diferentes en una población italiana y en otra ucraniana.]

Sex	Pronosticated sex					
	SPAIN			DENMARK		
	Males	Females	Total	Males	Females	Total
Males	244	36	280	824	94	918
Females	21	280	301	65	908	973
	ITALY			UKRAINE		
	Males	Females	Total	Males	Females	Total
Males	49	1	50	42	8	50
Females	8	42	50	6	44	50

## DISCUSSION

The use of three morphometric traits was enough for sexing individuals with a great precision and improved current discriminant meth-

ods proposed for this species (Calleja *et al.*, 2006). All biometric measurements can easily be taken in the field with great accuracy, because they show low variability when the same morphometric measure is taken by different ob-

servers (Møller *et al.*, 2006). Another advantage of the method proposed is that researchers can select the percentage of individuals correctly classified by selecting a narrower range of the discriminant scores, with the cost of leaving out a sample of individuals to be sexed by other methods. Because we proved that the discriminant functions were reliable enough for sexing Barns swallows from very distant geographical area, we encourage the use of the discriminant function obtained in this study by researchers working with this species in a distant geographical area. We proposed that researchers or naturalists working on populations placed at European southern latitudes should use the discriminant function obtained for the Spanish population, whereas studies on northern populations should use the discriminant function obtained for Danish populations.

ACKNOWLEDGMENTS.—Thanks to all the people who helped to obtain data in the field, especially F. Mateos, C. Navarro, P. Ninni, J. Cuervo, A. Barbosa, J. Moreno, S. Merino, D. Gil, L. Garamszegi and A. N. Dreiss. Special thanks to Nicola Saino and Anders P. Møller for providing us the Italian data and the Danish and Ukrainian data, respectively. This research was supported by the Spanish Ministry of Education and Science (CGL-2006-01937). AM was supported by post-doctoral (EX-2006-0557) grant from Spanish Ministry of Education and Science.

#### BIBLIOGRAPHY

- BALBONTIN, J., FERRER, J. and CASADO, E. 2001. Sex determination in Booted Eagles (*Hieraetus pennatus*) using molecular procedures and discriminant function analysis. *Journal of Raptor Research*, 35: 20-23.
- BALBONTIN, J., HERMOSELL, I. G., MARZAL, A., REVIRIEGO, M., LOPE, F. DE AND MØLLER, A. P. 2007. Age-related change in breeding performance in early life is associated with an increase in competence in the migratory barn swallow *Hirundo rustica*. *Journal of Animal Ecology*. 76: 915-925.
- BERTELLOTTI, M., TELLA, J. L., GODOY, J. A., BLANCO, G., FORERO, M. G., DONAZAR, J. A. and CEBALLOS, O. 2002. Determining sex of Magellanic Penguins using molecular procedures and discriminant functions. *Waterbirds*, 25: 479-484.
- BORTOLOTTI, G. R. 1984a. Age and sex size variation in Golden Eagles. *Journal of Field Ornithology*, 55: 54-66.
- BORTOLOTTI, G. R. 1984b. Sexual size dimorphism and age-related size variation in Bald Eagles. *Journal of Wildlife Management*, 48: 72-81.
- BOSCH, M. 1996. Sexual size dimorphism and determination of sex in Yellow-legged Gulls. *Journal of Field Ornithology*, 67: 534-541.
- CALLEJA, J. A., PONCE, C., DIAZ, A., LEAL, A. and CRUZ, A. 2006. Biometría de la Golondrina común (*Hirundo rustica*) en la colonia reproductora de la Universidad Autónoma de Madrid. *Revista de anillamiento*, 16-17: 59-62.
- CUERVO, J. J. and MØLLER, A. P. 2006. Experimental tail elongation in male Barn Swallows *Hirundo rustica* reduces provisioning of young, but only in second broods. *Ibis*, 148: 449-458.
- CUERVO, J. J., LOPE, F. DE. and MØLLER, A. P. 1996a. The function of long tails in female barn swallows (*Hirundo rustica*): An experimental study. *Behavioral Ecology*, 7: 132-136.
- CUERVO, J. J., MØLLER, A. P. and LOPE, F. DE. 2003. Experimental manipulation of tail length in female barn swallows (*Hirundo rustica*) affects their future reproductive success. *Behavioral Ecology*, 14: 451-456.
- CUERVO, J. J., LOPE, F. DE., MØLLER, A. P. and MORENO J. 1996b. Energetic cost of tail streamers in the barn swallow (*Hirundo rustica*). *Oecologia*, 108: 252-258.
- DELESTRADE, A. 2001. Sexual size dimorphism and positive assortative mating in Alpine Choughs (*Pyrrhocorax graculus*). *Auk*, 118: 553-556.
- DEVLIN, C. M., DIAMOND, A. W. and SAUNDERS, G. W. 2004. Sexing Arctic Terns in the field and laboratory. *Waterbirds*, 27: 314-320.
- EDWARDS, T. C. and KOCHERT, M. N. 1987. Use of body weight and length of footpad as predictors of sex in Golden Eagles. *Journal of Field Ornithology*, 58: 144-147.
- ELLEGREN, H. 1996. First gene on the avian W chromosome (CHD) provides a tag for universal sex-

- ing of non-ratite birds. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 263: 1635-1641.
- FERRER, M. and DELECOURT, C. 1992. Sex Identification in the Spanish Imperial Eagle. *Journal of Field Ornithology*, 63: 359-364.
- FIGUEROLA, J., GARCIA, L., GREEN, A. J., IBAÑEZ, F., MAÑEZ, F., VALLE, J. L. DEL, GARRIDO, H., ARROYO, J. L. and RODRIGUEZ, R. 2006. Sex Identification in Glossy Ibis chicks based on morphological characters. *Ardeola*, 53: 229-235.
- GARAMSZEGI, L. Z., HEYLEN, D., MØLLER, A. P., EENS, M. AND LOPE, F. DE. 2005. Age-dependent health status and song characteristics in the barn swallow. *Behavioral Ecology*, 16: 580-591.
- GARCELON, D. K., MARTELL, M. S., REDIG, P. T. and BUOEN, L. C. 1985. Morphometric, karyotypic, and laparoscopic techniques for determining sex in Bald Eagles. *Journal of Wildlife Management*, 49: 595-599.
- GRECIAN, V. D., DIAMOND, A. W. and CHARDINE, J. W. 2003. Sexing razorbills *Alca torda* at Machias Seal Island, New Brunswick, Canada, using discriminant function analysis. *Atlantic Sea Birds*, 5: 73-80.
- GRIFFITHS, R., DOUBLE, M. C., ORR, K. and DAWSON, R. J. G. 1998. A DNA test to sex most birds. *Molecular Ecology*, 7: 1071-1075.
- HUBERTY, C. J. and L. L. LOWMAN. 1997. Discriminant analysis via statistical packages. *Educational and Psychological Measurement*, 57: 759-784.
- KOSE, M. and MØLLER, A. P. 1999. Sexual selection, feather breakage and parasites: the importance of white spots in the tail of the barn swallow (*Hirundo rustica*). *Behavioral Ecology and Sociobiology*, 45: 430-436.
- KOSE, M., MÄND, R. and MØLLER, A. P. 1999. Sexual selection for white tail spots in the barn swallow in relation to habitat choice by feather lice. *Animal Behaviour*, 58: 1201-1205.
- LACHENBRUCH, P. A. and M. GOLDSTEIN. 1979. Discriminant analysis. *Biometrics*, 35: 69-85.
- LOPE, F. DE. 1983. La avifauna de las Vegas Bajas del Guadiana. *Doñana Acta Vertebrata*, 10: 91-121
- LOPE, F. DE. 1985. Pterilosis y dimorfismo sexual de *Hirundo rustica rustica* L. en Extremadura (España). *Ardeola*, 32: 3-8.
- LOPE, F. DE, and MØLLER, A. P. 1993. Female reproductive effort depends on the degree of ornamentation of their mates. *Evolution*, 47: 1152-1160.
- MØLLER, A. P. 1993. Female preference for apparently symmetrical male sexual ornaments in the Barn Swallow *Hirundo rustica*. *Behavioral Ecology and Sociobiology*, 32: 371-376.
- MØLLER, A. P. 1994a. Symmetrical male sexual ornaments, paternal care, and offspring quality. *Behavioral Ecology*, 5: 188-194.
- MØLLER, A. P. 1994b. Sexual selection and the Barn swallow. *Oxford University Press*, Oxford, U.K.
- MØLLER, A. P. 1995. Sexual selection in the Barn Swallow (*Hirundo rustica*). 5. Geographic variation in ornament size. *Journal of Evolutionary Biology*, 8: 3-19.
- MØLLER, A. P. 2002. Temporal change in mite abundance and its effect on barn swallow reproduction and sexual selection. *Journal of Evolutionary Biology*, 15: 495-504.
- MØLLER, A. P. 2003. Sexual selection and extinction: why sex matters and why asexual models are insufficient. *Annales Zoologici Fennici*, 40: 221-230.
- MØLLER, A. P. 2004. Protandry, sexual selection and climate change. *Global Change Biology*, 10: 2028-2035.
- MØLLER, A. P., LOPE, F. DE and SAINO, N. 1995. Sexual selection in the barn swallow *Hirundo rustica*. 6. Aerodynamic adaptations. *Journal of Evolutionary Biology*, 8: 671-687.
- MØLLER, A. P., CHABI, Y., CUERVO, J. J., LOPE, F. DE, KILPIMAA, J., KOSE, M., MATYJASIAK, P., PAP, P. L., SAINO, N., SAKRAOUI, R., SCHIFFERLI, L. and HIRSCHHEYDT, J. 2006. An analysis of continent-wide patterns of sexual selection in a Passerine bird. *Evolution*, 60: 856-868.
- SAINO, N. and MØLLER, A. P. 1995. Testosterone correlates of mate guarding, singing and aggressive behaviour in male Barn Swallows, *Hirundo rustica*. *Animal Behaviour*, 49: 465-472.
- SAMUEL, D. E. 1971. Field methods for determining the sex of Barn Swallows (*Hirundo rustica*). *Ohio Journal of Science*, 71: 125-128.
- SARASOLA, J. H., and NEGRO J. J. 2004. Gender determination in the Swainson's hawk (*Buteo swainsoni*) using molecular procedures and discriminant function analysis. *Journal of Raptor Research*, 38: 357-361.

TELLA, J. L. and TORRE, I. 1993. Sexual Size Dimorphism and Determination of Sex in the Chough (*Pyrrhonorax pyrrhonorax*). *Journal für Ornithologie*, 134: 187-190.

[Recibido: 07-05-07]

[Aceptado: 26-09-07]

**Ignacio G. Hermosell** is on his early research stage career and belongs to the research team working on evolutionary ecology and sexual selection. Nowadays he enjoys a research fellowship at University of Extremadura. **Javier Balbontín** presently investigate on the ecology and evolution

of behaviour of Barn swallow. He has previously done his Ph. D. in the ecology and dispersive behaviour of Bonelli's eagle in south Spain. **Alfonso Marzal** is a postdoctoral researcher at University of Lund. His present research is centered in the field of Molecular Ecology on host-parasite evolution of avian malaria parasites. **Maribel Reviriego** is a Phd student involved in study of blood parasite load effects on life history, migration and senescence in a colony of House martin. **Florentino de Lope** is Zoology Professor at University of Extremadura and the director of Evolutionary Biology and Ethology Research Group at the Animal Biology Department.

## Malaria infection and feather growth rate predict reproductive success in house martins

Alfonso Marzal · Maribel Reviriego · Ignacio G. Hermosell · Javier Balbontín · Staffan Bensch · Carmen Relinque · Laura Rodríguez · Luz Garcia-Longoria · Florentino de Lope

Received: 3 November 2011 / Accepted: 19 August 2012  
© Springer-Verlag 2012

**Abstract** Carry-over effects take place when events occurring in one season influence individual performance in a subsequent season. Blood parasites (e.g. *Plasmodium* and *Haemoproteus*) have strong negative effects on the body condition of their hosts and could slow the rate of feather growth on the wintering grounds. In turn, these winter moult costs could reduce reproductive success in the following breeding season. In house martins *Delichon urbica* captured and studied at a breeding site in Europe, we used ptilochronology to measure growth rate of tail feathers moulted on the winter range in Africa, and assessed infection status of blood parasites transmitted on the wintering grounds. We found a negative association between haemosporidian parasite infection status and inferred growth rate of tail feathers. A low feather growth rate and blood parasite infections were related to a delay in laying date in their European breeding quarters. In addition, clutch size and the number of fledglings were negatively related to a delayed laying date and blood parasite

infection. These results stress the importance of blood parasites and feather growth rate as potentially mechanisms driving carry-over effects to explain fitness differences in wild populations of migratory birds.

**Keywords** Carry-over effects · *Delichon urbica* · *Haemoproteus* · Host–parasite interaction · *Plasmodium*

### Introduction

Annual periodicity provokes seasonal changes in environmental conditions of temperate areas to which animals must adapt in order to reproduce and survive. Migration, the regular seasonal journey undertaken by many different groups of animals, is a response to such environmental hazards in search of mild ecological conditions like warmer temperatures and more food availability (Lack 1968; Gauthreaux 1982). In birds, this usually involves the movement of billions of individuals flying vast distances between a breeding site and another non-breeding area, where they spend the rest of the year (Greenberg and Marra 2005; Newton 2008). In consequence, migratory individuals spend different periods of their annual cycle in widely separated and ecologically disparate locations. These periods are linked, so individuals can carry over effects from one season to the next (Webster et al. 2002; Harrison et al. 2011). In this line, Marra et al. (1998) showed in songbirds that winter habitat can influence the departure dates from winter quarters and subsequent dates of arrival and condition on the breeding grounds. Also, Norris et al. (2004) showed that the reproductive success of long-distance migratory American redstarts (*Setophaga ruticilla*) is influenced by the quality of the winter habitat, which is located thousands of kilometres from the temperate

Communicated by Douglas Robinson.

A. Marzal (✉) · M. Reviriego · I. G. Hermosell · J. Balbontín · C. Relinque · L. Rodríguez · L. Garcia-Longoria · F. de Lope  
Departamento de Biología Animal,  
Universidad de Extremadura, 06071 Badajoz, Spain  
e-mail: amarzal@unex.es

A. Marzal · S. Bensch  
Department of Biology, Lund University, Ecology Building,  
22362 Lund, Sweden

J. Balbontín  
Departamento de Zoología, Facultad de Biología,  
Universidad de Sevilla, Edificio Verde, Avda. de Reina  
Mercedes s/n, 41013 Sevilla, Spain

Published online: 11 September 2012

 Springer

breeding grounds. Moreover, Saino et al. (2004) reported in barn swallows *Hirundo rustica* that higher winter food availability resulted in earlier arrival on the breeding grounds, increased frequency of second clutches and more fledged offspring per season.

In bird species, feathers are constantly exposed to degrading agents such as physical abrasion (Butler and Johnson 2004), bacterial activity (Burt and Ichida 1999) and ultraviolet light (Bergman 1982). Moulting, the regular replacement of feathers, has evolved as an important strategy in avian life-histories to repair this damage and to maintain plumage functions. The moulting process involves changes in nutritional and energetic demands of birds (Klaassen 1995; Murphy 1996), and important reallocation of resources among organs and functions (Murphy and Taruscio 1995; Murphy 1996; Nava et al. 2001). Accordingly, both the duration and timing of moulting is usually adjusted within the annual cycle of birds to minimise the overlap with other energetically demanding activities such as breeding and migration (Jenni and Winkler 1994; Yuri and Rohwer 1997; de la Hera et al. 2009a). In this context, Rohwer et al. (2011) have recently shown a trade-off between moulting and breeding in the black-footed albatross (*Phoebastria nigripes*), where adults with overly worn primary flight feathers suffered reduced fledging success in the current breeding season and were likely to skip the next breeding season. In addition, Nilsson and Svensson (1996) showed experimentally that a delay of breeding may compromise future reproduction and survival in blue tits (*Cyanistes caeruleus*), suggesting that these long-term effects may be the result of the late breeding following the clutch removal interfering with the moulting that occurs after breeding. Also, feather quality might play a key role in moulting strategies in birds. For instance, Dawson et al. (2000) demonstrated experimentally that newly grown primary feathers of male European starlings (*Sturnus vulgaris*) that re-grew quickly were of poorer quality. Similarly, Serra (2001) showed in grey plovers *Pluvialis squatarola* a higher quality of primaries that were re-grown at a slow speed.

Moulting patterns can be affected by parasite infection. In this sense, Langston and Hillgarth (1995) showed that Laysan albatrosses *Phoebastria immutabilis* infected with oesophageal parasites replaced fewer primaries, grew fewer primaries simultaneously and began their moulting later than uninfected individuals. Avian malaria and related haemosporidian parasites are known to provoke detrimental effects on their hosts by decreasing body condition (Valkiūnas 2005; Palinauskas et al. 2008). Moreover, some studies have shown blood parasite infections associated with moulting status of their avian hosts. Morales et al. (2007) showed that the proportion of female pied flycatchers *Ficedula hypoleuca* infected with *Haemoproteus* at an

initial breeding stage was higher among females with a moulting–breeding overlap compared with females with no overlap. Also, Tarello (2007) reported a stop or delayed moulting in falcons infected with *Haemoproteus*. In contrast, Allander and Sundberg (1997) did not find any relationship between moulting score and *Haemoproteus* intensity in captive male yellowhammers *Emberiza citrinella*. However, whether avian blood parasites affect the growth rate of an individual feather still remains unknown.

Here, we propose an easy and quick way of using ptilochronology under field conditions to measure growth rate in outermost tail feathers based on daily feather growth shown by the number of growth bars in tail streamers. The house martin *Delichon urbica* is a small colonial hirundine which moults its wing and tail feathers in Africa (Cramp 1988; Turner and Rose 1989). This migratory species gets most of its blood parasite infections in Africa, but carry them back to Europe as chronic infections. This assumption is based on the absence of blood parasite infection in more than 400 fledgling and juvenile house martins analysed before their first migration (Marzal, unpublished data). Here, we analyse whether the presence of malaria infection during the breeding season is associated with the feather growth rate during moulting the preceding winter in Africa. Also, we address the carry-over effects of these two events taking place at the African winter grounds (rectrix growth rate and haemosporidian infection) on the breeding success in Europe. If blood parasite infection provokes detrimental effects on daily elongation of rectrix of house martins, we would expect a lower rectrix growth rate in infected birds. In addition, since events on wintering grounds can affect breeding performance, we would also predict a positive effect of rectrix growth rate and a negative effect of haemosporidian infection on reproductive success of house martins.

## Materials and methods

### Study site and collecting samples

The study was carried out in a colony of house martins in the surroundings of Badajoz (38°52'N, 7°05'W), southwest Spain, during an 11-year period (2000–2010).

Since breeding parameters differ from the first to the second clutches in house martins (Christe et al. 2001), as well as the effects of malaria parasites (Christe et al. 2002), only data from first clutches were used in this study. From February to July, we followed reproductive events and collected blood samples from breeding individuals. Nests were inspected every second day to determine the start of laying. When the clutch was finished, as shown by no more eggs being added to the clutch, we captured adult house

martins at dawn in their nests and recorded body mass with a Pesola spring balance to the nearest 0.5 g. Feathers were inspected for the presence of chewing lice by holding the extended tail against the light and counting parasites (see Christe et al. 2002 for detailed information). We took a blood sample in a capillary tube for measurement of haematocrit. After centrifugation, blood cells were stored in 500  $\mu$ L of 96 % ethanol at room temperature.

#### Measurement of feather growth rate

In the field, the length of the right outermost tail feather was measured to the nearest 1 mm with a ruler. In addition, the number of growth bars was estimated by holding the extended tail against the light and counting the number of dark bands. Bird feathers have a series of light and dark bands perpendicular to the feather rachis. Each light and dark band taken together (one growth bar) represents 24 h of growth (Riddle 1908; Michener and Michener 1938; Brodin 1993; Grubb 2006). Thus, the number of dark bands indicates the number of days employed in moulting these feathers.

In order to assess the accuracy of data obtained in the field, we plucked the right rectrix of 89 randomly chosen house martins after field measurements. We measure the number of growth bars and the length of right outermost rectrix feather in a gel documentation system in the laboratory following the instructions from Shawkey et al. (2003). To visualise growth bars, we placed the feather in the light cabinet. We positioned a ruler near to the feather for use as a scale marker. Growth bars were most clearly visible when one of the two cabinet lights was shaded using a piece of black cardboard. Once contrast and resolution were optimised, a digital image of the feather was obtained. We measured the number of growth bars and the length of rectrix minus the calamus using ImageJ software (Abramoff et al. 2004).

#### Molecular detection of blood parasite infections

Haemosporidian parasites (*Plasmodium* spp. and *Haemoproteus* spp.) were detected from blood samples using molecular methods (Bensch et al. 2000; Waldenström et al. 2004). DNA from the avian blood samples were extracted in the laboratory using a standard chloroform/isoamylalcohol method (Sambrook et al. 2002). Diluted genomic DNA (25 ng/ $\mu$ l) was used as a template in a polymerase chain reaction (PCR) assay for detection of the parasites using nested-PCR protocols described by Waldenström et al. (2004). The amplification was evaluated by running 2.5  $\mu$ l of the final PCR on a 2 % agarose gel. All PCR experiments contained one negative control for every

eight samples. In the very few cases of negative controls showing signs of amplification (never more than faint bands in agarose gels), the whole PCR-batch was run again to make sure that all positives were true. Although we did not sequence positive samples, a previous study had revealed that *Haemoproteus* is the most prevalent blood parasite genus infecting house martins from this population (Marzal et al. 2008).

#### Statistical procedures

To assess the concordance in continuous data between paired measures of the same value, we calculated the concordance correlation coefficient,  $\rho_c$ , between the number of growth bars and length of outermost rectrix feather from field data and laboratory data (Zar 1996; Lin 2000). The concordance correlation coefficient was obtained using the online statistical calculator tool provided by NZ Ministry of Health, the Foundation for Research, Science and Technology (FRST), and Environmental Diagnostics Ltd (<http://www.niwa.co.nz/our-services/online-services/statistical-calculators>).

We used one-way ANOVA to test for significant differences between uninfected and infected house martins for the length of rectrix feather, the number of growth bars, breeding success and three individual measures of body condition (body mass, haematocrit value and the abundance of chewing lice on the tail feather).

We used general linear models (GLM) to investigate the effect of sex, year (i.e. environmental variation), body mass (body condition), haematocrit, the abundance of chewing lice and infection status (uninfected or infected) on the rectrix growth rate of house martins. Sex, year and infection status were included as fixed factors in the model. There was missing information for some individuals, which resulted in slightly varying sample sizes in the different analyses. The total dataset included each individual only once to avoid pseudoreplication (Hurlbert 1984).

We also used GLM with laying date, clutch size and number of fledglings as dependent variables. In these models, infection status (uninfected, infected), sex, year, haematocrit, body mass, number of chewing lice and rectrix growth rate were included as independent variables in each case. Since, timing of breeding could affect breeding success in hirundines (Balbontín et al. 2007, 2009) laying date was also included as predictor, when clutch size and number of fledglings were the response variables. We used a backward stepwise procedure to eliminate all non-significant terms ( $P > 0.05$ ) from our starting maximal model. Only the final models were presented. All analyses were performed using PASW Statistics 18 statistical package for Windows.

## Results

### Concordance between field and laboratory measurements

We measured the length of rectrix and the number of growth bars in the same feather both in the field and in the laboratory in order to assess the agreement between measurements. We evaluated the accuracy in these measurements by using the concordance correlation coefficient. The results showed a substantial agreement between measurements both in length of rectrix [sample concordance correlation coefficient ( $\rho_c$ ) = 0.956] and in the number of growth bars [sample concordance correlation coefficient ( $\rho_c$ ) = 0.962]. In addition, we found a substantial agreement between measurements of rectrix growth rate in field and in the laboratory [sample concordance correlation coefficient ( $\rho_c$ ) = 0.957].

### Blood parasites and body condition

We analysed 444 blood samples from house martins in search for blood parasites. We found 155 (35 %) uninfected individuals and 289 (65 %) individuals infected with blood parasites. The length of rectrix feather was smaller in infected house martins than in uninfected individuals [mean length (SD): uninfected = 60.90 (2.66); infected = 59.85 (2.71); ANOVA  $F_{1, 443} = 15.201, P < 0.001$ ]. Infected house martins had more growth bars than uninfected birds [mean number of growth bars (SD): uninfected = 19.20 (3.32); infected = 20.25 (2.53); ANOVA  $F_{1, 443} = 13.944, P < 0.001$ ]. Infected house martins had lower body mass than uninfected individuals [mean body mass (SD): uninfected = 16.73 (1.75); infected = 16.23 (1.47); ANOVA  $F_{1, 443} = 9.95, P = 0.002$ ]. In contrast, there were no differences between infected and no infected house martins in the abundance of chewing lice [mean intensity of chewing lice (SD): uninfected = 8.88 (10.11); infected = 8.70 (9.92); ANOVA  $F_{1, 443} = 0.03, P = 0.85$ ] or haematocrit levels [mean haematocrit (SD): uninfected = 53.97 (3.42); infected = 53.47 (3.71); ANOVA  $F_{1, 443} = 1.97, P = 0.16$ ].

### Blood parasites and rectrix growth rate

The GLM showed that infection status and year explained significant variation in rectrix growth rate, while controlling for other potentially influencing variables such as sex, abundance of chewing lice, haematocrit and body mass. Specifically, non-infected individuals had higher rectrix growth rate than infected individuals [mean feather growth rate (SD): uninfected = 3.24 (0.57) mm/day; infected = 2.99 (0.43) mm/day; estimate (SE) = 0.042 (0.12)].

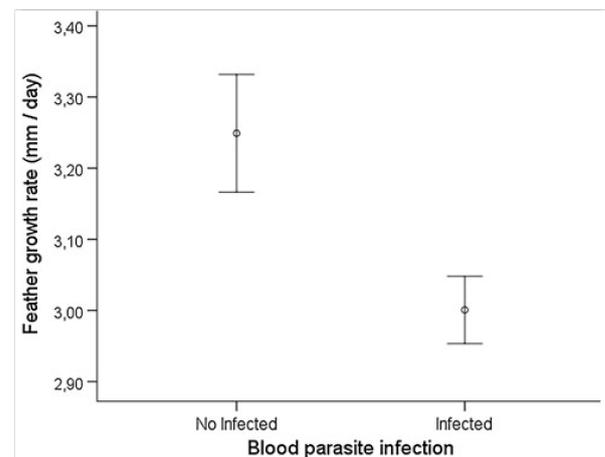
None of the interaction terms were statistically significant ( $P > 0.10$ ) in all models (Fig. 1; Table 1).

### Blood parasites, rectrix growth rate and reproduction investment

Prevalence of blood parasites, year and rectrix growth rate explained significant variation in laying date. In contrast, neither body mass, haematocrit, abundance of chewing lice or sex explained a significant variation in laying date (Table 2). Uninfected birds initiated clutches earlier than infected birds [mean laying date (SD) (Julian date): uninfected = 104.59 (19.30); infected = 114.47 (18.74); estimate (SE) =  $-5.087$  (5.762)] (Fig. 2a). Moreover, individuals that had moulted their rectrix feathers faster also initiated their clutches earlier [GLM; estimate slope (SE) =  $-5.78$  (2.19); Table 2; Fig. 3].

We found an effect of year, prevalence of blood parasites and laying date in clutch size (Table 3). Uninfected birds laid larger clutches compared with infected birds [mean clutch size (SD): uninfected = 4.56 (0.71); infected = 4.34 (0.72); estimate (SE) = 0.132 (0.26)] (Fig. 2b). Those birds that initiated their reproduction earlier also laid larger clutches [estimate slope (SE) =  $-0.007$  (0.003)].

Finally, we found that prevalence of blood parasites, year and laying date influenced the number of fledglings, while controlling for other confounding variables such as body mass, sex, haematocrit and abundance of chewing lice (Table 4). However, rectrix growth rate did not affect the number of fledging produced [GLM; estimate slope (SE) = 0.216 (0.17),  $F = 1.55, P > 0.05$ ]. Uninfected birds also fledged more nestlings than infected house martins [mean number of nestlings (SD): uninfected = 3.71 (1.06);



**Fig. 1** Feather growth rate for uninfected ( $n = 155$ ) and infected house martins *Delichon urbica* ( $n = 289$ ). Error bars show means  $\pm$  95 % of confidence interval

**Table 1** Results from the GLM explaining variation in the feather growth rate for individual house martins *Delichon urbica*

Independent variable	Square-sum III	df	F	P
Prevalence	0.463	1	3.945	0.048
Year	58.778	10	50.065	<0.001

Prevalence of blood parasites infection, year, sex, body mass, haematocrit and chewing lice were the predictor variables. Sample size was 444 individuals. Only statistical significant terms ( $P < 0.05$ ) are shown

**Table 2** Factors explaining variation in laying date of house martins

Independent variable	Square-sum III	df	F	P
Prevalence	3,422.827	1	14.841	<0.001
Year	29,391.602	10	12.744	<0.001
Feather growth rate	1,621.924	1	7.033	0.008

A backward stepwise procedure was used in a GLM analysis with prevalence of blood parasite, sex, year, body mass, haematocrit, number of chewing lice and feather growth rate as predictor variables. Only independent variables selected by the backward stepwise procedure are listed. Sample size was 439 individuals

infected = 3.08 (1.30); estimate (SE) = 0.532 (0.435)] (Fig. 2c). Those birds that initiated their reproduction earlier also bred more nestlings [estimate (SE) = -0.010 (0.004)]. Uninfected house martins also had higher breeding success (number of fledglings divided by clutch size) [mean breeding success (SD): uninfected = 0.82 (0.20); infected = 0.71 (0.28); ANOVA  $F_{1, 414} = 16.07$ ,  $P < 0.001$ ].

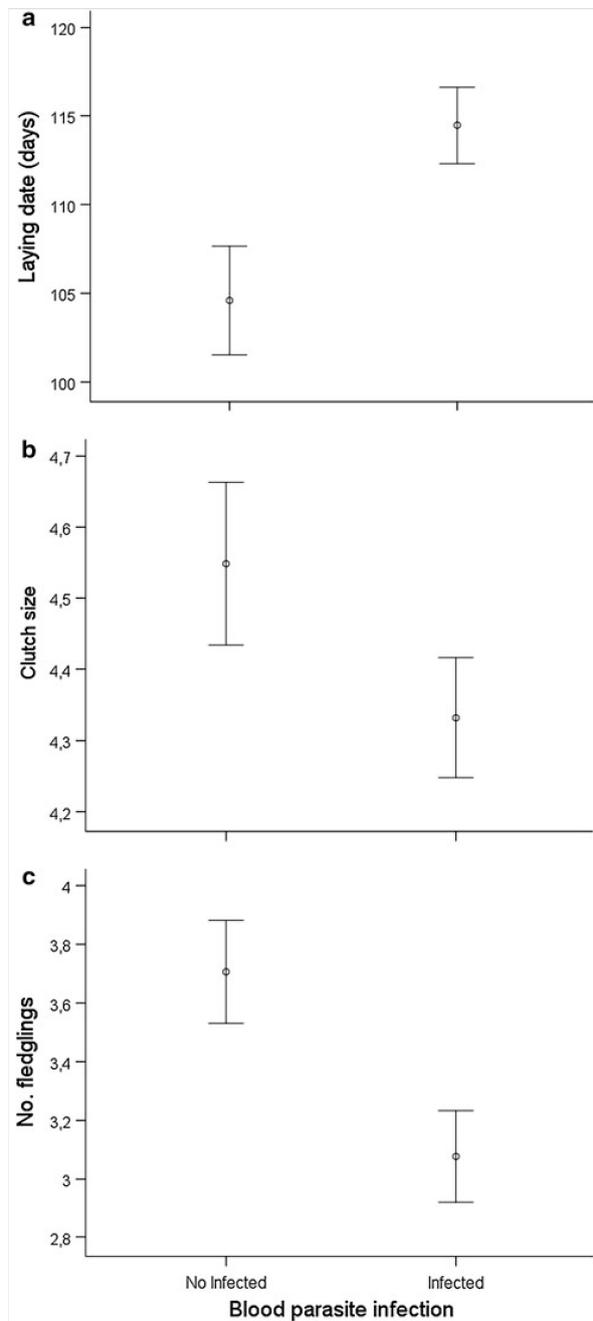
## Discussion

Here, we show for the first time an association between malaria and related haemosporidian parasites infection and the growth rate of rectrix feather of birds. Applying a ptilochronology-modified technique, the main findings of this study were that: (1) rectrix growth rate in wintering house martins was associated with higher occurrence of blood parasite infection, (2) a low rectrix growth rate and blood parasite infection was associated with a delay in laying date in their European breeding quarters, (3) clutch size and the number of reared chicks were negatively related to haemosporidian infection and a late laying date, and (4) rectrix growth rate and reproductive success varied significantly between the study years. Next, we will discuss these results in detail.

Previous studies have applied ptilochronology to estimate moult speed of both migratory and sedentary birds by measuring the width of growth bars (de la Hera et al. 2009b). Here, we used ptilochronology under field conditions to study the feather growth rate during the preceding winter to address its consequences for the reproductive performance. This non-invasive methodology requires only that individuals are captured once, and it is not limited to the moulting period. Given that a bird carries a regenerated

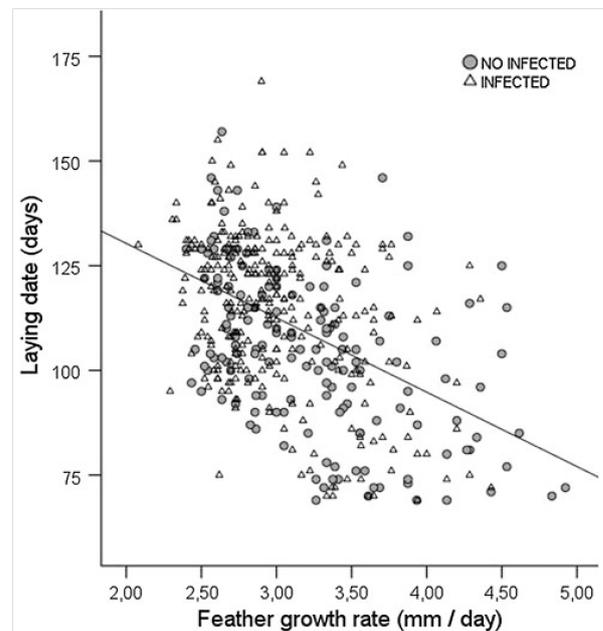
feather until the next moult, the capture and study of moult can be studied whenever and wherever the bird is capture.

Moult is an unfavourable period for birds, since it reduces flight performance (Williams and Swaddle 2003), increases the exposure to predators (Lind 2001) and increases costs of thermoregulation (Ginn and Melville 1983). We found that rate of rectrix growth was negatively related to blood parasite infection, where infected individuals had a lower daily feather elongation. To the best of our knowledge, this is the first study showing an association between blood parasite infection and the rate of feather elongation. We propose two non-mutually exclusive hypotheses to explain this observation. First, the association between feather growth rate and blood parasites infection could be a consequence of the nutrients directly consumed by the parasites. In this sense, it is known that malaria parasites obtain essential amino acids from host plasma and digestion of host haemoglobin (Sherman 1979; Martin and Kirk 2007). The renewal of the feathers is one of the most remarkable metabolic feats that birds undergo during the course of their annual cycle, involving large amounts of amino acids, the main chemical compound of feathers (Murphy et al. 1990). In haemosporidian species, the synthesis of new malaria proteins during infection requires substantial amounts of leucine, valine and isoleucine (Sherman 1977), which are three of the four most abundant essential amino acids in the composition of bird feathers (Murphy et al. 1990). In consequence, the removal of essential amino acids from the avian host by blood parasites could provoke this decrease in feather growth rate. Alternatively, these differences in daily feather elongation could be caused by the need to mount an immune response against the blood parasites. Because both avian moult and immune response are nutrient-demanding



**Fig. 2** Reproductive parameters for uninfected ( $n = 155$ ) and infected house martins ( $n = 289$ ) of **a** laying date, **b** clutch size and **c** number of fledglings. Error bars show means  $\pm$  95 % of confidence interval

processes (Murphy 1996; Martin et al. 2003; Klasing 2004), there may be a reallocation of resources and, consequently, trade-offs between physiological activities (Wikelski and Ricklefs 2001; Monaghan 2004), where the



**Fig. 3** Scatter plot showing the relationship between the feather growth rate and laying date for uninfected (open symbol,  $n = 155$ ) and infected house martins (closed symbol,  $n = 289$ )

rate of feather growth of infected house martins is decreased, given that it is necessary to assign resources to mount an immune response. In this line, Amat et al. (2007) experimentally showed that regenerated feathers of immune-challenged greenfinches *Carduelis chloris* were more asymmetric in length than regenerated feathers from control birds. Moreover, Sanz et al. (2004) showed that immunised male pied flycatchers *Ficedula hypoleuca* delayed the onset of post-nuptial primary moult compared to control males, suggesting that the activation of the immune system decreased the resources necessary for initiating post-nuptial moult. Recently, Moreno-Rueda (2010) showed in male house sparrows *Passer domesticus* that the stimulation of the immune system with an antigen affected the moult by reducing its speed to half, showing a trade-off between moulting and the immune response. Further experimental investigations are needed to demonstrate conclusively the detrimental effect of haemosporidian infection on the feather growth rate of birds.

A 'seasonal interaction' can occur if events and conditions in one region or season affect populations and individuals in another (Webster and Marra 2005). The pattern of feather moult during winter may affect departure schedules from the wintering grounds, and thus migratory dates. Because most African moulting species (i.e. house martin) do not start the northward migration until the moult is complete (Cramp 1988; Møller et al. 2011; Turner and Rose 1989), a lower speed in feather growth could lead to a

**Table 3** Factors explaining variation in clutch size of house martins

Independent variable	Square-sum III	df	F	P
Prevalence	2.818	1	5.742	0.017
Year	19.718	10	4.018	<0.001
Laying date	7.988	1	16.278	<0.001

A backward stepwise procedure was used in a GLM analysis with prevalence of blood parasite, sex, year, body mass, haematocrit, number of chewing lice, laying date and feather growth rate as predictor variables. Only independent variables selected by the backward stepwise procedure are listed. Sample size was 439 individuals

**Table 4** Factors explaining variation in the number of fledglings produced by house martins

Independent variable	Square-sum III	df	F	P
Prevalence	34.277	1	26.370	<0.001
Year	55.239	10	4.250	<0.001
Laying date	15.221	1	11.710	0.001

A backward stepwise procedure was used in a GLM analysis with prevalence of blood parasite, sex, year, body mass, haematocrit, number of chewing lice, laying date and feather growth rate as predictor variables. Only independent variables selected by the backward stepwise procedure are listed. Sample size was 410 individuals

delay in timing of migration (Marra et al. 1998) and, consequently, a late arrival and start of reproduction in Europe (Saino et al. 2004). We found that the rate of feather growth and blood parasite infection in Africa was associated with poorer breeding performance of house martins in their European breeding quarters. In agreement with our predictions, individuals moulting their outermost tail feathers more quickly advanced their laying date in their breeding areas. On the other hand, haemosporidian-infected birds laid their clutches later in the breeding season. Given that blood parasite infections decrease body condition of birds (Valkiūnas 2005; Palinauskas et al. 2008), and may slow down their feather replacement, only uninfected house martins could moult their feather quickly and advance their arrival at their breeding grounds. Similar results were reported by Møller et al. (2004) in migratory barn swallows, where those infected with blood parasites delayed their arrival at Spanish breeding quarters.

These carry-over effects should lead to variations in reproductive success, because birds tend to show declining prospects of reproductive success as the breeding season advances (Pajuelo et al. 1992; Verhulst et al. 1995; Hansson et al. 2000; Arnold et al. 2004). For instance, Brown and Brown (1999) reported a decline in clutch size and annual reproductive success across the season in cliff swallows *Petrochelidon pyrrhonota*. In the same line, Sakraoui et al. (2005) showed a clear negative relation between clutch size and laying date in barn swallows. These observations are in agreement with our findings, since the delay in laying date was related to a decrease in clutch size and in the number of nestlings produced. We also found that blood parasite infection was associated with a decrease in clutch size and a reduced number of fledged

chicks. Previous experimental studies on passerine birds reported similar detrimental effects of haematozoan parasites on breeding performance, negatively affecting brood size and breeding success (Merino et al. 2000; Marzal et al. 2005). In addition, the production of an immune response to fight against pathogens could be costly for house martins in terms of delay in egg laying and smaller brood sizes (Marzal et al. 2007).

Finally, we reported an inter-annual variation in rectrix growth rate, clutch size and the number of reared young. It is well established that variation in environmental factors can shape the evolution of life histories (Partridge and Harvey 1988). Previous studies have also shown between-year variation in estimated moult duration (Bensch and Grahm 1993; Smith and Sheeley 1993), while other studies have reported that moult speed did not vary across years (Borras et al. 2004). On the other hand, inter-annual variation in breeding success has also been reported in wild birds (Pajuelo et al. 1992; Cézilly et al. 1995; Forbes 2010). Such differences could be explained by annual variations in environmental factors such as climate or food availability that can affect moult phenology and/or key reproductive traits of birds (Bryant 1975; Murphy 1986; Smith and Sheeley 1993; Renfrew et al. 2011).

In conclusion, the results obtained in the present study reveal a negative association between haemosporidian parasite infection and the feather growth rate of house martins. Moreover, our findings also suggest carry-over effects between African winter quarters and European breeding sites, where timing of reproduction and reproductive success were negatively related to the rectrix growth rate and infection of blood parasites. It would be desirable that further studies would take into account blood

parasite infection and feather growth rate as potentially mechanisms driving carry-over effects between winter quarters and breeding areas to explain fitness differences in wild populations of migratory birds.

**Acknowledgments** We thank to W. Douglas Robinson and two anonymous referees for their constructive comments on the manuscript. This study was funded by research projects of the Spanish Ministry of Education and Science (CGL2009-08976), Junta of Extremadura (PRI08A116) and University of Extremadura (A7-26). A.M., I.G.H. and L.G.L. were supported by grants from Spanish Ministry of Education and Science (JC2011-0405, BES-2007-15549 and BES-2010-030295, respectively). S.B. was supported by a grant from the Swedish Research Council (621-2007-5193). L.R. was supported by a collaboration grant of UEx. All the experiments comply with the current laws of Spain, where the experiments were performed.

**Conflict of interest** The authors declare that they have no conflict of interest.

## References

- Abramoff MD, Magelhaes PJ, Ram SJ (2004) Image processing with image. *J Biophotonics Int* 11:36–42
- Allander K, Sundberg J (1997) Temporal variation and reliability of blood parasite levels in captive yellowhammer males *Emberiza citrinella*. *J Avian Biol* 28:325–330
- Amat JA, Aguilera E, Visser GH (2007) Energetic and developmental costs of mounting an immune response in greenfinches (*Carduelis chloris*). *Ecol Res* 22:282–287
- Arnold JM, Hatch JJ, Nisbet ICT (2004) Seasonal declines in reproductive success of the common tern *Sterna hirundo*: timing or parental quality? *J Avian Biol* 35:33–45
- Balbontín J, Hermosell IG, Marzal A, Reviriego M, de Lope F, Møller AP (2007) Age-related change in breeding performance in early life is associated with an increase in competence in the migratory barn swallow (*Hirundo rustica*). *J Anim Ecol* 76:915–925
- Balbontín J, Møller AP, Hermosell IG, Marzal A, Reviriego M, de Lope F (2009) Divergent patterns of impact of environmental conditions on life history traits in two populations of a long-distance migratory bird. *Oecologia* 159:859–872
- Bensch S, Grahn M (1993) A new method for estimating individual speed of moult. *Condor* 95:305–315
- Bensch S, Stjernman M, Hasselquist D, Östman Ö, Hansson B, Westerdahl H, Pinheiro RT (2000) Host specificity in avian blood parasites: A study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. *Proc R Soc Lond B* 267:1583–1589
- Bergman G (1982) Why are the wings of *Larus f. fuscus* so dark? *Ornis Fenn* 59:77–83
- Borras A, Cabrera T, Cabrera J, Senar JC (2004) Inter-locality variation in speed of moult in the citril finch *Serinus citrinella*. *Ibis* 146:14–17
- Brodin A (1993) Radio-ptilochronology tracing radioactively labelled food in feathers. *Ornis Scand* 24:167–173
- Brown CR, Brown MB (1999) Fitness components associated with laying date in the cliff swallow. *Condor* 101:230–245
- Bryant DM (1975) Breeding biology of the house martin, *Delichon urbica* in relation to aerial insect abundance. *Ibis* 117:180–221
- Burt EH Jr, Ichida JM (1999) Occurrence of feather-degrading bacilli in the plumage of birds. *Auk* 116:364–372
- Butler M, Johnson AS (2004) Are melanized feather barbs stronger? *J Exp Biol* 207:285–293
- Cézilly F, Boy V, Green RE, Hiron GH, Johnson AR (1995) Interannual variation in greater flamingo breeding success in relation to water levels. *Ecology* 76:20–26
- Christe P, de Lope F, González G, Saino N, Møller AP (2001) The influence of environmental conditions on immune responses, morphology and recapture probability of nestling house martins (*Delichon urbica*). *Oecologia* 126:333–338
- Christe P, Møller AP, González G, de Lope F (2002) Intra-seasonal variation in immune defence, body mass and hematocrit in adult house martin *Delichon urbica*. *J Avian Biol* 33:321–325
- Cramp S (1988) The birds of the western palearctic, vol 5. Oxford University Press, Oxford
- Dawson A, Hinsley SA, Ferns PN, Bonser RHC, Eccleston L (2000) Rate of moult affects feather quality: a mechanism linking current reproductive effort to future survival. *Proc R Soc Lond B* 267:2093–2098
- De la Hera I, Díaz JA, Pérez-Tris J, Tellería JL (2009a) A comparative study of migratory behaviour and body mass as determinants of moult duration in passerines. *J Avian Biol* 40:461–465
- De la Hera I, Pérez-Tris J, Tellería JL (2009b) Migratory behaviour affects the trade-off between feather growth rate and feather quality in a passerine bird. *Biol J Linn Soc* 97:98–105
- Forbes S (2010) Family structure and variation in reproductive success in blackbirds. *Behav Ecol Sociobiol* 64:475–483
- Gauthreaux JSA (1982) The ecology and evolution of avian migration systems. In: Farner DS, King JR, Parker KC (eds) *Avian biology*. Academic, New York, pp 93–163
- Ginn HB, Melville DS (1983) Moulting in birds (BTO Guide 19). British Trust for Ornithology, Hertfordshire
- Greenberg R, Marra PP (2005) *Birds of two worlds; the ecology and evolution of migration*. Johns Hopkins University Press, Portland
- Grubbs TC Jr (2006) *Ptilochronology: feather time and the biology of birds*. Oxford University Press, New York
- Hansson B, Bensch S, Hasselquist D (2000) The quality and the timing hypotheses evaluated using data on great reed warblers. *Oikos* 90:575–581
- Harrison XA, Blount JD, Inger R, Norris DR, Bearhop S (2011) Carry-over effects as drivers of fitness differences in animals. *J Anim Ecol* 80:4–18
- Hurlbert SH (1984) Pseudo-replication and the design of ecological field experiments. *Ecol Monogr* 54:187–211
- Jenni L, Winkler R (1994) Moulting and ageing of European passerines. Academic, London
- Klaassen M (1995) Moulting and basal metabolic costs in males of two subspecies of stonechats: the European *Saxicola torquata rubicula* and the east African *S. t. axillaris*. *Oecologia* 104:424–432
- Klasing KC (2004) The cost of immunity. *Acta Zool Sin* 50:961–969
- Lack D (1968) *Ecological adaptations for breeding in birds*. Methuen, London
- Langston NE, Hillgarth N (1995) The extent of primary molt varies with parasites in Laysan albatrosses: a possible role in life history tradeoffs between current and future reproduction. *Proc R Soc Lond B* 261: 239–243
- Lin LI-K (2000) A note on the concordance correlation coefficient. *Biometrics* 56:324–325
- Lind J (2001) Escape flight in moulting tree sparrows (*Passer montanus*). *Funct Ecol* 15:29–35
- Marra PP, Hobson KA, Holmes RT (1998) Linking winter and summer events in a migratory bird by using stable-carbon isotopes. *Science* 282:1884–1886
- Martin RE, Kirk K (2007) Transport of the essential nutrient isoleucine in human erythrocytes infected with the malaria parasite *Plasmodium falciparum*. *Blood* 109:2217–2224

- Martin LB II, Scheuerlein A, Wikelski M (2003) Immune activity elevates energy expenditure of house sparrows: a link between direct and indirect costs? *Proc R Soc Lond B* 270:153–158
- Marzal A, de Lope F, Navarro C, Møller AP (2005) Malarial parasites decrease reproductive success: an experimental study in a passerine bird. *Oecologia* 142:541–545
- Marzal A, Reviriego M, de Lope F, Møller AP (2007) Fitness costs of an immune response in the house martin (*Delichon urbica*). *Behav Ecol Sociobiol* 61:1573–1580
- Marzal A, Bensch S, Reviriego M, Balbontin J, de Lope F (2008) Effects of malaria double infection in birds: one plus one is not two. *J Evol Biol* 21:979–987
- Merino S, Moreno J, Sanz JJ, Arriero E (2000) Are avian blood parasites pathogenic in the wild? A medication experiment in blue tits (*Parus caeruleus*). *Proc R Soc Lond B* 267:2507–2510
- Michener H, Michener JR (1938) Bars in flight feathers. *Condor* 40:149–160
- Møller AP, de Lope F, Saino N (2004) Parasitism, immunity, and arrival date in a migratory bird, the barn swallow. *Ecology* 85:206–219
- Møller AP, Nuttall R, Piper SE, Szép T, Vickers EJ (2011) Migration, moult and climate change in barn swallows *Hirundo rustica* in South Africa. *Clim Res* 47:201–205
- Monaghan P (2004) Resource allocation and life history strategies in birds. *Acta Zool Sin* 50:942–947
- Morales J, Moreno J, Merino S, Sanz JJ, Tomás G, Lobato E, Martínez-de la Puente J (2007) Early moult improves local survival and reduces reproductive output in female pied flycatcher. *Ecoscience* 14:31–39
- Moreno-Rueda G (2010) Experimental test of a trade-off between moult and immune response in house sparrows *Passer domesticus*. *J Evol Biol* 23:2229–2237
- Murphy MT (1986) Temporal components of reproductive variability in eastern kingbirds (*Tyrannus tyrannus*). *Ecology* 67:1483–1492
- Murphy ME (1996) Energetics and nutrition of molt. In: Carey C (ed) *Avian energetics and nutritional ecology*. Plenum, New York, pp 158–198
- Murphy ME, Taruscio TG (1995) Sparrows increase their rates of tissue and whole-body protein synthesis during the annual molt. *Comp Biochem Physiol A Mol Integr Physiol* 111:385–396
- Murphy ME, King JR, Taruscio TG, Geupel GR (1990) Amino acid composition of feather barbs and rachises in three species of pygoscelid penguins: nutritional implication. *Condor* 92:913–921
- Nava MP, Veiga JP, Puerta M (2001) White blood cell counts in house sparrows (*Passer domesticus*) before and after moult and after testosterone treatment. *Can J Zool* 79:145–148
- Newton I (2008) *The migration ecology of birds*. Academic, London
- Nilsson JÅ, Svensson E (1996) The cost of reproduction: a new link between current reproductive effort and future reproductive success. *Proc R Soc Lond B* 263:711–714
- Norris DR, Marra PP, Kyser TK, Sherry TW, Ratcliffe LM (2004) Tropical winter habitat limits reproductive success on the temperate breeding grounds in a migratory bird. *Proc R Soc Lond B* 271:59–64
- Pajuelo L, de Lope F, da Silva E (1992) Biología de la reproducción del avión común (*Delichon urbica*) en Badajoz, España. *Ardeola* 39:15–23
- Palinauskas V, Valkiunas G, Bensch S, Bolshakov VC (2008) Effects of *Plasmodium relictum* (lineage P-SGS1) on experimentally infected passerine birds. *Exp Parasitol* 120:372–380
- Partridge L, Harvey PH (1988) The ecological context of life history evolution. *Science* 241:1449–1455
- Renfrew RB, Frey SJK, Klavins J (2011) Phenology and sequence of the complete prealternate molt of bobolinks in South America. *J Field Ornithol* 82:101–113
- Riddle O (1908) The genesis of fault-bars in feathers and the cause of alternation of light and dark fundamental bars. *Biol Bull* 14:328–370
- Rohwer S, Viggiano A, Marzluff JM (2011) Reciprocal tradeoffs between molt and breeding in Albatrosses. *Condor* 113:61–71
- Saino N, Szép T, Romano M, Rubolini D, Spina F, Møller AP (2004) Ecological conditions during winter predict arrival date at the breeding quarters in a trans-saharan migratory bird. *Ecol Lett* 7:21–25
- Sakraoui R, Dacqi W, Bañbura J, Chabi y (2005) Breeding biology of barn swallows *Hirundo rustica* in Algeria, North Africa. *Ornis Fenn* 82:33–43
- Sambrook J, Fritsch FJ, Maniatis T (2002) *Molecular cloning, a laboratory manual*. Cold Spring Harbor Laboratory Press, New York
- Sanz JJ, Moreno J, Merino S, Tomás G (2004) A trade-off between two resource-demanding functions: post-nuptial moult and immunity during reproduction in male pied flycatcher. *J Anim Ecol* 73:441–447
- Serra L (2001) Duration of primary moult affects primary quality in grey plovers *Pluvialis squatarola*. *J Avian Biol* 32:377–380
- Shawkey MD, Beck ML, Hill GE (2003) Use of a gel documentation system to measure feather growth bars. *J Field Ornithol* 74:125–128
- Sherman IW (1977) Amino acid metabolism and protein synthesis in malarial parasites. *Bull World Health Org* 55:265–276
- Sherman IW (1979) Biochemistry of *Plasmodium* (malarial parasites). *Microbiol Rev* 43:453–495
- Smith L, Sheeley D (1993) Molt patterns of wintering northern pintails in the southern high plains. *J Wildl Manag* 57:229–238
- Tarello W (2007) Clinical signs and response to primaquine in falcons with *Haemoproteus tinnunculi* infection. *Vet Rec* 161:204–205
- Turner AK, Rose C (1989) *A handbook to the swallows and martins of the world*. Christopher Helm, London
- Valkiunas G (2005) *Avian malaria parasites and other haemosporidia*. CRC, Boca Raton
- Verhulst S, Balen JHV, Tinbergen JM (1995) Seasonal decline in reproductive success of the great tit: variation in time or quality? *Ecology* 76:2392–2403
- Waldenström J, Hasselquist D, Östman Ö, Bensch S (2004) A new nested PCR method very efficient in detecting *Plasmodium* and *Haemoproteus* infections from avian blood. *J Parasitol* 90:191–194
- Webster MS, Marra PP (2005) Importance of understanding migratory connectivity In: Greenberg Russell S. *Birds of two worlds: ecology and evolution of migration*, Johns Hopkins University Press, Baltimore, pp 199–209
- Webster MS, Marra PP, Haig SM, Bensch S, Holmes RT (2002) Links between worlds: unraveling migratory connectivity. *Trends Ecol Evol* 17:76–83
- Wikelski M, Ricklefs R (2001) The physiology of life-histories. *Trends Ecol Evol* 16:479–481
- Williams EV, Swaddle JP (2003) Molt, flight performance, and wingbeat frequency during take-off in european starlings. *J Avian Biol* 34:371–378
- Yuri T, Rohwer S (1997) Molt and migration in the northern rough-winged swallow. *Auk* 114:249–262
- Zar JH (1996) *Biostatistical analysis*, 3rd edn. Prentice Hall, Upper Saddle River

## Variation in sperm morphometry and sperm competition among barn swallow (*Hirundo rustica*) populations

Terje Laskemoen · Tomas Albrecht · Andrea Bonisoli-Alquati · Jaroslav Cepak · Florentino de Lope · Ignacio G. Hermosell · Lars Erik Johannessen · Oddmund Kleven · Alfonso Marzal · Timothy A. Mousseau · Anders P. Møller · Raleigh J. Robertson · Geir Rudolfson · Nicola Saino · Yoni Vortman · Jan T. Liffeld

Received: 7 September 2012 / Revised: 2 November 2012 / Accepted: 7 November 2012  
© Springer-Verlag Berlin Heidelberg 2012

**Abstract** Spermatozoa vary greatly in size and shape among species across the animal kingdom. Postcopulatory sexual selection is thought to be the major evolutionary force driving this diversity. In contrast, less is known about how sperm size

varies among populations of the same species. Here, we investigate geographic variation in sperm size in barn swallows *Hirundo rustica*, a socially monogamous passerine with a wide Holarctic breeding distribution. We included samples

Communicated by S. Pruett-Jones

**Electronic supplementary material** The online version of this article (doi:10.1007/s00265-012-1450-0) contains supplementary material, which is available to authorized users.

T. Laskemoen (✉) · L. E. Johannessen · O. Kleven · J. T. Liffeld  
Natural History Museum, University of Oslo,  
P.O. Box 1172, Blindern, 0318 Oslo, Norway  
e-mail: terje.laskemoen@nhm.uio.no

T. Albrecht  
Institute of Vertebrate Biology, v.v.i., Academy of Sciences of the  
Czech Republic, Brno, Czech Republic

T. Albrecht  
Department of Zoology, Faculty of Science, Charles University in  
Prague, Prague, Czech Republic

A. Bonisoli-Alquati · T. A. Mousseau  
Department of Biological Sciences, University of South Carolina,  
Columbia, SC, 29208, USA

J. Cepak  
Bird-ringing Station, National Museum, Prague,  
Czech Republic

F. de Lope · I. G. Hermosell · A. Marzal  
Departamento de Anatomía, Biología Celular y Zoología,  
Universidad de Extremadura, Badajoz, Spain

O. Kleven  
Norwegian Institute for Nature Research-NINA,  
P. O. Box 5685, Sluppen, 7485 Trondheim, Norway

A. P. Møller  
Laboratoire d'Ecologie, Systématique et Evolution, CNRS UMR  
8079, Université Paris-Sud,  
Orsay Cedex, France

R. J. Robertson  
Department of Biology, Queen's University, Kingston,  
Ontario, K7L 3N6, Canada

G. Rudolfson  
Department for Environmental Radioactivity, Norwegian  
Radiation Protection Authority, Fram Center,  
9296 Tromsø, Norway

N. Saino  
Dipartimento di Biologia, Università degli Studi di Milano,  
via Celoria 26,  
20133 Milano, Italy

Y. Vortman  
Department of Zoology, Faculty of Life Sciences,  
Tel-Aviv University,  
Ramat Aviv, Tel-Aviv, 69978, Israel

from seven populations and three subspecies: five populations of ssp. *rustica* in Europe (Czech, Italy, Norway, Spain, and Ukraine), one population of ssp. *transitiva* in Israel, and one population of ssp. *erythrogaster* in Canada. All sperm traits (head length, midpiece length, tail length, and total length) varied significantly among populations. The variation among the European *rustica* populations was much lower than the differences among subspecies, indicating that sperm traits reflect phylogenetic distance. We also performed a test of the relationship between the coefficient of between-male variation in total sperm length and extrapair paternity levels across different populations within a species. Recent studies have found a strong negative relationship between sperm size variation and extrapair paternity among species. Here, we show a similar negative relationship among six barn swallow populations, which suggests that the variance in male sperm length in a population is shaped by the strength of stabilizing postcopulatory sexual selection.

**Keywords** Barn swallow · Extrapair paternity · *Hirundo rustica* · Sperm competition · Sperm size

## Introduction

Spermatozoa show enormous variation in size and shape across animal taxa (e.g., Cohen 1977). The evolutionary forces shaping this variation have received increasing attention but are not fully understood (Birkhead et al. 2009). Polyandry, i.e., females mating with more than one male, is common across the animal kingdom, and this constitutes a potentially powerful source of postcopulatory sexual selection on sperm traits, either through sperm competition (Parker 1970) or cryptic female choice (Eberhard 1996).

It is well documented that spermatozoa vary considerably in size both within (e.g., Ward 1998; Morrow and Gage 2001b; Laskemoen et al. 2007) and among species (e.g., Gage 1998; Calhim et al. 2007; Kleven et al. 2008; Lüpold et al. 2009). Although geographical variation in sperm size within species has been studied in a range of species, the patterns are complex and not clear. For example, in guppies (*Poecilia reticulata*), Elgee et al. (2010) found that males from populations with high risk of predation had faster swimming sperm and sperm with longer midpieces than males from populations with lower risk of predation. However, total sperm length did not differ between guppy populations (Elgee et al. 2010). Studies of several drosophilid flies have found significant variation in sperm size among populations (Snook 2001; Pitnick et al. 2003; Joly et al. 2004). Yellow dung flies (*Scatophaga stercoraria*) have been shown to have longer sperm at high temperature when raised experimentally in laboratory conditions (Blanckenhorn and Hellriegel 2002). However, another study of the same

species did not find differences in sperm length in three natural populations (Hosken et al. 2003). A comparative study of sperm morphology of rhacophorid frogs included samples of the species (*Chirixalus eiffingeri*) from both Japan and Taiwan and documented significant differences in sperm size between these two populations (Kuramoto 1996). A study of quacking frogs (*Crinia georgiana*) documented significant variation in sperm size and relative number of sperm among four populations (Hettyey and Roberts 2006). The sea urchin (*Strongylocentrotus droebachiensis*) was found to have high diversity in sperm traits, except total sperm length, among three different populations (Manier and Palumbi 2008). Minoretti and Baur (2006) documented significant differences in sperm size among four populations of the land snail (*Arianta arbustorum*). In birds, two recent studies have investigated geographic variation in sperm size, both documenting significant differences among populations (Lüpold et al. 2011; Schmoll and Kleven 2011). In red-winged blackbirds (*Agelaius phoeniceus*), sperm morphology varied significantly among the 17 study sites in continental United States (Lüpold et al. 2011). Lüpold et al. (2011) also found a gradual increase in sperm size from southwest to northeast of the breeding range, and further, a negative relationship between sperm length and body size. Schmoll and Kleven (2011) investigated variation in sperm size within and between two populations of coal tits (*Periparus ater*) and documented significant variation in total sperm length and sperm head length between the two populations. These two species are characterized by little or moderate gene flow, which could help explain geographical differences in sperm traits with high heritability. A call is thus made for investigation of variation in sperm size among populations in species with a higher degree of gene flow among populations.

Recently, comparative studies of passerine birds have shown that the intraspecific variation in total sperm length and other sperm components are negatively associated with the risk of sperm competition (Calhim et al. 2007; Immler et al. 2008; Kleven et al. 2008; Lifjeld et al. 2010). This can be interpreted as evidence of stronger stabilizing selection on males producing an optimal sperm type when the risk of sperm competition increases. However, this relationship has not been investigated among populations of the same species. It is well known that levels of extrapair paternity vary among populations (Petrie and Kempenaers 1998), and in this study, we ask if this variation amongst populations in sperm competition could also be reflected in between-male variation in sperm size.

The barn swallow (*Hirundo rustica*) is a socially monogamous passerine with a broad Holarctic breeding distribution (Møller 1994b; Turner 2006). The barn swallow has been thoroughly studied over the last decades and is considered a model species for studies on sexual selection, mate choice, and sperm competition (e.g., Møller 1988; Møller

1994a; Møller 1994b; Saino et al. 1997; Safran et al. 2005; Kleven et al. 2006; Lifjeld et al. 2011). Currently, six different subspecies of barn swallows are recognized (Turner 2006; Dor et al. 2010). However, some of these subspecies are geographically isolated, preventing gene flow (Turner 2006). A recent phylogeny of barn swallows and other *Hirundo* species found that the European *H. rustica rustica* and East-Mediterranean barn swallows *H. rustica transitiva* cluster together, as do the Asian and American barn swallows (Dor et al. 2010). Dor et al. (2010) also reported pairwise molecular distances within and between these clades, showing low distance within the European-Mediterranean clade and substantially greater distance between the Asian-American and the European-Mediterranean clades. Furthermore, there is evidence from barcoding of the mitochondrial cytochrome c oxidase I (CO1) gene showing that the North American barn swallow *H. rustica erythrogaster* differs almost 2 % in genetic distance from the European barn swallow (Johnsen et al. 2010).

Here, we investigate geographic variation in sperm morphometry in barn swallows. We examined sperm morphometry in seven different barn swallow populations representing three subspecies: five populations of European (Czech, Italy, Norway, Spain and Ukraine), one population of East-Mediterranean (Israel), and one North American population (Canada). Based on the prior knowledge of genetic variation (Dor et al. 2010; Johnsen et al. 2010), we predicted that European and Mediterranean barn swallows would exhibit more similar sperm morphometry when compared to North American barn swallows. We also tested the hypothesis that sperm size variation is negatively related to the risk of sperm competition across populations.

## Methods

### Field procedures

We captured and sampled male barn swallows from seven different populations; Czech Republic (2009), Italy (2011), Norway (2008 and 2009), Spain (2010 and 2011), Ukraine (2010), Israel (2010 and 2011), and Canada (2006). See supplementary Table S1 for detailed sampling location information. Notably, European barn swallows migrate through the breeding areas of East-Mediterranean barn swallows in Israel, which could potentially cause sampling errors in this area. However, all birds sampled in Israel were earlier banded and belonged to a monitored population of East-Mediterranean barn swallows. To prevent pseudo-replication of already sampled birds, all unbanded birds were banded with unique aluminium bands. We obtained ejaculate samples either by gently massaging the males' cloacal protuberance following a modified method from Wolfson (1952) or from fecal samples

following Immler and Birkhead (2005). The ejaculate or fecal sample was immediately fixed in a 5 % formalin solution and stored until slide preparation. After sampling, all birds were released in the immediate vicinity of their respective colonies.

### Sperm morphometry

For each sperm sample, a small aliquot of approximately 15  $\mu\text{l}$  was applied on a microscope slide, allowed to air-dry, and subsequently gently rinsed with distilled water and air-dried again. We measured the head, midpiece, and tail ( $\pm 0.1 \mu\text{m}$ ) of ten intact spermatozoa per male. Measuring ten sperms per male has been shown to give representative estimations of an individual's mean sperm length (Laskemoen et al. 2007). Total length is the sum of all three separate sperm traits. For one individual, we measured the same ten sperms twice to establish the repeatability of our measurements, following Lessells and Boag (1987). The measurements were highly repeatable (head:  $F_{9,10}=6.83$ ,  $P=0.003$ ,  $r=0.74$ ; midpiece:  $F_{9,10}=114.0$ ,  $P<0.001$ ,  $r=0.98$ ; tail:  $F_{9,10}=105.8$ ,  $P<0.001$ ,  $r=0.98$ ). For the samples of North American barn swallows, we used a Zeiss AxioCam HRc camera mounted on a Zeiss Axioplan 2 light microscope to obtain digital images of spermatozoa at a magnification of 200 $\times$ . Further, the morphometric measurements were conducted using the software Zeiss AxioVision 4.1 (© Carl Zeiss Vision GmbH, Germany). For all other samples, we used a Leica DFC420 camera mounted on a Leica DM6000 B digital light microscope to obtain digital images at magnifications of 200 $\times$ . The morphometric measurements were conducted using Leica Application Suite (version 2.6.0 R1). The use of two different microscope setups could potentially cause artificial differences. In order to establish whether the different microscope systems gave different results, we measured ten randomly chosen individuals of the North American barn swallows on the Leica microscope system as well. No significant differences were observed (average head length, 14.3 vs. 14.3  $\mu\text{m}$ ; average midpiece length, 59.8 vs. 59.9  $\mu\text{m}$ ; average tail length, 14.4 vs. 14.2  $\mu\text{m}$ , Zeiss vs. Leica systems, respectively; paired  $t$  tests: all  $t<0.57$ , all  $P>0.58$ ). To avoid observer effects, one person (TL) conducted all morphometric measurements. In an earlier study of sperm characteristics of Ukrainian barn swallows, Møller et al. (2008) found differences in both sperm size and proportion of abnormal sperm between birds sampled near the Chernobyl area and those sampled at control areas southwest of Chernobyl. Therefore, we tested for differences in sperm traits between the four Ukrainian localities in the present dataset. We found no significant differences in any of the measured sperm traits (ANOVA, all  $F_{3,21}<1.46$ , all  $P>0.25$ ). Hence, we pooled all individuals from Ukraine as one population. Notably, the Ukrainian samples included in the present study are not the same as in Møller et al. (2008).

## Extrapair paternity data

We extracted data on extrapair paternity from five populations from the literature (Canada, Italy, Israel, Spain, and Ukraine). In addition, we obtained unpublished data on extrapair paternity from the Czech population (T. Albrecht, J. Kreisinger, and R. Michalkova unpublished data). Notably, all extrapair paternity data originate from the same populations from which we obtained sperm samples. A summary of paternity data and sample sizes are presented in Table 1.

## Statistical analyses

We included all sperm measurements, i.e., ten sperms per male, and used General Linear Mixed Models (GLMM) with population as fixed factor and individual as random factor in our tests for differences in sperm traits among populations. For the traits that showed significant variation among populations, we estimated marginal means and conducted all possible pairwise comparisons, Bonferroni-adjusted for multiple comparisons. We calculated the coefficient of variation in sperm length between males ( $CV_{bm}$ ) for all populations using the formula:  $CV_{bm} = SD/mean \times 100$ . Furthermore, as the coefficient of variation for small sample sizes tends to be underestimated, we applied the formula recommended by Sokal and Rohlf (1995):  $CV_{adj} = CV \times (1 + (1/4n))$ . Hence, all  $CV_{bm}$  values reported here are adjusted for sample size. Statistical tests were conducted using Statistica v7.1 (StatSoft Inc), SPSS v19.0.0 (SPSS Inc.), and figures were drawn using Origin v7.0300 (OriginLab Corporation).

## Results

## Sperm morphometry

Sperm head length, midpiece length, and total length differed significantly among populations (GLMMs: head

length:  $F_{6, 183} = 17.4$ ,  $P < 0.001$ ; midpiece length:  $F_{6, 183} = 15.9$ ,  $P < 0.001$ ; total length:  $F_{6, 183} = 10.3$ ,  $P < 0.001$ ). Thus, for these traits, we estimated marginal means and pairwise comparisons to reveal which populations differed (Table S2–S4). Descriptive statistics of sperm morphometry from all seven populations are presented in Table 2. Sperm tail length did not differ significantly among populations ( $F_{6, 183} = 2.0$ ,  $P = 0.07$ ). Generally, the North American barn swallows differed most from the other populations in sperm morphometry, with longer sperm heads than individuals from all the other populations and shorter midpieces than all but the Italian and East-Mediterranean populations (Fig. 1a, b; Table S2, S3). Considering total sperm length, North American barn swallows generally had shorter sperm than the European populations but did not differ from the population of East-Mediterranean barn swallows (Fig. 1c; Table S4).

Two of our populations (Norway and Spain) included samples from two different seasons, and thus we tested for potential year-effects on sperm size. However, we found no significant effect of sample year on any of the sperm traits measured (Norway: all  $F_{1,24} < 0.69$ ,  $P > 0.42$ ; Spain: all  $F_{1,11} < 2.48$ , all  $P > 0.14$ ).

## Among-male variation in sperm size and extrapair paternity levels

The populations showed somewhat different values of among male variation in sperm size ( $CV_{bm}$ ) (Table 2). When examining the relationship between  $CV_{bm}$  and percentage broods containing one or more extrapair young (EPB), we found a significant negative relationship (Pearson's:  $r = -0.96$ ,  $N = 6$ ,  $P = 0.003$ ) (Fig. 2a). The relationship between  $CV_{bm}$  and percentage extrapair young (EPY) also went in the predicted negative direction, although this was not statistically significant (Pearson's:  $r = -0.62$ ,  $N = 6$ ,  $P = 0.19$ ) (Fig. 2b). Hence, both tests were in the predicted direction with decreasing  $CV_{bm}$

**Table 1** Summary of extrapair paternity data from six populations of barn swallows *Hirundo rustica* used in the present study

Country	EPY (95 % CI) <sup>a</sup>	EPB <sup>b</sup>	N young/N broods	Source
Czech	23.1 (15.2–31.1)	60.0	108/25	(T. Albrecht, J. Kreisinger and R. Michalkova unpublished data)
Italy	29.0 (22.9–35.1)	52.0	214/52	(Saino et al. 1999)
Spain	17.8 (14.9–20.7)	32.4	674/170	(Møller et al. 2003)
Ukraine	30.0 (20.0–40.0)	50.0	80/18	(Ellegren et al. 1997)
Israel	15.3 (9.7–20.9)	43.9	161/41	(Vortman et al. 2011)
Canada	28.8 (25.9–31.7)	48.1	917/210	(Kleven et al. 2005)

<sup>a</sup> Percentage extrapair young with the 95 % lower and upper confidence limits of the estimate indicated

<sup>b</sup> Percentage broods containing one or more extrapair young

**Table 2** Descriptive statistics of sperm morphometry in seven populations of barn swallows *Hirundo rustica* (see supplementary Table S2–S4 for pairwise comparisons between all populations)

	Head length ( $\mu\text{m}$ )		Midpiece length ( $\mu\text{m}$ )		Tail length ( $\mu\text{m}$ )		Total length ( $\mu\text{m}$ )		CV <sub>bm</sub> <sup>a</sup>	CV <sub>wm</sub> <sup>b</sup>
	Mean $\pm$ SD	Range	Mean $\pm$ SD	Range	Mean $\pm$ SD	Range	Mean $\pm$ SD	Range		
Czech ( $n=20$ )	13.1 $\pm$ 0.5	11.7–13.9	62.2 $\pm$ 2.8	57.3–66.9	14.7 $\pm$ 2.2	9.7–18.7	89.9 $\pm$ 2.1	86.4–94.2	2.34	1.52
Italy ( $n=8$ )	13.0 $\pm$ 0.3	12.6–13.4	64.4 $\pm$ 1.3	62.9–66.4	13.0 $\pm$ 2.6	10.2–18.7	90.3 $\pm$ 2.2	88.0–94.8	2.54	1.65
Norway ( $n=26$ )	13.2 $\pm$ 0.5	12.1–14.2	61.6 $\pm$ 3.1	54.2–65.9	15.8 $\pm$ 3.4	11.6–25.6	90.6 $\pm$ 2.4	86.4–94.6	2.63	1.67
Spain ( $n=13$ )	13.4 $\pm$ 0.5	12.8–14.6	62.5 $\pm$ 2.5	58.3–66.0	15.0 $\pm$ 2.2	11.5–20.3	91.0 $\pm$ 2.9	85.3–96.9	3.29	1.66
Ukraine ( $n=25$ )	13.2 $\pm$ 0.4	12.2–14.2	62.6 $\pm$ 2.3	57.7–66.6	15.4 $\pm$ 2.9	11.0–24.5	91.2 $\pm$ 2.5	86.2–96.8	2.76	1.92
Israel ( $n=8$ )	13.2 $\pm$ 0.6	12.4–14.0	61.7 $\pm$ 2.9	57.5–66.0	13.6 $\pm$ 1.2	11.5–15.5	88.5 $\pm$ 2.8	84.3–92.2	3.13	2.24
Canada ( $n=90$ )	14.0 $\pm$ 0.7	12.5–15.5	59.0 $\pm$ 2.1	52.3–63.3	14.8 $\pm$ 2.1	10.2–20.1	87.9 $\pm$ 2.5	81.4–93.0	2.82	1.74

<sup>a</sup> Coefficient of variation of total sperm length calculated as  $\text{SD}/\text{mean} \times 100$  and adjusted for sample size following the formula  $(\text{CV}^*(1+(1/4n)))$  (Sokal and Rohlf 1995)

<sup>b</sup> Average coefficient of variation of total sperm length within males, based on ten sperms per male

being associated with increasing level of extrapair paternity (Lifjeld et al. 2010).

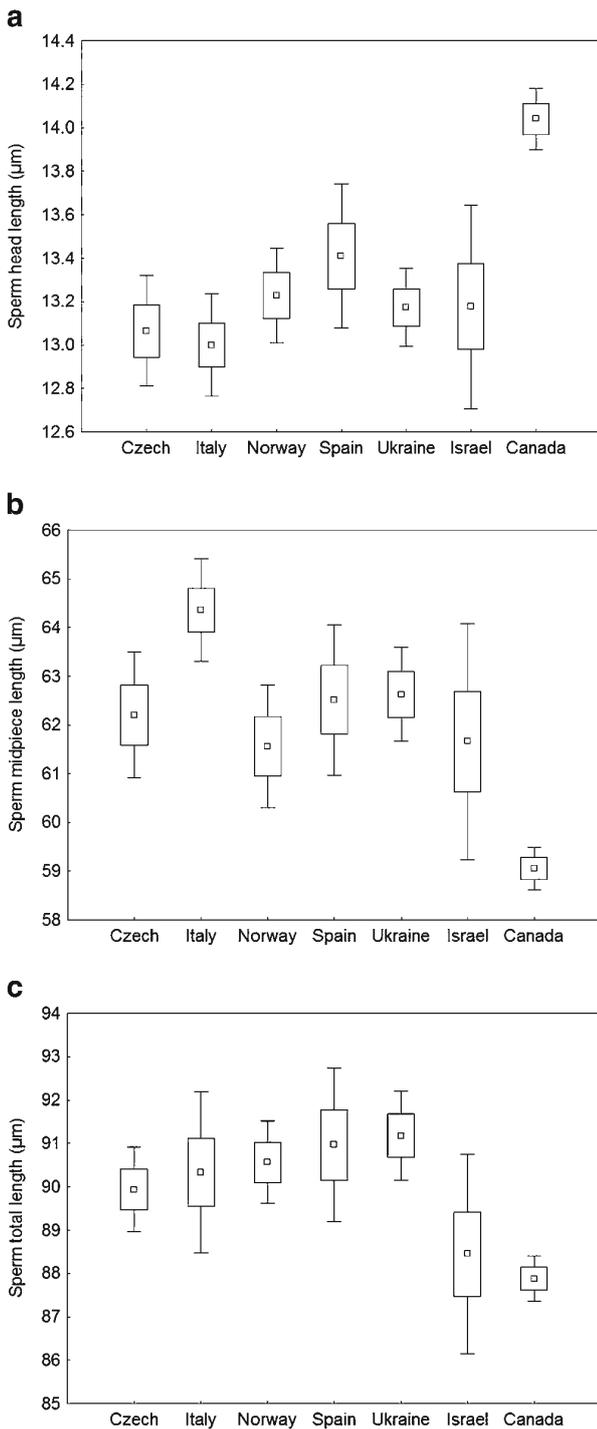
## Discussion

Our analysis revealed significant differences in sperm morphometry among barn swallow populations and subspecies. North American barn swallows from Canada had significantly shorter sperm than European barn swallows from Czech, Norway, Spain, and Ukraine but did not differ significantly from European barn swallows from Italy and East-Mediterranean barn swallows from Israel. When examining the three different sperm traits included in our analyses (head length, midpiece length, and tail length), North American barn swallows were characterized by longer sperm heads than all other populations and shorter midpieces than all other populations except the East-Mediterranean one. Further, we found that between male variation in sperm length (CV<sub>bm</sub>) was negatively related to levels of extrapair paternity, both expressed as percentage broods containing extrapair young (EPB) and percentage extrapair young (EPY).

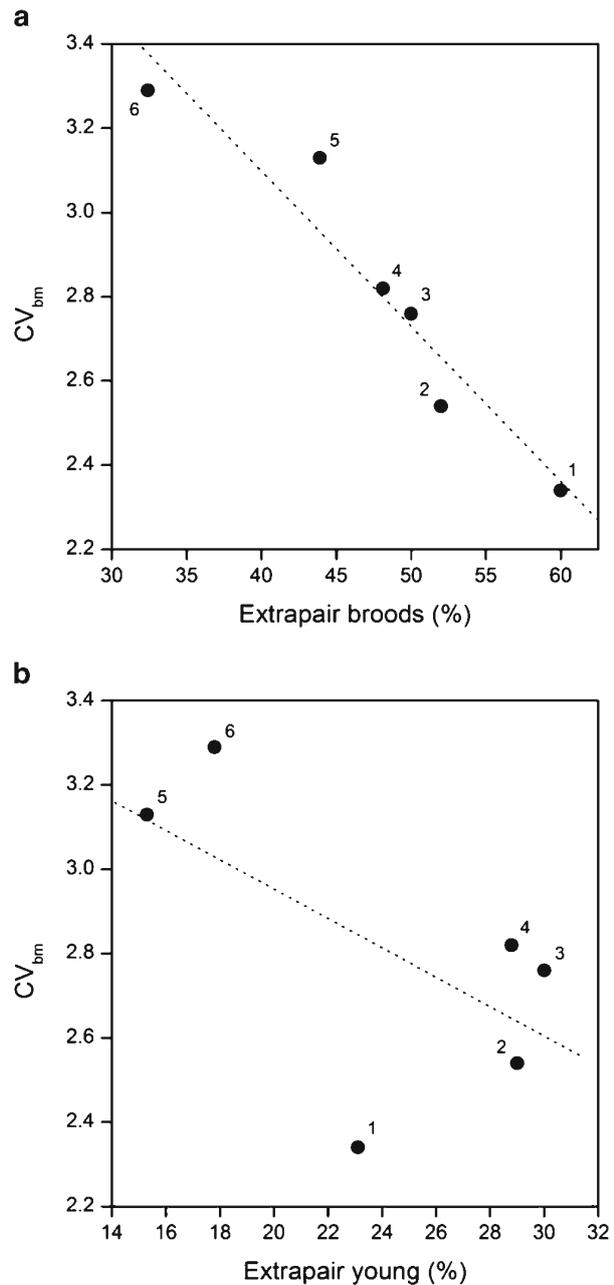
Based on the phylogenetic relationships between the three subspecies of barn swallows included in our analyses (Dor et al. 2010), we predicted that European and East-Mediterranean barn swallows should be more similar to each other in sperm morphometry compared to North American barn swallows. Although our findings were not clear, some of the sperm traits coincided with this prediction, e.g., spermatozoa of North American barn swallows had longer heads and shorter midpieces than all other populations. Total sperm length showed a different pattern, with European barn swallows having significantly longer sperm than both East-Mediterranean and North American barn

swallows, whereas the two latter did not differ significantly for this trait. Interestingly, sperm head size seems to be a trait that often differs among populations. It was the most variable sperm trait in the coal tit study (Schmoll and Kleven 2011). Also, in a recent comparison of wild and domesticated zebra finches (*Taeniopygia guttata*), sperm head length showed the greatest difference between the two groups (Immler et al. 2012). We do not know if genetic drift or selection is causing the differentiation in sperm morphometry among populations. Both factors can however be important for differentiation in sperm morphometry and are not mutually exclusive. Whereas the studies of coal tits and red-winged blackbirds documented significant differentiation in sperm size between relatively close populations (Lüpold et al. 2011; Schmoll and Kleven 2011), this study documented little differentiation in sperm size across the five European barn swallow populations. Another study on pied flycatchers (*Ficedula hypoleuca*) found a similar pattern as the present study, with no difference in sperm size between one German and two Norwegian populations (Lifjeld et al. 2012). The discrepancy between the coal tit and red-winged blackbird on one hand and the pied flycatcher and barn swallow studies on the other, might be explained by differences in gene flow among populations. Both coal tits and red-winged blackbirds are resident or short distance migratory birds, whereas pied flycatchers and barn swallows are long distance migrants with clear differences in dispersal distance (Paradis et al. 1999). Notably, the East-Mediterranean barn swallow is sedentary but still is subject to high levels of gene flow supposedly from migrating European barn swallows (Dor et al. 2012). Thus, gene flow could be more prominent in the two latter species.

Notably, it has been shown in Gouldian finches (*Erythrura gouldiae*) that males can exhibit plasticity in



**Fig 1** Sperm **a** head, **b** midpiece, and **c** total length, in seven populations of barn swallows *Hirundo rustica*. Average values are indicated as *small squares*, boxes indicate  $\pm$ SE, and *whiskers* indicate  $\pm$ 95 % confidence intervals. Sample sizes are; Czech  $n=20$  males, Italy  $n=8$  males, Norway  $n=26$  males, Spain  $n=13$  males, Ukraine  $n=25$  males, Israel  $n=8$  males, and Canada  $n=90$  males. See “Results” section for test statistics



**Fig 2** Relationship between **a** percentage broods containing extrapair young and coefficient of variation (adjusted for sample size) ( $CV_{bm}$ ) in sperm length between males and **b** percentage extrapair young and  $CV_{bm}$ , in six populations of barn swallows. Numbers represent the following populations: (1) Czech, (2) Italy, (3) Ukraine, (4) Canada, (5) Israel, and (6) Spain. See “Results” section for test statistics

sperm morphometry within the same breeding season when the social environment is altered (Immler et al. 2010). Males were found to increase the relative size of the sperm mid-piece when placed in intermediate to high competitive environments and increase the size of the sperm tail when facing

low to intermediate competitive environments (Immler et al. 2010). This could imply that the relative size of the sperm midpiece and tail could change across the breeding season in barn swallows as well. Similarly, a recent study of sperm size variation in house wrens (*Troglodytes aedon*) found that total sperm length was highly consistent across the breeding season, whereas the flagellum:head length ratio increased during the season, indicating phenotypic plasticity or adjustment in the relative size of sperm components (Cramer et al. 2012). However, repeated samples across the breeding season of North American and Czech barn swallows have shown very high repeatability of all sperm traits (own unpublished data).

Sperm head, midpiece, and flagellum length have been shown to be heritable in the zebra finch (Birkhead et al. 2005) and in several other taxa (e.g. Ward 2000; Morrow and Gage 2001a; Baer et al. 2006). Thus, plasticity in sperm morphometry within the same breeding season might be rather marginal and not biasing the population differences reported here.

To the best of our knowledge, this is the first study examining the relationship between sperm size variation and extrapair paternity levels across populations within the same species. A few studies have previously demonstrated that this relationship is significantly negative in passerine birds (Calhim et al. 2007; Kleven et al. 2008; Lifjeld et al. 2010). Recently, a similar negative relationship between sperm size variation and polyandry has been demonstrated in social insects (Fitzpatrick and Baer 2011). As extrapair paternity levels are known to vary among populations of the same species (e.g., Griffith et al. 2002), with barn swallows being one of the species showing considerable variation (Saino et al. 1999; Møller et al. 2003; Kleven et al. 2005; Vortman et al. 2011), we tested associations between  $CV_{bm}$  and levels of extrapair paternity data across populations of this species. Both EPB and EPY were negatively related to  $CV_{bm}$ , as predicted from the aforementioned interspecific studies. The relationship between  $CV_{bm}$  and EPY was, however, not statistically significant. Although our sample size in the present dataset is small, we argue that this indicates that  $CV_{bm}$  and risk of sperm competition are negatively related also within species, further supporting the notion that risk of sperm competition induces stabilizing selection on sperm size.

The present study shows little variation in sperm size/morphometry among European and East-Mediterranean barn swallows, and somewhat larger variation between European-East-Mediterranean and North American barn swallows. These results make sense in the light of the genetic difference and the large geographic distance between populations. The subspecies *rustica* and *transitiva* are genetically closely related (Dor et al. 2010), and they do not differ much in sperm morphometry. The subspecies *erythrogaster* is genetically quite distant from both *rustica*

and *transitiva* (Dor et al. 2010; Johnsen et al. 2010), and this is also reflected in the observed sperm morphometry. In conclusion, sperm morphometry might give an indication of genetic distance, not only between species, but also between phylogenetic lines within species. Further, we found negative relationships between  $CV_{bm}$  and two measures of extrapair paternity, EPB and EPY, indicating that the same pattern as have been found among species (Calhim et al. 2007; Kleven et al. 2008; Lifjeld et al. 2010; Fitzpatrick and Baer 2011), also is present intraspecifically, and which strengthens the explanation that sperm competition exerts a stabilizing selection pressure on sperm size variation.

**Acknowledgments** We are grateful to all people that assisted with field work, especially Frode Fossoy in Canada, Bjørn Aksel Bjerke in Norway, and Luz Garcia-Longoria in Spain. A special thanks to Gustav Thorsø Mohr for allowing us to trap inside the barn at Thorsø Herregård. We thank two anonymous reviewers for helpful comments on an earlier draft of the manuscript. This study was supported by funding from the Czech Science Foundation (to TA, project no. P506/12/2472), a Fondazione Cariplo grant (to NS, grant no. 2009–3496), the Ministry of Culture of the Czech Republic (to JC, grant no. DKRVO 00023272), the Natural Sciences and Engineering Research Council of Canada (to RJR), the Norwegian Research Council (to JTL, OK, LEJ and TL), the Samuel Freeman Charitable Trust (to TAM), and the Spanish Ministry of Economy and Competitiveness (to IGH, FdL and AM, grant no. CGL 2012–36665).

**Ethical standards** All authors declare that the present study complies with the current laws and ethical standards of animal research in Canada, Czech Republic, Israel, Italy, Norway, Spain, and Ukraine.

**Conflict of interest** The authors declare that they have no conflict of interest

## References

- Baer B, de Jong G, Schmid-Hempel R, Schmid-Hempel P, Høeg JT, Boomsma JJ (2006) Heritability of sperm length in the bumblebee *Bombus terrestris*. *Genetica* 127:11–23
- Birkhead TR, Pellatt EJ, Brekke P, Yeates R, Castillo-Juarez H (2005) Genetic effects on sperm design in the zebra finch. *Nature* 434:383–387
- Birkhead TR, Hosken DJ, Pitnick S (2009) Sperm biology: an evolutionary perspective. Academic Press, Oxford
- Blanckenhorn WU, Hellriegel B (2002) Against Bergmann's rule: fly sperm size increases with temperature. *Ecol Lett* 5:7–10
- Calhim S, Immler S, Birkhead TR (2007) Postcopulatory sexual selection is associated with reduced variation in sperm morphology. *PLoS One* 2:e413
- Cohen J (1977) *Reproduction*. Butterworths, London
- Cramer ERA, Laskemoen T, Kleven O, Lifjeld JT (2012) Sperm length variation in House Wrens *Troglodytes aedon*. *J Ornithol* DOI: 10.1007/s10336-012-0878-3
- Dor R, Safran RJ, Sheldon FH, Winkler DW, Lovette IJ (2010) Phylogeny of the genus *Hirundo* and the barn swallow subspecies complex. *Mol Phyl Evol* 56:409–418
- Dor R, Safran RJ, Vortman Y, Lotem A, McGowan A, Evans MR, Lovette IJ (2012) Population genetics and morphological

- comparisons of migratory European (*Hirundo rustica rustica*) and sedentary East-Mediterranean (*Hirundo rustica transitiva*) barn swallows. *J Hered* 103:55–63
- Eberhard WG (1996) Female control: sexual selection by cryptic female choice. Princeton University Press, Princeton, NJ
- Elgee KE, Evans JP, Ramnarine IW, Rush SA, Pitcher TE (2010) Geographic variation in sperm traits reflects predation risk and natural rates of multiple paternity in the guppy. *J Evol Biol* 23:1331–1338
- Ellegren H, Lindgren G, Primmer CR, Møller AP (1997) Fitness loss and germline mutations in barn swallows breeding in Chernobyl. *Nature* 389:593–596
- Fitzpatrick JL, Baer B (2011) Polyandry reduces sperm length variation in social insects. *Evolution* 65:3006–3012
- Gage MJG (1998) Mammalian sperm morphometry. *Proc R Soc Lond B* 265:97–103
- Griffith SC, Owens IPF, Thuman KA (2002) Extra pair paternity in birds: a review of interspecific variation and adaptive function. *Mol Ecol* 11:2195–2212
- Hettyey A, Roberts JD (2006) Sperm traits of the quacking frog, *Crinia georgiana*: intra- and interpopulation variation in a species with a high risk of sperm competition. *Behav Ecol Sociobiol* 59:389–396
- Hosken DJ, Garner TWJ, Blanckenhorn WU (2003) Asymmetry, testis and sperm size in yellow dung flies. *Funct Ecol* 17:231–236
- Immler S, Birkhead TR (2005) A non-invasive method for obtaining spermatozoa from birds. *Ibis* 147:827–830
- Immler S, Calhim S, Birkhead TR (2008) Increased postcopulatory sexual selection reduces the intramale variation in sperm design. *Evolution* 62:1538–1543
- Immler S, Pryke SR, Birkhead TR, Griffith SC (2010) Pronounced within-individual plasticity in sperm morphometry across social environments. *Evolution* 64:1634–1643
- Immler S, Griffith SC, Zann R, Birkhead TR (2012) Intra-specific variance in sperm morphometry: a comparison between wild and domesticated Zebra Finches *Taeniopygia guttata*. *Ibis* 154:480–487
- Johnsen A, Rindal E, Ericson PGP, Zuccon D, Kerr KCR, Stoeckle MY, Lifjeld JT (2010) DNA barcoding of Scandinavian birds reveals divergent lineages in trans-Atlantic species. *J Ornithol* 151:565–578
- Joly D, Korol A, Nevo E (2004) Sperm size evolution in *Drosophila*: inter- and intraspecific analysis. *Genetica* 120:233–244
- Kleven O, Jacobsen F, Robertson RJ, Lifjeld JT (2005) Extrapair mating between relatives in the barn swallow: a role for kin selection? *Biol Lett* 1:389–392
- Kleven O, Jacobsen F, Izadnegahdar R, Robertson RJ, Lifjeld JT (2006) Male tail streamer length predicts fertilization success in the North American barn swallow (*Hirundo rustica erythrogaster*). *Behav Ecol Sociobiol* 59:412–418
- Kleven O, Laskemoen T, Fossøy F, Robertson RJ, Lifjeld JT (2008) Intraspecific variation in sperm length is negatively related to sperm competition in passerine birds. *Evolution* 62:494–499
- Kuramoto M (1996) Generic differentiation of sperm morphology in treefrogs from Japan and Taiwan. *J Herpetol* 30:437–443
- Laskemoen T, Kleven O, Fossøy F, Lifjeld JT (2007) Intraspecific variation in sperm length in two passerine species, the bluethroat *Luscinia svecica* and the willow warbler *Phylloscopus trochilus*. *Ornis Fenn* 84:131–139
- Lessells CM, Boag PT (1987) Unrepeatable repeatabilities: a common mistake. *Auk* 104:116–121
- Lifjeld JT, Laskemoen T, Kleven O, Albrecht T, Robertson RJ (2010) Sperm length variation as a predictor of extrapair paternity in passerine birds. *PLoS One* 5:e13456
- Lifjeld JT, Kleven O, Jacobsen F, McGraw KJ, Safran RJ, Robertson RJ (2011) Age before beauty? Relationships between fertilization success and age-dependent ornaments in barn swallows. *Behav Ecol Sociobiol* 65:1687–1697
- Lifjeld JT, Laskemoen T, Kleven O, Pedersen ATM, Lampe HM, Rudolfsen G, Schmoll T, Slagsvold T (2012) No evidence for pre-copulatory sexual selection in a passerine bird. *PLoS One* 7:e32611
- Lüpold S, Linz GM, Birkhead TR (2009) Sperm design and variation in the New World blackbirds (Icteridae). *Behav Ecol Sociobiol* 63:899–909
- Lüpold S, Westneat DF, Birkhead TR (2011) Geographical variation in sperm morphology in the red-winged blackbird (*Agelaius phoeniceus*). *Evol Ecol* 25:373–390
- Manier MK, Palumbi SR (2008) Intraspecific divergence in sperm morphology of the green sea urchin, *Strongylocentrotus droebachiensis*: implications for selection in broadcast spawners. *BMC Evol Biol* 8:283
- Minoretti N, Baur B (2006) Among- and within-population variation in sperm quality in the simultaneously hermaphroditic land snail *Arianta arbustorum*. *Behav Ecol Sociobiol* 60:270–280
- Møller AP (1988) Female choice selects for male sexual tail ornaments in the monogamous swallow. *Nature* 332:640–642
- Møller AP (1994a) Repeatability of female choice in a monogamous swallow. *Anim Behav* 47:643–648
- Møller AP (1994b) Sexual selection and the barn swallow. Oxford University Press, Oxford
- Møller AP, Brohede J, Cuervo JJ, de Lope F, Primmer C (2003) Extrapair paternity in relation to sexual ornamentation, arrival date, and condition in a migratory bird. *Behav Ecol* 14:707–712
- Møller AP, Mousseau TA, Lynn C, Ostermiller S, Rudolfsen G (2008) Impaired swimming behaviour and morphology of sperm from barn swallows *Hirundo rustica* in Chernobyl. *Mutat Res Genet Tox Environ Mutagen* 650:210–216
- Morrow EH, Gage MJG (2001a) Artificial selection and heritability of sperm length in *Gryllus bimaculatus*. *Heredity* 87:356–362
- Morrow EH, Gage MJG (2001b) Consistent significant variation between individual males in spermatozoal morphometry. *J Zool Lond* 254:147–153
- Paradis E, Baillie SR, Sutherland WJ, Gregory RD (1999) Dispersal and spatial scale affect synchrony in spatial population dynamics. *Ecol Lett* 2:114–120
- Parker GA (1970) Sperm competition and its evolutionary consequences in the insects. *Biol Rev* 45:525–567
- Petrie M, Kempnaers B (1998) Extra-pair paternity in birds: explaining variation between species and populations. *Trends Ecol Evol* 13:52–58
- Pitnick S, Miller GT, Schneider B, Markow TA (2003) Ejaculate-female coevolution in *Drosophila mojavensis*. *Proc R Soc Lond B* 270:1507–1512
- Safran RJ, Neumann CR, McGraw KJ, Lovette IJ (2005) Dynamic paternity allocation as a function of male plumage color in barn swallows. *Science* 309:2210–2212
- Saino N, Primmer CR, Ellegren H, Møller AP (1997) An experimental study of paternity and tail ornamentation in the barn swallow (*Hirundo rustica*). *Evolution* 51:562–570
- Saino N, Primmer CR, Ellegren H, Møller AP (1999) Breeding synchrony and paternity in the barn swallow (*Hirundo rustica*). *Behav Ecol Sociobiol* 45:211–218
- Schmoll T, Kleven O (2011) Sperm dimensions differ between two coal tit *Parus ater* populations. *J Ornithol* 152:515–520
- Snook RR (2001) Absence of latitudinal clines in sperm characters in North American populations of *Drosophila subobscura* (Diptera: Drosophilidae). *Pan-Pac Entomol* 77:261–271

- Sokal RR, Rohlf FJ (1995) Biometry: the principles and practice of statistics in biological research. Freeman, New York
- Turner AK (2006) The barn swallow. T. & A. D. Poyser, London
- Vortman Y, Lotem A, Dor R, Lovette IJ, Safran RJ (2011) The sexual signals of the East-Mediterranean barn swallow: a different swallow tale. Behav Ecol 22:1344–1352
- Ward PI (1998) Intraspecific variation in sperm size characters. Heredity 80:655–659
- Ward PI (2000) Sperm length is heritable and sex-linked in the yellow dung fly (*Scathophaga stercoraria*). J Zool Lond 251:349–353
- Wolfson A (1952) The cloacal protuberance: a means for determining breeding condition in live male passerines. Bird Band 23:159–165

Research



**Cite this article:** Hermosell IG, Laskemoen T, Rowe M, Møller AP, Mousseau TA, Albrecht T, Lifjeld JT. 2013 Patterns of sperm damage in Chernobyl passerine birds suggest a trade-off between sperm length and integrity. *Biol Lett* 9: 20130530.  
<http://dx.doi.org/10.1098/rsbl.2013.0530>

Received: 8 June 2013

Accepted: 7 September 2013

**Subject Areas:**  
evolution

**Keywords:**  
acrosome, radiation,  
sperm evolution, sperm size

**Author for correspondence:**  
Jan T. Lifjeld  
e-mail: [j.t.lifjeld@nhm.uio.no](mailto:j.t.lifjeld@nhm.uio.no)

Electronic supplementary material is available at <http://dx.doi.org/10.1098/rsbl.2013.0530> or via <http://rsbl.royalsocietypublishing.org>.



Evolutionary biology

## Patterns of sperm damage in Chernobyl passerine birds suggest a trade-off between sperm length and integrity

Ignacio G. Hermosell<sup>1</sup>, Terje Laskemoen<sup>2</sup>, Melissah Rowe<sup>2</sup>, Anders P. Møller<sup>3</sup>, Timothy A. Mousseau<sup>4</sup>, Tomáš Albrecht<sup>5,6</sup> and Jan T. Lifjeld<sup>2</sup>

<sup>1</sup>Departamento de Anatomía, Biología Celular y Zoología, Universidad de Extremadura, Avenida de Elvas s/n, 06071 Badajoz, Spain

<sup>2</sup>Natural History Museum, University of Oslo, PO Box 1172, Blindern, 0318 Oslo, Norway

<sup>3</sup>Laboratoire d'Ecologie, Systématique et Evolution, CNRS UMR 8079, Université Paris-Sud, Bâtiment 362, 91405 Orsay Cedex, France

<sup>4</sup>Department of Biological Sciences, University of South Carolina, Columbia, SC 29208, USA

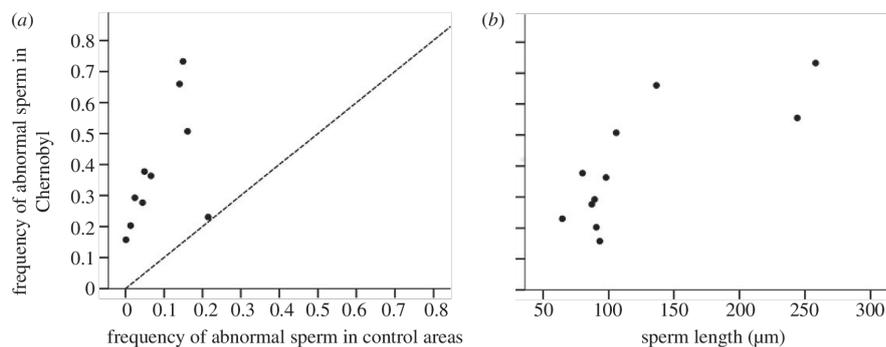
<sup>5</sup>Academy of Sciences of the Czech Republic, Institute of Vertebrate Biology, Kvetna 8, Brno 603 65, Czech Republic

<sup>6</sup>Faculty of Sciences, Charles University in Prague, Vinicna 7, Praha 12844, Czech Republic

Interspecific variation in sperm size is enigmatic, but generally assumed to reflect species-specific trade-offs in selection pressures. Among passerine birds, sperm length varies sevenfold, and sperm competition risk seems to drive the evolution of longer sperm. However, little is known about factors favouring short sperm or constraining the evolution of longer sperm. Here, we report a comparative analysis of sperm head abnormalities among 11 species of passerine bird in Chernobyl, presumably resulting from chronic irradiation following the 1986 accident. Frequencies of sperm abnormalities varied between 15.7 and 77.3% among species, more than fourfold higher than in uncontaminated areas. Nonetheless, species ranked similarly in sperm abnormalities in unpolluted areas as in Chernobyl, pointing to intrinsic factors underlying variation in sperm damage among species. Scanning electron microscopy of abnormal spermatozoa revealed patterns of acrosome damage consistent with premature acrosome reaction. Sperm length, but not sperm competition risk explained variation in sperm damage among species. This suggests that longer spermatozoa are more susceptible to premature acrosome reaction. Therefore, we hypothesize a trade-off between sperm length and sperm integrity affecting sperm evolution in passerine birds.

### 1. Introduction

Spermatozoa display tremendous diversity in size and shape across the animal kingdom [1]. Fertilization mode and post-copulatory sexual selection are two main factors assumed to shape this diversity among taxa [1–3]. Passerine birds have spermatozoa adapted to internal fertilization and sperm storage, with a sevenfold variation in length, i.e. approximately 40–290  $\mu\text{m}$  [2,4,5]. Post-copulatory sexual selection seems to promote the evolution of longer sperm [2,4,6] although the adaptive function of longer sperm in sperm competition is not well understood. Longer sperm have longer mid-pieces, which contain the fused mitochondrion and hence more energy resources [7]. However, energy does not seem to translate directly into higher swimming speeds [6,7], so other energy-dependent traits like longevity, could be important. Post-copulatory sexual selection also increases total investment in sperm production [8,9], which is manifested in a disproportionate increase in sperm number compared with the increase in sperm size across species [2]. This suggests that there is no clear trade-off between sperm size and number in



**Figure 1.** Frequency of sperm abnormality in Chernobyl passerine species plotted against (a) the frequency of sperm abnormality in uncontaminated areas and (b) mean sperm length for the species. The line in (a) is  $y = x$ .

**Table 1.** PGLS analyses of the proportion of abnormal sperm in relation to total sperm length and sperm competition risk ( $CV_{bm}$  of sperm length) among passerine species in Chernobyl, Ukraine and uncontaminated areas (Norway and Czech Republic). In all tests, the phylogenetic parameter ( $\lambda$ ) was approximately 0 and significantly different from 1, suggesting there was no strong phylogenetic signal in the relationships.

area	predictor	slope	s.e.	t	p
Chernobyl ( $n = 11$ species)	total sperm length	1.02	0.24	4.32	0.002
	sperm competition risk	0.08	0.09	0.89	0.40
uncontaminated ( $n = 10$ species)	total sperm length	0.16	0.29	0.55	0.60
	sperm competition risk	0.04	0.07	0.63	0.54

passerine birds, owing to some significant cost or constraint to the evolution of longer sperm.

Here, we report a comparative study of sperm abnormalities in passerine birds providing evidence for reduced functional performance of longer sperm. Radioactive contamination following the Chernobyl accident in 1986 had a series of negative environmental effects, including sperm damage in mammals [10] and birds [11]. We examined the frequency of abnormal sperm among 11 passerine species sampled around Chernobyl in 2010 and 2011. We also scored sperm abnormalities in samples from 10 of these species from uncontaminated areas elsewhere in Europe. We tested whether the patterns of sperm damage covaried with species-specific sperm lengths and sperm competition risk, as sperm competition enhances both sperm quantity and quality among passerine birds [9]. Thus, we predicted that species with a high risk of sperm competition should have more robust sperm, in this case lower frequencies of radiation-induced damage. As sperm length is positively correlated with sperm competition risk [2,4,6], we would also expect species with longer sperm to have less damaged sperm. Finally, we used scanning electron microscopy (SEM) to study the nature of sperm abnormalities.

## 2. Material and methods

We obtained sperm samples from 102 individuals belonging to 11 species of passerine birds in Chernobyl, Ukraine, during May–June 2010–2011, and 84 individuals from 10 of the same species in Norway and the Czech Republic (uncontaminated areas) during 2007–2013. A detailed sample list is given in the electronic supplementary material, table S1. Ejaculates were obtained by cloacal massage, diluted in saline and immediately fixed in 5% formaldehyde [6]. From each sample, an approximately 3  $\mu$ l aliquot

was spread on a microscope slide and air-dried, then gently rinsed with distilled water and air-dried again. Digital images of the spermatozoa were captured (160 $\times$  magnification) using a Leica DFC420 digital camera attached to a LEICA DM6000 B microscope, and analysed with Leica APPLICATION software. We typically examined 100 spermatozoa per sample, unless fewer sperm were available, and scored them as ‘normal’ or ‘abnormal’ (i.e. different head shape or reduced head length). All images were scored by one person (I.G.H.) to reduce observer error. Additional scoring of images from 10 samples (one sample randomly drawn from each species in uncontaminated areas), done blindly with respect to the first scorings, gave a high repeatability of the scored frequencies of abnormal sperm ( $R = 0.98$ ,  $F_{9,10} = 101.6$ ,  $p < 0.001$ ).

For SEM, we selected samples from three species with relatively high frequencies of abnormal sperm. Formalin-fixed spermatozoa were prepared and examined as in Lifjeld *et al.* [12]. Mean sperm lengths for each species were obtained from the avian sperm collection database at the Natural History Museum, Oslo, only using measurements of sperm with no sign of damage from the control populations (10 species) or Chernobyl (*Luscinia luscinia*). The coefficient of variation in mean sperm length ( $CV_{bm}$ ) among males from the same populations was used as an index of sperm competition risk [4]. Species-specific values for sperm length and sperm competition risk are listed in the electronic supplementary material, table S2.

Comparative analyses of the proportion of abnormal sperm were conducted using a generalized least-squares approach controlling for phylogenetic relatedness among species (i.e. PGLS; electronic supplementary material, methods). For all parametric tests, proportions were arcsine square-root transformed, and sperm lengths were  $\log_{10}$ -transformed to approach normality.

## 3. Results

Frequencies of abnormal sperm varied from 15.7 to 73.3% among Chernobyl species ( $N = 11$ ), with an overall mean

frequency of 39.6%. Among uncontaminated populations of the same species ( $N = 10$ ), frequencies varied from 0.1 to 21.1%, with an overall mean frequency of 8.6%. The frequency of abnormal sperm was always higher in Chernobyl than that in uncontaminated populations (paired  $t_9 = 7.68$ ,  $p < 0.001$ ; figure 1a), although for one outlier species, *Coccothraustes coccothraustes*, frequencies were quite similar. Sperm abnormalities tended to be positively correlated between contaminated and uncontaminated areas (Pearson  $r = 0.629$ ,  $p = 0.051$ ,  $n = 10$ ; figure 1a, with the outlier species excluded:  $r = 0.928$ ,  $p < 0.001$ ,  $n = 9$ ), which implies an intrinsic, species-specific factor underlying the consistent variation in sperm abnormalities between environments.

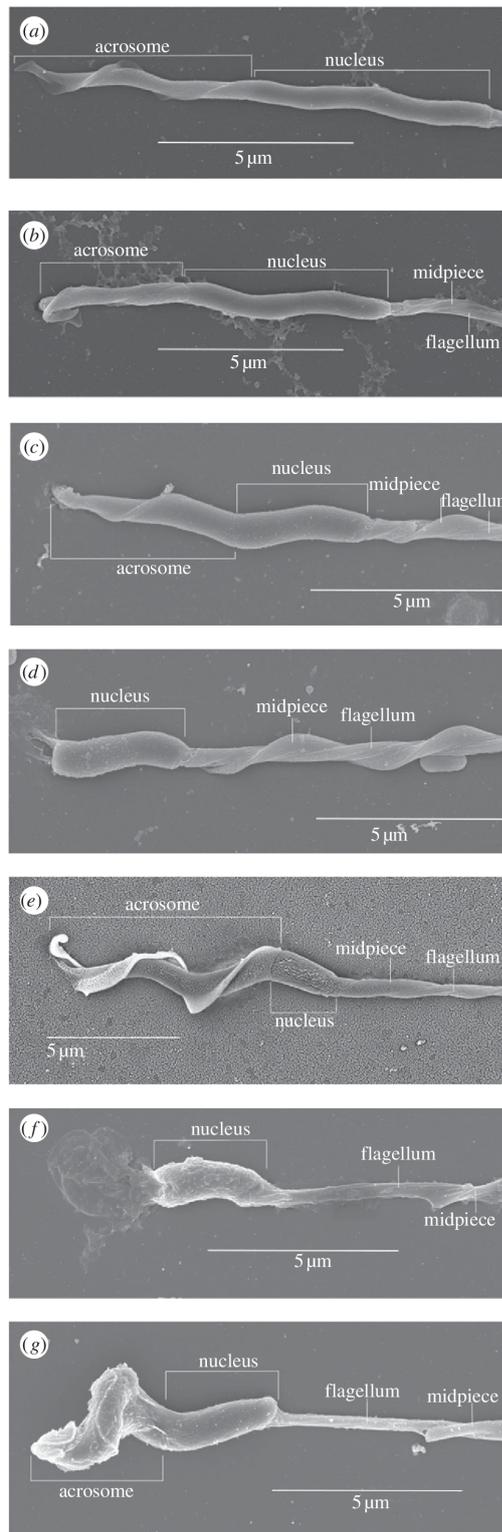
Variation in sperm abnormalities among Chernobyl populations was significantly associated with sperm length, i.e. species with longer sperm had more abnormal sperm (table 1 and figure 1b). By contrast, there was no significant association with sperm competition risk (table 1). Among uncontaminated populations, neither sperm length nor sperm competition risk explained significant variation in sperm abnormalities (table 1), but sperm length had a significant effect when the outlier was excluded (PGLS,  $t = 2.77$ ,  $n = 9$ ,  $p = 0.028$ ).

SEM images of spermatozoa (figure 2) revealed that abnormalities were restricted to the acrosome, i.e. the anterior part of the head. The types of damage varied between a bent acrosomal tip (figure 2b), a reduced acrosome and acrosomal microtubule helix (figure 2g), an 'emptied' acrosome with remnants of the plasma membrane (figure 2f) and an entirely missing acrosome (figure 2d). These changes reflect the various stages of the acrosome reaction, which normally takes place when the sperm penetrates the inner perivitelline layer of the ovum [13]. Hence, we interpret the sperm head abnormalities as evidence of premature acrosome reaction.

#### 4. Discussion

Our study documented three novel patterns of sperm damage. First, all studied passerine birds in the Chernobyl area have elevated frequencies of sperm abnormality, even 25 years after the radioactive fallout. Background radiation in this environment has had significant negative impact on many birds and other taxa [14,15]. Studies of local barn swallow *Hirundo rustica* populations have revealed a series of physiological and morphological defects [16,17], including increased sperm abnormalities and reduced sperm swimming speed [11,18]. Our results indicate that most passerine birds in the area are significantly affected by radiation-induced sperm damage.

Second, our SEM analyses identified premature acrosome reaction as the likely mechanism of the observed sperm damage. Correct timing of the acrosome reaction is crucial for fertilizing success of an ejaculate, and spermatozoa without an intact acrosome are unable to penetrate the inner perivitelline layer of the ovum [13]. Our sperm samples were fresh ejaculates, so the conditions causing the defect must be related to the physiology of the male. Several factors are known to induce premature acrosome reaction in birds and mammals, including mutations [19], extracellular calcium and lead [20,21], and oxidative stress [22]. Interestingly, a link between oxidative stress and poor sperm performance has already been indicated in barn swallows from Chernobyl [11,23], so



**Figure 2.** Scanning electron micrographs of normal and abnormal spermatozoa of three passerine species: (a,b) barn swallow *Hirundo rustica*, (c,d) common blackbird *Turdus merula* and (e–g) common chaffinch *Fringilla coelebs*.

we speculate that premature acrosome reaction is caused by oxidative stress and accelerated sperm senescence [24]. There was no seasonal trend in the proportion of abnormal sperm (see electronic supplementary material, methods).

Finally, species with longer sperm had more damaged sperm. This implies that longer sperm, under otherwise similar extracellular conditions, has a lower structural integrity of the acrosome, and may be more susceptible to lipid peroxidation of the plasma membranes [22]. Oscine passerine birds are characterized by the acrosome being longer than the nucleus [5], and longer acrosomes could be structurally less stable. However, it is not yet known whether species with longer

sperm also have longer acrosomes. Regardless of the actual mechanism underlying the inferred premature acrosome reaction of longer sperm, the pattern reflects a possible trade-off between sperm length and sperm integrity, and a possible explanation for why there is no run-away selection for longer sperm in passerine birds as compared with other taxa [2].

**Acknowledgement.** We thank various field assistants for help with sperm sampling and Antje Hoenen for help with SEM analyses.

**Funding statement.** The work was supported by grants from the Research Council of Norway, the Czech Science Foundation (P506/12/2472), the Samuel Freeman Charitable Trust, the University of South Carolina, the Fulbright Program and NATO.

## References

- Pitnick S, Hosken DJ, Birkhead TR. 2009 Sperm morphological diversity. In *Sperm biology: an evolutionary perspective* (eds TR Birkhead, DJ Hosken, S Pitnick), pp. 69–149. Oxford, UK: Elsevier.
- Immler S, Pitnick S, Parker GA, Durrant KL, Lüpold S, Calhim S, Birkhead TR. 2011 Resolving variation in the reproductive tradeoff between sperm size and number. *Proc. Natl Acad. Sci. USA* **108**, 5325–5330. (doi:10.1073/pnas.1009059108)
- Snook RR. 2005 Sperm in competition: not playing by the numbers. *Trends Ecol. Evol.* **20**, 46–53. (doi:10.1016/j.tree.2004.10.011)
- Lifjeld JT, Laskemoen T, Kleven O, Albrecht T, Robertson RJ. 2010 Sperm length variation as a predictor of extrapair paternity in passerine birds. *PLoS ONE* **5**, e13456. (doi:10.1371/journal.pone.0013456)
- Jamieson BGM. 2006 Avian spermatozoa: structure and phylogeny. In *Reproductive biology and phylogeny of birds. Part A.* (ed. BGM Jamieson), pp. 249–511. Enfield, NH: Science Publishers Inc.
- Kleven O, Fossoy F, Laskemoen T, Robertson RJ, Rudolfsen G, Lifjeld JT. 2009 Comparative evidence for the evolution of sperm swimming speed by sperm competition and female sperm storage duration in passerine birds. *Evolution* **63**, 2466–2473. (doi:10.1111/j.1558-5646.2009.00725.x)
- Rowe M, Laskemoen T, Johnsen A, Lifjeld JT. 2013 Evolution of sperm structure and energetics in passerine birds. *Proc. R. Soc. B* **280**, 20122616. (doi:10.1098/rspb.2012.2616)
- Pitcher TE, Dunn PO, Whittingham LA. 2005 Sperm competition and the evolution of testes size in birds. *J. Evol. Biol.* **18**, 557–567. (doi:10.1111/j.1420-9101.2004.00874.x)
- Rowe M, Pruett-Jones S. 2011 Sperm competition selects for sperm quantity and quality in the Australian Maluridae. *PLoS ONE* **6**, e15720. (doi:10.1371/journal.pone.0015720)
- Pomerantseva MD, Ramaiya LK, Chekhovich AV. 1997 Genetic disorders in house mouse germ cells after the Chernobyl catastrophe. *Mutation Res.* **381**, 97–103. (doi:10.1016/S0027-5107(97)00155-3)
- Møller AP, Surai P, Mousseau TA. 2005 Antioxidants, radiation and mutation as revealed by sperm abnormality in barn swallows from Chernobyl. *Proc. R. Soc. B* **272**, 247–252. (doi:10.1098/rspb.2004.2914)
- Lifjeld JT, Hoenen A, Johannessen LE, Laskemoen T, Lopes RJ, Rodrigues P, Rowe M. 2013 The Azores bullfinch (*Pyrrhula pyrrhula*) has the same unusual and size-variable sperm morphology as the Eurasian bullfinch (*Pyrrhula pyrrhula*). *Biol. J. Linn. Soc.* **108**, 677–687. (doi:10.1111/j.1095-8312.2012.02040.x)
- Stepinska U, Bakst MR. 2006 Fertilization. In *Reproductive biology and phylogeny of birds. Part A.* (ed. BGM Jamieson), pp. 553–587. Enfield, NH: Science Publishers Inc.
- Møller AP, Mousseau TA. 2007 Species richness and abundance of forest birds in relation to radiation at Chernobyl. *Biol. Lett.* **3**, 483–486. (doi:10.1098/rsbl.2007.0226)
- Møller AP, Mousseau TA. 2009 Reduced abundance of insects and spiders linked to radiation at Chernobyl 20 years after the accident. *Biol. Lett.* **5**, 356–359. (doi:10.1098/rsbl.2008.0778)
- Møller AP, Mousseau TA, de Lope F, Saino N. 2007 Elevated frequency of abnormalities in barn swallows from Chernobyl. *Biol. Lett.* **3**, 414–417. (doi:10.1098/rsbl.2007.0136)
- Bonisoli-Alquati A, Mousseau TA, Møller AP, Caprioli M, Saino N. 2010 Increased oxidative stress in barn swallows from the Chernobyl region. *Comp. Biochem. Physiol. A* **155**, 205–210. (doi:10.1016/j.cbpa.2009.10.041)
- Møller AP, Mousseau TA, Lynn C, Ostermiller S, Rudolfsen G. 2008 Impaired swimming behaviour and morphology of sperm from barn swallows *Hirundo rustica* in Chernobyl. *Mutation Res.* **650**, 210–216. (doi:10.1016/j.mrgentox.2007.12.006)
- Brown J, Cebra-Thomas JA, Bleil JD, Wassarman PM, Silver LM. 1989 A premature acrosome reaction is programmed by mouse *t* haplotypes during sperm differentiation and could play a role in transmission ratio distortion. *Development* **106**, 769–773.
- Lemoine M, Grasseau I, Brillard JP, Blesbois E. 2008 A reappraisal of the factors involved in *in vitro* initiation of the acrosome reaction in chicken spermatozoa. *Reproduction* **136**, 391–399. (doi:10.1530/REP-08-0094)
- Oliveira H, Spanò M, Santos C, Pereira M. 2009 Lead chloride affects sperm motility and acrosome reaction in mice. *Cell Biol. Toxicol.* **25**, 341–353. (doi:10.1007/s10565-008-9088-4)
- Bansal AK, Bilaspuri GS. 2011 Impacts of oxidative stress and antioxidants on semen functions. *Vet. Med.* **2011**, 686137. (doi:10.4061/2011/686137)
- Bonisoli-Alquati A, Møller AP, Rudolfsen G, Saino N, Caprioli M, Ostermiller S, Mousseau TA. 2011 The effects of radiation on sperm swimming behavior depend on plasma oxidative status in the barn swallow (*Hirundo rustica*). *Comp. Biochem. Physiol. A* **159**, 105–112. (doi:10.1016/j.cbpa.2011.01.018)
- Pizzari T, Dean R, Pacey A, Moore H, Bonsall MB. 2008 The evolutionary ecology of pre- and post-meiotic sperm senescence. *Trends Ecol. Evol.* **23**, 131–140. (doi:10.1016/j.tree.2007.12.003)

